Novel antibody-based assays for disease and food contaminant detection

A thesis submitted for the degree of Ph.D.

By

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July 2007

Based on research carried out at

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy, is entirely my own work, and has not been taken from work of others, save and to the extent that such work is cited and acknowledged within the text of my work.

Signed: _____ **ID No:** 98329278

Date: 3/10/'07

Table of Contents

Abbreviations

A	Absorbance
AFB_1	aflatoxin B_1
AFB_1-BSA	aflatoxin B_1-bovine serum albumin
AMP	ampicillin
AMP-BSA	ampicillin-bovine serum albumin
AMP-OVA	ampicillin-ovalbumin
AMP-THY	ampicillin-thyroglobulin
AMV RT	Avian Myeloblastosis Virus Reverse Transcriptase
APA	(+)-6-aminopenicillanic acid
BCA	bicinchoninic acid
B-cells	Bursa-equivalent-derived small lyphocytes
BIA	biomolecular interaction analysis
bp	base pairs
BSA	bovine serum albumin
CAT	Cambridge Antibody Technology
cDNA	complementary DNA
CDR	complementarity determining regions
C_H	constant region of heavy chain
C_L	constant region of light chain
cfu	colony forming unit
CM	carboxymethylated
CR	cross-reactivity
CV	coefficient of variation
cDNA	complementary deoxyribonucleic acid
d	degeneracy
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidyl triphosphates
DSn	diagnostic sensitivity
DSp	diagnostic specificity
EDC	*N*-ethyl-*N'*- (dimethylaminopropyl)-carbodiimide
EDTA	ethylenediaminetetra-acetic acid

EEC	European Economic Community
ELISA	enzyme-linked immunosorbent assay
EU	European Union
Fab	antigen-binding fragment
Fc	crystallisable fragment
FCA	Freund's complete adjuvant
FIA	fluorescence immunoassay
FICA	Freund's incomplete adjuvant
Fv	variable fragment
FWR	framework region
GC	gas chromatography
GLC	gas-liquid chromatography
HAT	hypoxanthine aminopterin thymidine
HBS	hepes buffered saline
HGPRT	hypoxanthine guanidine phosphoribosyl transferase
His	histidine
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
IFN	interferon
IgG	immunoglobulin class G
IgA	immunoglobulin class A
IgD	immunoglobulin class D
IgE	immunoglobulin class E
IgM	immunoglobulin class M
IL	interleukin
IMAC	immobilised metal affinity chromatography
IPTG	isopropyl-β-D-galactopyranoside
KLH	keyhole limpet haemocyanin
LB	Luria Bertani media
LC	liquid chromatography
LED	light emitting diode
LFIA	lateral flow immunoassay
LOD	limit of detection
Log	logarithmic

mAb	monoclonal antibody
Marvel™	dried skimmed milk powder (Premier Foods Ltd., UK)
MHC	major histocompatibility complex
MRL	maximum residue limit
mRNA	messenger RNA
MS	mass spectroscopy
MW	molecular weight
ND	not determined
NHS	*N*-hydroxysuccinimide
NK	natural killer
NTA	nitrilotriacetic acid
OD	optical density
o-PD	*o*-phenylenediamine dihydrochloride
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin binding protein
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.05% (v/v) Tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	log of the hydrogen ion concentration
pI	isoelectric point
RNA	ribonucleic acid
Rnasin®	ribonuclease inhibitor
RT-PCR	reverse transcriptase PCR
RU	response unit
SAS	saturated ammonium sulphate
scFv	single chain variable fragment
SD	standard deviation
SDS	sodium dodecyl sulphate
SOE-PCR	splice by overlap extension- polymerase chain reaction
SPR	surface plasmon resonance
TAE	tris acetic acid-ethylene diamine tetra acetic acid
TLC	thin layer chromatography

TMB	3, 3', 5, 5'-tetramethylbenzidine
USDA	United States Department of Agriculture
UV	ultraviolet
V_H	variable region of heavy chain
V_L	variable region of light chain

Units

°C	degrees Celsius
µg	microgram
µl	microlitre
µM	micromolar
cm	centimetre
Da	Dalton(s)
g	gram
g	centrifugal force
kg	kilogram
kbp	kilo base pair
kDa	kilo Dalton(s)
L	litre
M	molar
m	metre
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mol	moles
msec	millisecond
nM	nanomolar
ng	nanogram
pg	picogram
rpm	revolutions per minute
RU	response units
sec	second
V	volts
v/v	volume per unit volume
w/v	weight per unit volume

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Courses

Successful completion of Nanobiotechnology graduate programme (2003) delivered by Cornell University Nanobiotechnology Centre (NBTC) in conjugation with the NCSR. January – May.

Abstract

This thesis describes the development of immunoassays for the diagnosis of caseous lymphadenitis, a disease of sheep caused by *Corynebacterium pseudotuberculosis;* and for the detection of the food contaminants, aflatoxin B_1 (AFB_1), and the β-lactam antibiotic, ampicillin.

Immunoassays based on the detection of antibodies to a phospholipase D (PLD) exotoxin of *Corynebacterium pseudotuberculosis,* the causative agent of caseous lymphadenitis (CLA), were investigated. A previously cloned recombinant form of PLD (Menzies *et al.,* 1994) was expressed in *E. coli,* purified and subsequently used in an indirect ELISA and Biacore assay for the detection of antibodies to *C. pseudotuberculosis* in sheep serum samples. These assays were optimised using a variety of positive and negative controls and were then applied to a range of clinical samples from Irish herds.

A previously isolated AFB_1-specific single chain variable fragment (scFv) was converted to a chimeric Fab format by the addition of human constant regions, using PCR assembly methods. A mutant Fab fragment was also isolated and demonstrated improved assay performance when compared to the parent scFv. The antibody fragments were successfully applied to the development of several immunoassay formats and compared with regard to sensitivity, specificity, and stability. The scFv fragment was also successfully biotinylated *in vivo* and applied to the development of an ELISA.

Rabbit polyclonal antibodies to ampicillin were produced, characterised and subsequently applied to ELISA and Biacore-based assays for the detection of ampicllin in 'spiked' samples of PBS and whole milk. The antibodies were then employed in the development of a fluorescence-based immunoassay for subsequent incorporation into a disposable biochip senor. Naïve and immune phage displayed libraries were also screened against a number of ampicillin conjugates for the isolation of an ampicillin-specific scFv.

Chapter 1

Introduction

1.1 Section overview

The main aim of this research was the development of immunoassays for the detection of the food contaminants, aflatoxin B_1 and ampicillin, and for the diagnosis of caseous lymphadenitis, a disease of sheep caused by *Corynebacterium pseudotuberculosis* infection. Immunoassays exploit the specific and sensitive interaction of an antibody to its corresponding antigen and thus, this introductory chapter gives a brief overview of antibody structure and function. Antibody production by both conventional and genetic means and their applications are also discussed. Additional information on target antigens and current methods for their detection is also presented at the beginning of each of the relevant chapters.

1.2 The immune system

The term immunity, derived from the Latin term 'immunis' (meaning exempt), refers to all the mechanisms used by the body as protection against environmental agents that are foreign to the body (Benjamini *et al.*, 2000). This immune response involves a complex interaction between both cell-mediated and humoral immunity in association with components of innate immunity.

1.2.1 Innate immunity

Innate or 'non-specific' immunity is present from birth and refers to the body's natural ability to protect itself from invasion of foreign bodies. It allows primary defence through physiological and chemical barriers, while various specialised cells facilitate secondary responses. The skin acts as the major physical barrier and when intact, is impermeable to most infectious agents. Microbial or other foreign particles are also trapped within the mucous membranes present on respiratory and gastrointestinal tracts, which can be subsequently transported to external openings by epithelial cells or removed from the body by ciliary movement (*i.e.* coughing and sneezing). The acidic pH of sweat and sebaceous secretions and the presence of lysozymes in tears, nasal secretions and saliva also confer an antimicrobial effect. If an invading microorganism penetrates these physiological and chemical barriers, various internal components act as the next line of defence. Interferons and a variety of serum proteins (beta-lysin, lysozymes, polyamines, kinins), phagocytotic cells (granulocytes, macrophages and microglial cells) facilitate the destruction and elimination of foreign material from the body.

1.2.2 Acquired immunity

In contrast to innate immunity, acquired immunity is more specialised and specific and as the name suggests, is acquired following contact with a foreign invader. Following contact with this foreign agent (antigen), a chain of events is triggered which subsequently lead to the activation of cells (lymphocytes) and protein synthesis. This type of response can be sub-divided into two discriminate types, humoral and cell-mediated response, and involves three major cell types, B and T-lymphocytes and antigen-presenting cells (APCs). The function of APCs (macrophages and dendritic cells) is to process and present the antigen, in association with major histocompatibility complex (MHC) class I or II molecules, to specific receptors on T lymphocytes. These T lymphocytes, which mature in the thymus, are involved in cell-mediated response and there are several subpopulations, including $CD4^+$ helper (T_H1 and T_H2), $CD8^+$ cytotoxic (T_C) and natural killer T cells (T_{NK}). The activation of T cells requires both the binding of T cell receptors (TCRs) to antigen and the ligation of co-stimulatory molecules on T cells and APCs. Antigen presented on MHC class I participates in the activation of cytotoxic T cells (T_C), while antigen expressed on MHC class II results in activation of helper T cells (T_H). The resulting activated cells synthesise and release cytokines, which in turn come in contact with the appropriate receptors on different cells leading to a cascade of events.

The humoral immune response is mediated by Bursa-derived lymphocytes (B cells), which originate in the bone marrow. Approximately 10^5 B cell receptors (*i.e.* immunoglobulins (Ig)) of the same specificity are expressed on each B cell with each cell specific to a particular antigen or epitope (Benjamini *et al.*, 2000). This vast repertoire of antigenic specificities allows the immune system to respond to many different antigenic determinants. Specific antibodies are secreted into the circulation following binding of BCRs with their respective antigen and subsequent B cell activation. Thymus-independent (TI) antigens (*i.e* large proteins or antigens that contain multiple repeating epitopes) have the ability to crosslink BCRs and directly activate B cells independent of T cell activation. This type of response results in the synthesis of predominantly low affinity IgM antibodies, which do not give rise to memory cells (Roitt, 1994). However, thymus-dependent (TD) antigens (*i.e.* antigens that contain small discrete single epitopes) cannot crosslink BCRs by themselves and thus, require the co-operation of both B and T cells to elicit an immune response. This

is referred to as T-cell mediated humoral response, as illustrated in *Figure 1.1*. T cells are activated by APCs (*e.g.* dendritic cells and macrophages) in association with MHC class II complexes. B cells can also process and present antigens to helper T cells (T_H), following binding of their BCRs (*i.e.* Ig) to their specific antigen. This Ig-antigen complex is taken up by the B cell by phagocytosis, processed and presented on its extracellular surface in association with MHC class II molecules. This MHC II/antigen complex then interacts with $CD4^+$ T cells (T_H) bearing the appropriate TCR resulting in B cell activation. Other paired interactions at the surface of B and T cells, including LFA-1/ICAM-1, C5-5/CD72 and CD2/CD58 strengthen T-B cell interaction and enhance activation (Benjamini *et al.*, 2000). Subsequent to antigen binding T cells secrete cytokines, which in turn activate B cells to synthesise and differentiate into either antibody-secreting plasma cells or memory B cells. In contrast to plasma B cells, memory B cells are long-lived and can thus, respond quickly upon re-exposure to an antigen. The nature of these cytokines secreted also determines which Ig isotype the B cell synthesises, referred to as isotype-switching (*e.g.* cytokines released by T_H2 cells result in B cell class switch to IgE synthesis).

1.3 Antibody structure

Antibodies are soluble serum glycoproteins expressed in both secreted and membrane-bound forms in response to an invading foreign antigen. The molecules are also referred to as immunoglobulins (Ig) because of their globular structure. All immunoglobulins consist of a basic four-polypeptide chain structure of two identical heavy chains (each approx. 440 amino acids long) and two identical light chains (each approx. 220 amino acids long), which are held together by disulphide bonds. The heavy and light chains also contain intrachain disulphide bonds, which stabilise their folding into 110-amino acid domains. Sequence analysis has revealed that antibodies have one of two types of light chain, kappa (κ) or lambda (λ), and their ratio varies with species (mouse 95% κ, humans 60% κ) (Benjamini *et al.*, 2000). Different classes of heavy chains also exist and it is this variation in heavy chain that confers on the molecule its unique biological properties. The five major types of heavy chain are mu (μ), delta (δ), alpha (α), epsilon (ε) and gamma (γ), which give rise to the five different classes (isotypes) of immunoglobuins, IgM, IgD, IgA, IgE and IgG, respectively (Roitt, 1980).

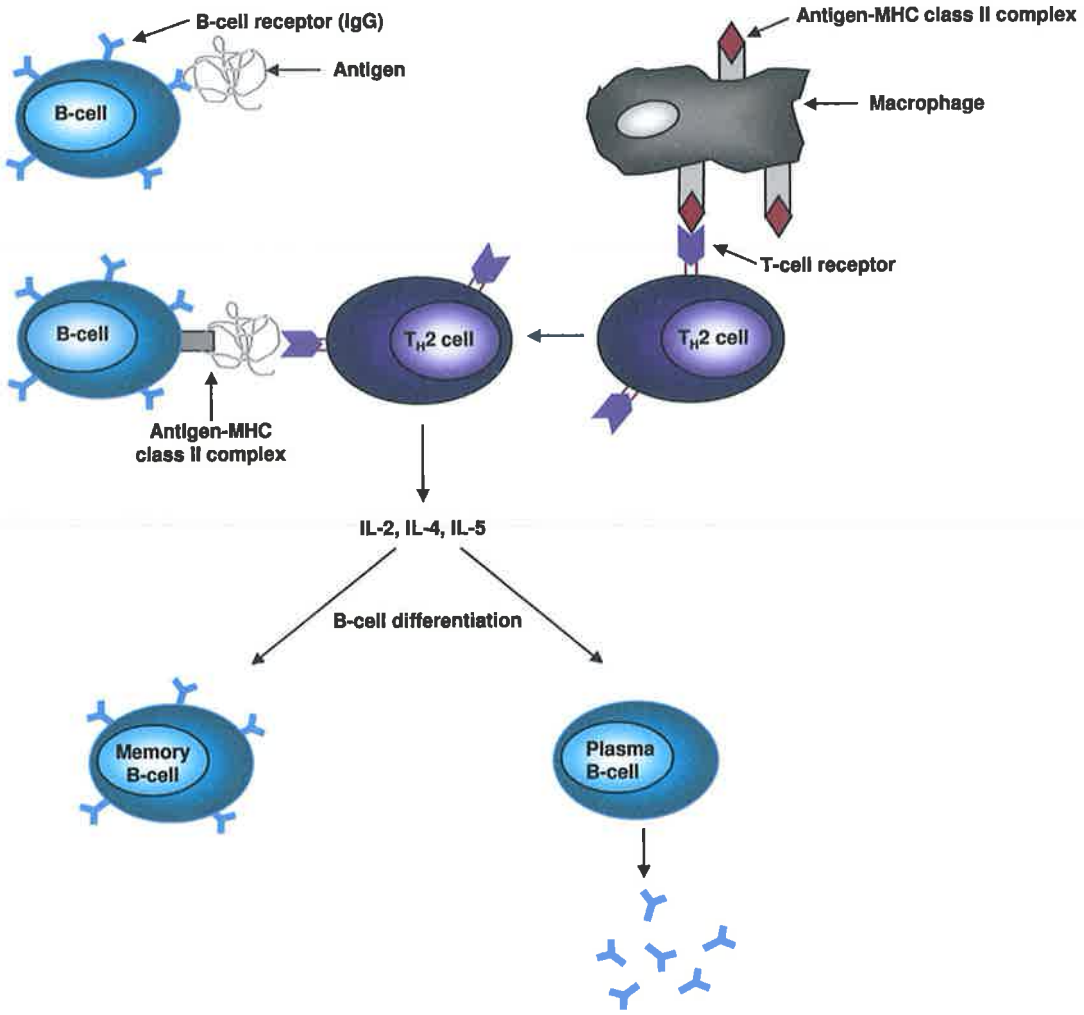


Figure 1.1. *Schematic illustration of T- cell dependent humoral immunity. Antigen is taken up by APCs (e.g. dendritic cells and macrophages) by phagocytosis, processed and presented on the cell surface in conjunction with MHC class II complex. B cells can also act as APCs to helper T cells (T_H), following binding of their BCRs (i.e. Ig) to their specific antigen. This MHC II/antigen complex then interacts with $CD4^+$ T cells (T_H) bearing the appropriate TCR resulting in B cell activation. Subsequent to antigen binding T cells secrete cytokines, which in turn activate B cells to synthesise and differentiate into either antibody-secreting plasma cells or memory B cells. In contrast to plasma B cells, memory B cells are long-lived and can thus, respond quickly upon re-exposure to an antigen.*

The IgM, is the first immunoglobulin synthesised following immunisation or exposure to T-independent antigens and, thus, elevated levels of IgM are generally an indication of either recent infection or recent exposure to antigen. Although they are associated with primary response, secondary response shifts from IgM to synthesis of IgG and other isotypes. The molecule, with a molecular weight of 900kDa and a half-life of five days, is a pentameric molecule composed of five subunits. Each of the subunits, containing two heavy and two light chains, are joined together by disulphide bonds between their Fc portions and a polypeptide chain, referred to as the J chain (Davey, 1992). IgM molecules have ten antigen-binding sites, as opposed to the other immunoglobulin classes, which have two (with the exception of dimeric IgA, which has four). However, the molecule has a valency of only five as a result of conformational constraints imposed by the polymerisation of the subunits. IgD (180kDa), a monomer, is not secreted by plasma cells and is therefore present at low concentrations (<0.4mg/ml) in serum (Roitt *et al.*, 1980). It is the most susceptible of the immunoglobulin to proteolytic degradation with a half-life of approx. 2.8 days (Benjamini *et al.*, 2000). IgD molecules are co-expressed with IgM on surface of mature B cells where they function as antigen-specific B cell receptor for the control of lymphocyte activation and suppression. The IgA molecule is the major immunoglobulin found in external secretions (*e.g.* saliva, mucus, sweat, gastric fluid and tears) and its purpose is to defend exposed external body surfaces against attack by microorganisms (Roitt *et al.*, 1980). It is also the major class of immunoglobulin found in colostrum and milk and it is thought to be responsible for the intestinal protection against pathogens in neonates (Benjamini *et al.*, 2000). The molecule has a half-life of 5.5 days and is present in both monomeric (160kDa) and dimeric forms (Benjamini *et al.*, 2000). The IgE (200kDa) has the shortest half-life (approx. two days) of all immunoglobulins and is present at the lowest concentrations (0.002% of total immunoglobulin) (Roitt *et al.*, 1980). It binds with very high affinity to receptors found on mast cells and basophils and plays an important role in immediate hypersensitivity reactions (allergic reactions).

The IgG molecule is the most abundant class of immunoglobulin in the serum of mammals and it is the major Ig synthesised after the secondary immune response. It accounts for 80% of the total immunoglobulins (Male *et al.*, 1996) and 15% of total protein found in humans (Benjamini *et al.*, 2000). It is also the most versatile of the

immunoglobulins and is capable of executing numerous biological functions, including neutralisation of toxins and viral activity, immobilisation of microorganisms and activation of complement. It is also the only class of Ig that has the ability to cross the placenta and, thus, enables a mother to confer her immunity to the foetus. The molecule, with a molecular weight of 150kDa and a half-life of approximately twenty-three days (Roitt *et al.*, 1980), is found in both intravascular and extravascular spaces. There are five subclasses of IgG. They differ in the arrangement of their disulphide bonds and also in additional structural features. The differences in the heavy chains ($\gamma1$, $\gamma2$, $\gamma3$, $\gamma4$) give rise to the IgG_1, IgG_2, IgG_3 and IgG_4 subclasses.

Although antibodies can vary in structure, they are generally typified by the immunoglobulin G (IgG) molecule. The IgG molecule consists of two Fab (antigen binding fragment) fragments and the Fc (crystallisable fragment). This Fc portion is linked to the two Fab domains by a short segment of amino acids between the C_H1 and C_H2 domains of the heavy chains called the hinge region. This region permits flexibility of the two Fab arms of the Y-shaped molecule, facilitating them to move to accommodate binding to two epitopes. The hinge region comprises of cysteine residues, which are involved in the formation of interchain disulphide bonds and proline residues, which prevent the region from folding into a globular structure (Benjamini *et al.*, 2000). The number of disulphide bonds varies within the IgG subclasses, from two in human IgG_1 (illustrated in *Figure 1.2*) to eleven in IgG_3 (Barbas *et al.*, 2001). Intrachain disulphide bonds are also present within the chains and facilitate stabilisation of domain folding.

The heavy and light chains of the antibody can be further divided into constant (C) and variable (V) regions based on their amino acid variability. While specificity is derived from variable regions, the constant regions are involved in effector functions of the immunoglobulins (*e.g.* binding to phagocytes, activation of complement). It is both this specificity and biological activity that makes their role crucial for the immune system. The variable regions, which are located at the N-terminal portions of both the heavy and light chains, form the antigen-binding fragment (Fab). Comparison of these variable (V_L and V_H) gene sequences showed the greatest amount of variability in regions referred to as the hypervariable regions (Kabat and Wu, 1971). These regions, which participate in antigen binding, are also referred to as

complementarity-determining regions (CDRs), as they form regions complementary in structure to antigen epitopes. It is the variation in the amino acid sequence of the CDRs that allows for the generation of a repertoire of antibodies that exhibit great diversity, each specific to a particular antigen. The CDRH3, which displays the greatest variation in terms of length and sequence, is considered (in conjunction with the CDRL3) to make the most significant contributions to affinity and specificity (Barbas *et al.*, 2001). The remainder of the variable heavy and light domains, referred to as framework regions (FRs), exhibit far less variation, and fold into relatively rigid beta strands that maintain the overall Ig structure.

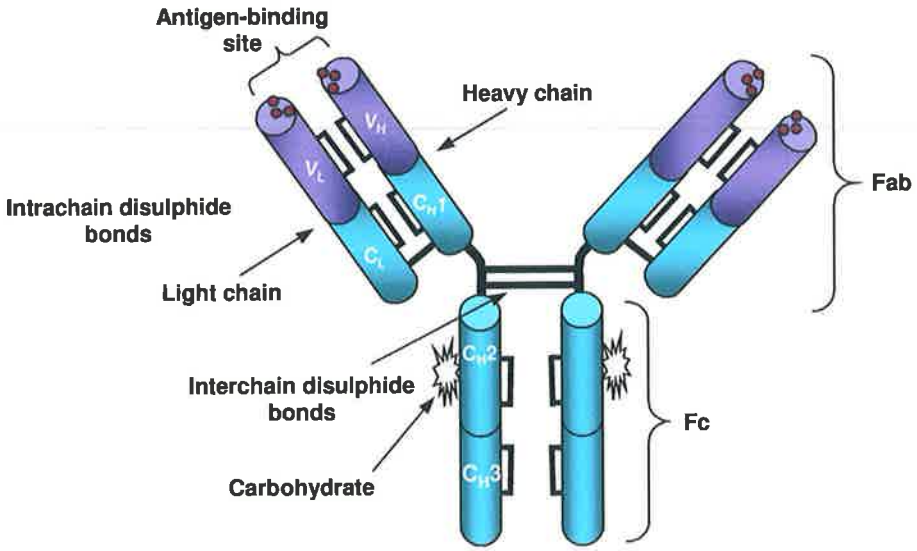


Figure 1.2. *Schematic representation of the basic structure of an immunoglobulin G (IgG_1) molecule. The molecule, with a molecular weight of approx. 150kDa, consists of two identical γ heavy chains (50kDa each) and two identical (κ or λ) light chains (25kDa each) held together by disulphide bonds. The antibody chains can be further divided into constant (C) and variable (V) regions based on their amino acid variability. The variable regions, which are located at the N-terminal portions of both the heavy and light chains, form the antigen-binding fragment (Fab). The crystallisable fragment (Fc) of the molecule, which contains the C_H2 and C_H3 domains, plays a number of important physiological roles in immunological responses. The two carbohydrate-binding domains, located on the C_H2 domain, also protect against proteolytic degradation and affect protein assembly, secretion and functional activity.*

1.4 Antibody synthesis and diversity

In humans, there is an estimated 10^{11} antibodies with different antigenic specificities (Janeway and Travers, 1999). This diversity is achieved without utilising an enormous number of germ line genes and instead is attained by a number of mechanisms, which allow for a small number of genes to generate a vast number of antibody (or Ig) molecules with different antigenic specificities. This method, referred to as genetic recombination, was first described by Dryer and Bennett (1965) and involves the movement and rearrangement of antibody genes within the genome of a differentiating B cells.

The light chain (κ or λ) of an antibody consists of two major domains, V_L and C_L. The amino-terminal V_L region (approx. 108 residues) is coded by two separate gene segments, a variable (V) segment that codes for 95 residues and a small joining (J) segment, which codes for 13 residues (Benjamini *et al.*, 2000). During DNA rearrangement, one of these V genes is combined with one J gene to produce a gene unit, which together with the C region codes for the entire Ig light chain. This unique gene rearrangement is referred to as V(D)J recombination. The genes coding for the κ chains, referred to as the κ locus, are located on chromosome two and there are approximately 40 different κ variable genes, each of which can code for the 95 N-terminal amino acids of the κ variable region. These Vκ genes are arranged linearly, each with its own leader sequence and are separated by introns. Downstream of this region there are a series of 5 Jκ gene segments, each encoding the remaining 13 amino acids of the κ variable region. At the carboxy-terminal is a single gene segment coding for a single constant region of the κ chain (Cκ). In the early stages of B cell lineage, a cell selects one of Vκ genes from its DNA and combines it to one of the Jκ segments in a random process. Following transcription, the primary RNA transcript is spliced to remove all intervening non-coding sequences to join the Vκ, Jκ and Cκ exons together. The resulting mature mRNA is then translated into the κ polypeptide chain and following cleavage of the leader sequence, is free to join with a heavy (H) chain to form an Ig molecule (*Figure 1.3*). The synthesis of λ chains is similar to that of the κ chains and involves the rearrangement of DNA, which joins a V_λ gene with a J_λ gene segment. The genes coding for the λ chains, referred to as the λ locus, are located on chromosome 22 in humans and consists of approximately 40 different λ V genes and 4 J_λ genes (Benjamini *et al.*, 2000). The J_λ genes are associated with a

different C_λ gene, however there are four different types of C_λ polypeptides in humans.

In contrast to the light chain, the variable region of a heavy chain is constructed from three (V_H, D_H, and J_H) rather than two gene segments. The D (diversity) and J segment code for the amino acid residues in the third complementary determining region (CDR3) of the heavy chain. The heavy chain locus is located on chromosome 14 in humans and consists of approximately 50 V_H, 20 D_H and 6 J_H gene segments (Benjamini *et al.*, 2000). In the early stages of the life of a particular B cell, two rearrangements of the germ line DNA must occur, the first joining one D segment with one J segment and the second bringing one V segment next to a DJ unit. The rearranged DNA is then transcribed with the closest C regions genes μ and δ. The primary RNA transcript is then alternatively spliced to form either a μ or δ polypeptide.

In addition to the rearrangement of V, D and J genes in different combinations, diversity in antibody specificity is also achieved by random assortment of heavy and light chains, junctional and insertional diversity and somatic hypermutation following stimulation of B cells by antigen. Further DNA rearrangement of VDJ genes with different C regions genes occurs during Ig class (or isotype) switching. Primary RNA transcripts are made from rearranged DNA and non-coding regions are spliced out resulting in translation of the resulting mRNA into L and H chains of IgM and IgD. Following antigenic stimulation in mature B cells and the synthesis of cytokines by T cells, a B cell with a VDJ unit linked to a constant gene (μ and δ) can further arrange its DNA to link to an S (switch) region in front of another C region (Cγ, Cα, or Cε) and the intervening C region is removed (Li *et al.*, 2004). This is referred to as isotype-switching and the resulting B cell that was synthesising either IgM or IgD switches to synthesise antibodies of a different isotype (IgG, IgA or IgE) but with the same antigenic specificity.

The lack of precision by which DNA is rearranged leads to deletions or substitutions of amino acids (referred to as junctional diversity) and thus, resultant changes in the antigen-binding site (Feeney *et al.*, 1994). Insertional diversity (the insertion of nucleotides), mediated by the enzyme terminal deoxynucleotidyltransferase (TdT) can

also occur at the VD and DJ junctions. Mutations that occur in heavy or light chain V genes during the lifetime of a B cell can also increase the variety of antibodies synthesised by the B cell population. Subsequent to secondary stimulation by an antigen, an increase in antigen affinity occurs resulting from point mutations in the V(D)J recombined unit. This is referred to as somatic hypermutation as it occurs at a rate at least 10,000-fold higher than the normal rate of mutation (Jacob *et al.*, 1993).

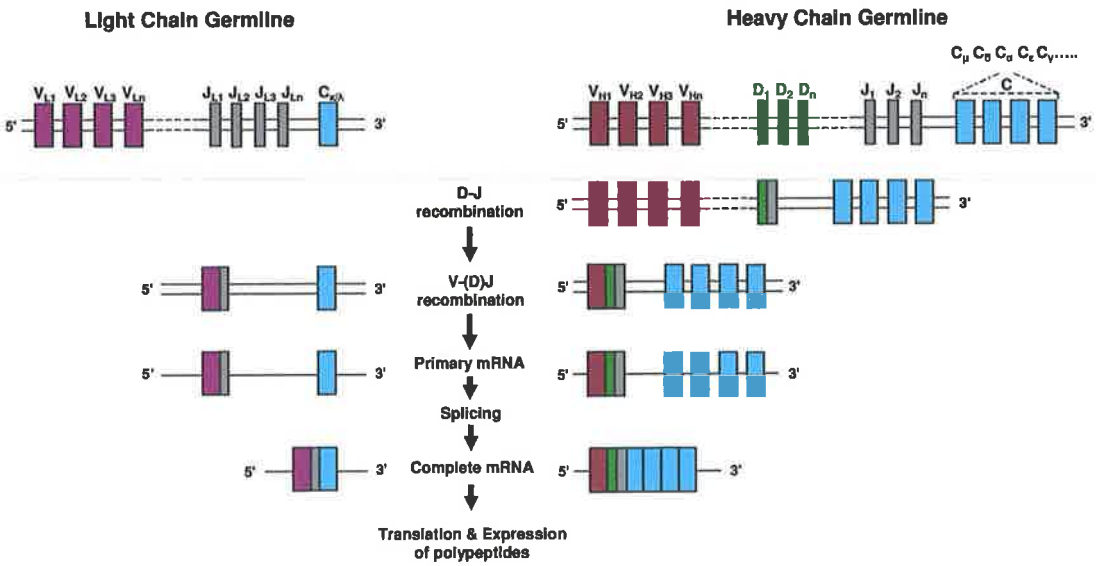


Figure 1.3. *Schematic representation of somatic recombination in humans. The variable region of an Ig light chain is constructed by joining one V_L gene segment to one J_L gene segment. This VJ unit is joined to the constant regions (κ or λ) following processing of primary RNA transcript. The variable region of the heavy chain is constructed from three separate genes, referred to as V_H (variable), D_H (diversity) and J_H (joining) gene segments, while a distinct gene segment codes for the constant region of the heavy chain (C_H). The VDJ unit, which codes for the entire variable region of the heavy chain, is linked to the C_H region. Primary RNA transcripts are made from the rearranged DNA and non-coding RNA is spliced out, resulting in mRNA for light and heavy chains, which are then translated into the H and L chain polypeptides that associate to form a complete antibody (Ig) (adapted from Janeway et al., 1999).*

1.5 Antibody production

1.5.1 Antigens

An antigen or immunogen is defined as a molecule that contains distinct sites or epitopes that are recognised and interact with various components of the immune system. Forces involved in antibody-antigen binding are weak, noncovalent interactions involving hydrogen bonds, ionic bonds, hydrophobic interactions and Van der Waals interactions (Tijssen, 1985). The strength of interaction of an antibody with its corresponding antigen (*i.e.* affinity) generally increases with repeated exposure to antigen. However, not all antigens elicit the same immune response and the degree of response varies from one antigen to another. In general, proteins and large molecules are capable of eliciting good immune responses with the aid of non-specific immune stimulants, referred to adjuvants. The most commonly used adjuvants are Freund's complete and incomplete adjuvants, FCA and FICA, respectively. FCA, which is only used for initial immunisation, consists of heat-killed *Mycobacterium tuberculosis*, non-biodegradable mineral oils and an emulsifier. The mycobacteria cause an inflammation, which attracts macrophages and other cells to the site of injection, where the antigen (emulsified in the oil) is released slowly to give a prolonged exposure to the immune system. To minimise side effects in the animal, FICA, which contains no bacteria, is used for subsequent boosts. Other adjuvants include Hunter's TiterMax, liposomes, the RIBI adjuvant system (RAS) and *Bordetella pertussis*.

Unlike larger proteins, smaller molecules or haptens, (<5kDa) are often too small to elicit an immune response on their own and, therefore, require chemical conjugation to larger carrier molecules to render them immunogenic. Various carrier proteins, including bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH) and thyroglobulin (THY), are commonly used for this purpose. However, other carriers composed of synthetic or natural polymers (*e.g.* dextran, poly-L-lysine), lipid bilayers or synthetic organic molecules have also been used. Regardless of the type of molecule, a carrier must be highly immunogenic, have the required solubility properties, be non-toxic *in vivo* and possess suitable functional groups for coupling to the hapten (Hermanson *et al.*, 1996).

Conjugation is a crucial step in antibody production as the specificity of the resultant antibodies is dependent on the hapten used to produce them. The choice of method is governed by the functional groups available on both the hapten and the carrier and the orientation of the hapten desired for presentation to the immune system. However, a major problem associated with the use of conjugates for antibody production is that antibodies will be produced against the hapten, the carrier and the linker molecule. To reduce the production of non-specific antibodies and direct the production of antibodies to the hapten rather than the carrier, the number of haptens conjugated to each carrier molecule can be optimized. The use of conjugation procedures employing EDC (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride), does not introduce a bridging molecule between the hapten and carrier, thus, eliminating the potential of antibodies being generated against the coupling reagent.

1.5.2 Polyclonal and monoclonal antibodies

Repeated immunisation of an animal with a multivalent antigen yields multispecific or polyspecific antiserum. This resulting antiserum contains a heterogeneous mixture of antibodies, each recognising different epitopes of the immunogen with different specificities and affinities (Davey, 1992). These antibodies are referred to as polyclonal antibodies. Animals, such as rabbits, goats, guinea pigs and sheep, are generally used for polyclonal antibody production, as they are relatively easy to handle for immunisation and bleeding purposes. Polyclonal antibodies can be produced quickly and relatively cheaply and do not require the same amount of expertise or time as monoclonal antibody production. They can also be very specific and high concentrations can be purified from relatively small amounts of serum. Due to the heterogenous nature of the population, the affinity of the antibodies cannot be determined. Instead the avidity, which gives an indication of average affinity value for the mixture of antibodies in a polyclonal population, can be estimated (Davey, 1992). This heterogenicity of the antibody population may lead to high levels of cross-reactivity with structurally related molecules and can also be hard to reproduce in subsequent immunisations. Unlike monoclonal antibody production, a consistent source of antibodies cannot be generated.

The use of hybridoma technology to produce monoclonal antibodies was first reported by Köhler and Milstein (1975). The antibody molecule is derived from a single clone

of B-cells with each secreted antibody having a single defined specificity and affinity for the target antigen. The hybridoma technique involves the repeated immunisation of an animal with the antigen of interest, the spleen is then removed and the splenocytes are harvested and fused with myeloma cells (tumour B-cells). These tumourigneic B-lymphocytes can survive for long periods in culture and confer immortality to the resulting hybrid cell, while the spleenocytes provide the antigen specificity. This resulting fusion product or hybridoma is thus, an immortal cell line, which has the ability to secrete a homogenous antibody population with the desired specificity. The selection of only antibody-secreting hybridomas is achieved with the use of HAT (hypoxanthine, aminopterin and thymidine)-supplemented media and myeloma cells that are deficient in the enzyme hypoxanthine guanidine phosphoribosyl transferase (HGPRT). The HGPRT-deficient myeloma cells are unable to proliferate in the presence of HAT medium. The presence of aminopterin blocks the cells ability to synthesis DNA through the *de novo* synthesis pathway and the cells cannot utilise the salvage pathways for DNA production because of their HGPRT deficiency (Campbell, 1984). This enzyme deficiency, therefore leads to the elimination of unfused myeloma cells. However, spleenocytes possess the required enzyme to produce DNA through the salvage pathway but are unable to proliferate outside the body and thus die off naturally. Fused cells (hybridomas) will proliferate in HAT media as they have the ability to use the salvage pathway for DNA synthesis and the characteristic to grow continuously from the myeloma cell.

An initial ELISA screening method allows for the identification of hybridomas secreting the desired antibody and then individual hybridomas are isolated from the heterogenous mixture by a technique referred to as cloning by limiting dilution. This is a critical step in monoclonal antibody production as it facilitates the elimination of non-secreting hybridomas so they cannot outgrow their antibody secreting counterparts. Limiting dilution involves seeding the cells at very low densities (≤ 1 cell/ml) in a 96-well plates. The resulting supernatants from each well are then tested for specific antibody activity and positive wells are divided and sub-divided to ensure monoclonality of the cell line.

1.5.3 Recombinant antibodies

Hybridoma technology has facilitated the production of large amounts of homogenous antigen-specific rodent-derived antibodies for use as diagnostic and therapeutic agents. This technique has indeed proven invaluable but there are a number of drawbacks. Hybridomas are sometimes difficult and expensive to maintain in culture. However, the main disadvantage with this technology is the difficulty in producing large quantities of stable human monoclonal antibodies, due to the absence of suitable myeloma cell lines. These disadvantages and other factors prompted the development of recombinant antibody technology.

Genetic engineering allowed the development of the first generation of humanized monoclonal antibodies, in which the antigen binding loops (CDRs) of the murine monoclonal antibodies were grafted onto a human IgG molecule (Riechmann *et al.*, 1988). A second generation of humanized antibodies, referred to as chimeric antibodies, soon followed. The chimeric antibodies consist of variable regions of a murine monoclonal antibody linked to the constant regions of a human IgG molecule (Morrison *et al.*, 1989). These constructs were generated in an attempt to circumvent the problem of inducing human anti-mouse antibody (HAMA) responses when murine monoclonal antibodies are used as therapeutic agents in human patients. However, they did not fully overcome this problem and it was not till much later with the development of recombinant antibody technology that the production of genetically-derived human monoclonal antibodies was possible.

Recombinant antibody technology has allowed the generation of monoclonal antibodies from several species including humans (Griffiths *et al.*, 1993; Vaughan *et al.*, 1996), chickens (Andris-Widhopf, *et al.*, 2000; Finlay *et al.*, 2005), sheep (Li *et al.*, 2000), camels (Arbabi Ghahroudi *et al.*, 1997), sharks (Dooley *et al.*, 2003) and mice (Krebber *et al.*, 1997). Advances in molecular biology, facilitated by the introduction of techniques such as polymerase chain reaction (PCR), have allowed the creation of large repertoires of antibodies from antibody variable genes bypassing hybridoma technology and sometimes, even immunisation. These libraries, combined with phage display techniques, attempt to reproduce the diversity of the immune system by displaying millions or even billions of antibody fragments on the surface of phage (Hoogenboom *et al.*, 1998). This technology has now made it possible to select

an antibody to almost any antigen from an antibody repertoire with a large number (greater than 10^8) of clones.

Prior to the development of recombinant technologies, antibody fragments could only be generated by proteolytic cleavage. Pepsin digestion of the IgG molecule produced a $(Fab')_2$ fragment (Nisonoff, 1960), while papain digestion resulted in the generation of single Fab fragments (Porter, 1959). However, with the advent of recombinant antibody technology years later, the generation of a number of antibody fragments, including single chain variable fragments (scFv) (McCafferty *et al.*, 1990) and Fab fragments (Kang *et al.*, 1991) was possible. The Fv fragment is the smallest fragment of an antibody that still contains the complete antigen site. However, the fragment, consisting of the variable domains of the light and heavy chain, can be quite unstable, as it lacks the disulphide bond present in the Fab fragment (Killard *et al.*, 1995). This problem has been overcome by protein engineering. Disulphide bonds can be formed by the introduction of cysteine residues into the variable domains producing a disulphide-stabilized Fv fragment (dsFv) (Brinkmann *et al.*, 1993). Stability problems were also overcome by incorporating a flexible peptide linker of approximately 15-20 amino acids into the Fv fragment resulting in a more stable single chain variable fragment (scFv) (Freund *et al.*, 1993). Smaller antibody fragments, which have the ability to bind antigen, can also be engineered; these include the Fd fragment, consisting of a V_H and C_H1 domain, and the complementarity determining region (CDR). A schematic representation of the antibody fragments that can be produced by enzymatic or genetic means is shown in *Figure 1.4.*

Recombinant antibody technology has several advantages including the speed of antibody production, the possibility of altering affinity and specificity and the ability to generate novel functionalities. Once the system has been set up in the laboratory it can take weeks rather than months to produce an antibody fragment of the desired specificity. The emergence of this technology has made it possible to generate high binding affinity molecules against any chosen target, resulting in potentially limitless applications. Recombinant antibodies have already contributed greatly in the analytical and diagnostic fields. They are also ideal for therapeutics where small rapidly penetrating but high affinity molecules are essential (Arakawa *et al.*, 2002; Suzuki *et al.*, 2003).

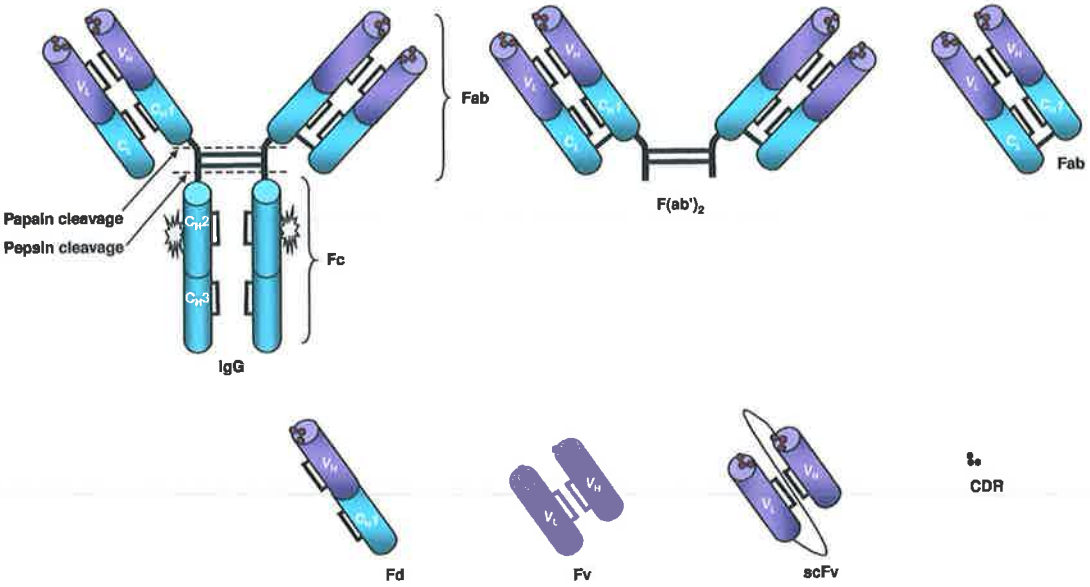


Figure 1.4. *Schematic illustration of an antibody molecule (IgG_1) and its various fragments produced through enzymatic or genetic means. Pepsin cleavage of the molecule produces a $F(ab')_2$ fragment, which consists of two disulphide-linked antigen-binding fragments. Papain digestion yields two single Fab fragment with each containing one antigen-binding fragment. Recombinant antibody technology has facilitated the generation of Fab fragments and smaller Fv fragments, consisting of the variable domains of the antibody. Incorporation of a hydrophilic peptide linker into the fragment by genetic means produces a single chain variable fragment (scFv). Other fragments include the Fd fragment, consisting of a V_H and C_H1 domain, and the CDRs, the smallest fragment capable of binding antigen.*

1.5.3.1 Phage display technology

The most widely used methodology for recombinant antibody library production utilises filamentous phage, a bacteriophage that infects *Escherichia coli*. The principle of phage display technology was first introduced by Smith (1985), who showed that foreign DNA fragments could be inserted into non-lytic filamentous bacteriophage for the display of specific binding peptides on the surface of the phage. The filamentous bacteriophages (genus *Inovirus*) are a group of viruses, which contain a circular single-stranded DNA (ssDNA) genome encased in a long protein capsid cylinder (Barbas *et al.*, 2001). The Ff class of filamentous phage (f1, fd and M13) are routinely used in phage display. These phage particles are covered by approximately 2,700 molecules of the major coat protein pVIII, as illustrated in *Figure 1.5*. At one end of the particle there are approximately five copies of the minor hydrophobic coat proteins pVII and pIX. The other end of the particle contains approximately five copies of each of the minor coat proteins pIII and pVI. (Barbas *et al.*, 2001). The filamentous phage infect male *E. coli* containing the F conjugative plasmid by attaching to the tip of the F pilus, encoded by the plasmid, and translocating the phage genome into the bacterial cytoplasm.

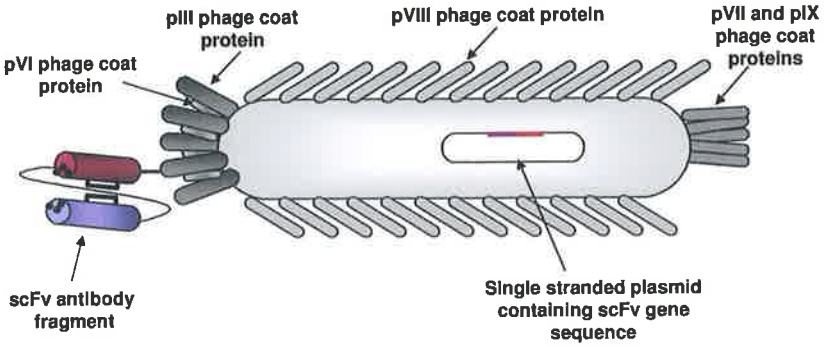


Figure 1.5. *A diagrammatic representation of the Ff filamentous bacteriophage particle routinely used in phage display. The phage particle is covered by approximately 2,700 molecules of the major coat protein pVIII. At one extremity of the particle there are approximately five copies of the minor hydrophobic coat proteins pVII and pIX, while at the other extremity there are approximately five copies of each of the minor coat proteins pIII and pVI. The antibody fragment is displayed as a fusion to the pIII protein at the tip of the phage.*

The phage display method involves cloning the genes encoding an antibody fragment into a particular vector, as a fusion to the gene encoding one of the phage coat proteins (pIII or pVIII). Originally complete phage vectors were used as the display vector but now small plasmid vectors or phagemids, which contain the appropriate packaging signal and cloning sites, are more commonly used (Daly *et al.*, 2001). Phagemids encoding the antibody fragment-pIII fusion are then transformed into male *E. coli* cells harbouring the F pilus (*e.g.* XL-1 blue or TG1) and packaged into phage particles using a particular helper phage (*e.g.* M13K07 or VCS-M13). The resulting antibody fragment is expressed on the surface of the phage, while its genetic material resides within the phage particle, providing a direct link between antibody genotype and phenotype.

1.5.3.2 Phage-display library production

The first step in the construction of a combinatorial antibody display library is to obtain the genes encoding for an antibody fragment. These genes can be isolated from B cells of immunised animals or from conventional hybridomas (immune libraries) (Krebber *et al.*, 1997) or from non-immunised donors (naïve libraries) (Vaughan *et al.*, 1996). Alternatively, antibodies may be constructed artificially, by the *in vitro* assembly of V-gene segments and D/J segments (synthetic libraries) (Knappik *et al.*, 2000). Naïve or semi-synthetic libraries offer the possibility of selection of antibodies without the need for immunisation. A number of very diverse, semi-synthetic, non-immune human antibody libraries have been generated by Cambridge Antibody Technology (Cambridge, UK) and MorphoSys AG (Munich, Germany) and many others exist. If human antibodies are required for therapeutic purposes, these human libraries are the obvious choice. However, if a range of antibodies against a particular molecule is desired, immunisation may be the best option. Mice (in particular the Balb/c strain) have become very popular for immunisation and an extensive range of PCR primers, in addition to methods for construction of murine libraries have been widely reported (*e.g.* the Krebber system). However, the considerable sequence variation in the amino terminus of the murine heavy chain requires the use of numerous PCR primers to capture a good representation of the antibody repertoire. Chicken and rabbits use very few germ-line genes and, thus, cloning of the antibody repertoire reduces the number of PCR primers required.

The variable antibody genes, which should contain a full repertoire of antigen specificities, are first amplified using suitable primers. They are then randomly combined (to yield a combinatorial antibody library) by splice by overlap extension-PCR (SOE-PCR), inserted onto a suitable plasmid and applied to one of a variety of expression systems, including yeast (*e.g. Pichia pastoris*) (Freyre *et al.*, 2000), bacterial cells (*e.g. E. coli*) (Krebber *et al.*, 1997), plant cells (*e.g. Nicotiana tabacum*) (Owen *et al.*, 1992), mammalian cells (*e.g.* COS cells) (Jost *et al.*, 1994) and Bacullovirus-infected insect cells (Kretzschmar *et al.*, 1996). *E. coli* is one of the most popular expression hosts used as it is easily manipulated and its fast growth allows for the large-scale production of proteins. Although many phage display vectors are available, those vectors used in the research presented in this thesis (*i.e.* the pAK and pComb3 vector series) are discussed in detail.

1.5.3.3 The pAK vector series

A phage display system for the generation of single chain antibody fragments (scFv's) from hybridoma or spleen cells was developed by Krebber *et al.* (1997). The Krebber system has been successfully employed for the generation of scFv's specific to a range of analytes, including, aflatoxins (Daly *et al.*, 2002; Dunne *et al.*, 2005), illicit drugs (Brennan *et al*, 2003), antibiotics (Darmanin Sheehan *et al.*, 2006) and *Listeria monocytogenes* (Leonard *et al.*, 2003).

The system was optimised for robustness, vector stability, tight control of scFv-Δgene III expression, primer usage for PCR amplification of variable genes, scFv assembly strategy and subsequent directional cloning using a rare single cutting restriction enzyme (*Sfi*I). A schematic representation of the Krebber system is given in *Figure 1.6.* The mRNA, encoding the antibody genes, is isolated from spleen cells of mice immunised with the target antigen or from hybridomas cell secreting specific antibodies. This mRNA is then reverse transcribed to complementary DNA (cDNA) using random hexamer primers. This cDNA serves as a PCR template for the subsequent amplification of variable and heavy light chain genes using sets of mouse primers. The set of primers described by Krebber et al. (1997) incorporates all murine V_H, V_κ and V_λ sequences from the Kabat database (Kabat *et al.*, 1991) and combines extended primer sets described by Kettleborough *et al.* (1993). Amplified variable

genes are then randomly combined by splice by overlap extension-PCR (SOE-PCR) in the format of V_L-$(Gly_4Ser)_4$-V_H, where the four (Gly_4Ser) repeats encode the 20-amino acid flexible linker linking the variable domains of the scFv fragments. Antibody fragments are then cloned into the optimised phage display vector pAK100 with the rare-cutting enzyme *Sfi*I. The restriction enzyme recognises eight bases, interrupted by five non-recognised nucleotides (5' GGCCNNNNNGGCC 3') and always cuts two sites at once, leaving a 3 base pair over-hang, to prevent self-annealing and facilitate directional cloning of digested fragments in the correct orientation. *Sfi*I restriction sites are virtually never found in immunoglobulin sequences and thus, eliminate the potential of internal digestion of sequences. Following ligation of the digested fragments into a previously digested phage display pAK100 vector, the vector is then transformed into male *E. coli* cells harbouring the F pilus (*e.g.* XL1-Blue). High ligation and transformation yields are essential in phage display library production and the size of an antibody library can be restricted by the efficiency of its introduction into *E. coli*. Thus, electroporation, the most efficient method for transforming bacterial cells with plasmid DNA, is generally used. The transformed cells are then amplified and the phagemid DNA is rescued following infection with helper phage (*e.g.* M13K07 or VCS-M13). This complex process, referred to as phage rescue, involves the entry of helper phage into the bacterial cell where it packages the DNA from the phagemid (encoding the antibody genes) and emerges from the cell displaying antibody fragments.

A compatible vector series (pAK100-600) (*Figure 1.7*) was also constructed by Krebber and colleagues to facilitate expression of the scFvs and their subsequent purification, detection, multimerisation and modification. All vectors contain the *lac*I repressor gene (to ensure independent *lac* promoter repression), the *pel*B (pectate lyase gene of *Erwinia carotovora*) leader sequence (which allows for proper folding of proteins and relocation of expressed antibody to periplasm), the *lac* promoter/operator (which allows for repression of translation by the addition of glucose), a strong upstream promoter (t_{HP}) (which in combination with the *lac* promoter eliminates background expression prior to induction) and a downstream termintor (t_{lpp}). Vectors also contain a chloramphenicol resistance gene (camR) and a tetracycline resistance 'stuffer' gene, which is replaced with the genes encoding the scFv fragment.

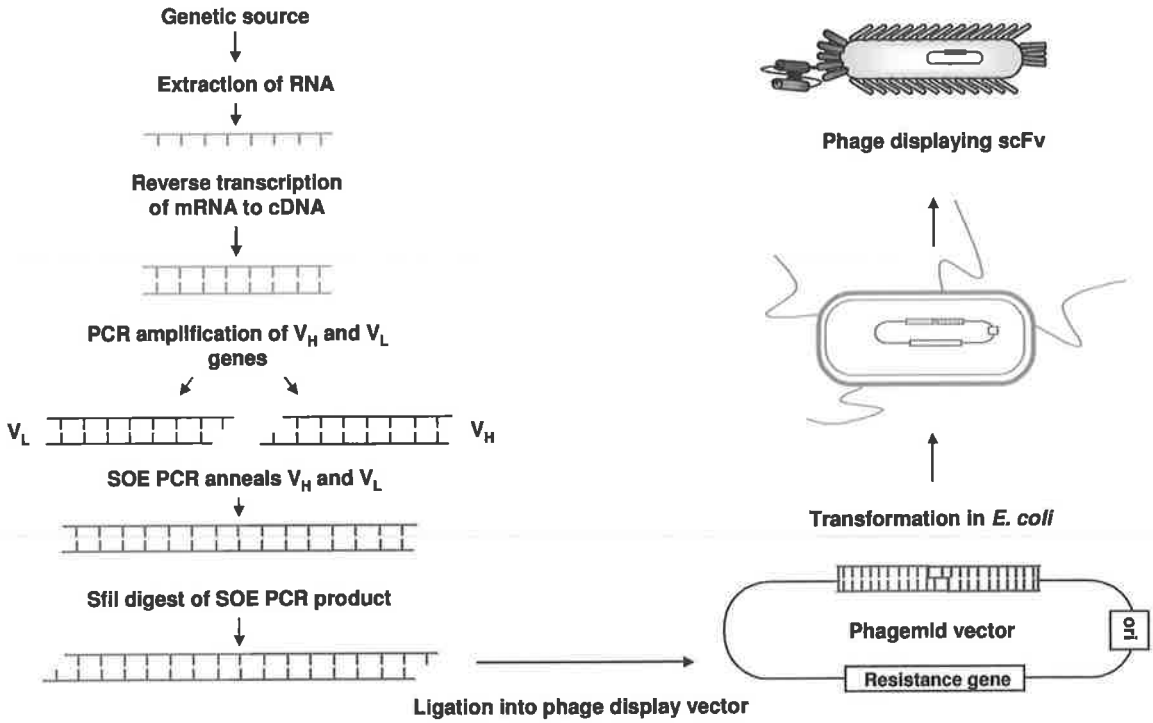


Figure 1.6. *Schematic representation of the steps involved in the generation of a phage display antibody library. Isolated mRNA is reverse transcribed to cDNA, which serves as a template for subsequent amplification of variable heavy (V_H) and light (V_L) chain genes. Amplified variable genes are then randomly combined by splice by overlap extension-PCR (SOE-PCR) and cloned into a phage display vector. The phagemid vector encoding the scFv gene sequence is transformed into E. coli and following infection with helper phage is displayed on the surface of the phage particles.*

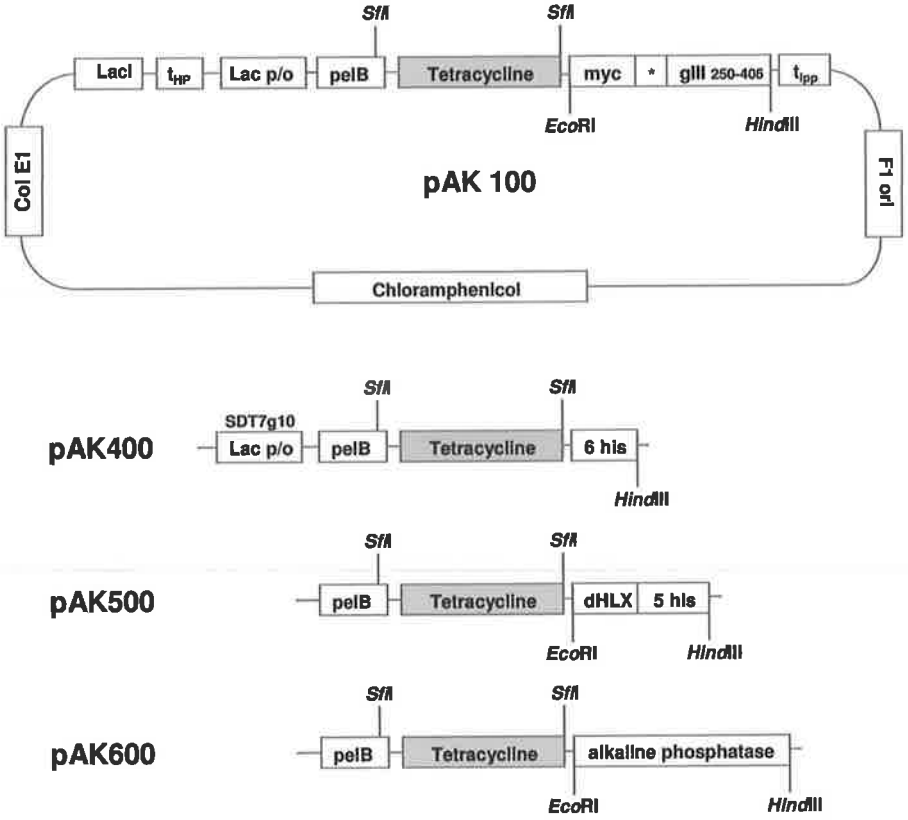


Figure 1.7. *The antibody fragment is cloned into the pAK100 vector, in frame with gene$III_{250-406}$, to facilitate phage display. The vector also contains a myc tag for detection and an amber codon, which allows switching between phage-bound and soluble expression by simply changing bacterial strain (suppressor or non-suppressor E. coli). The pAK400 facilitates enhanced scFv expression levels due to the strong Shine Dalgarno sequence SDT7g10 (from T7 phage gene 10) and a C-terminal hexahistidine tag allows for purification using immobilised metal affinity chromatography (IMAC) (Wels et al., 1992; Lindner et al., 1997) or for detection using an anti-his tag antibody. The pAK500 vector contains a single chain double helix (dHLX) for dimerisation, which can result in a homodimerised minibody with increased avidity for antigen (Pack et al., 1993). The pAK 600 vector encodes the bacterial alkaline phosphatase (AP) gene to enable direct detection and facilitate dimerisation by scFv-AP fusions (Lindner et al., 1997; Wels et al., 1992).*

1.5.3.4 The pComb3 vector series

The pComb3 phage display system (Rader and Barbas III, 1997; Barbas *et al.*, 2001) facilitates the generation of human Fab or mouse, chicken or rabbit scFv fragments. The choice of antibody construct is generally dependent on its final application. In comparison to the generation of Fab fragments, the production of scFv fragments requires fewer PCR steps. The smaller scFv molecules also tend to yield higher expression levels in *E. coli* than larger Fab fragments (Barbas *et al.*, 2001). Shortening the length of the peptide linker between the variable heavy and light chains of the scFv fragment can also lead to dimer formation (diabodies) and resulting enhanced avidity for antigen. While Fab fragments in some cases are more stable and do not generally show a tendency to multimerise (Borrebaeck *et al.*, 1992). The system, as described by Barbas *et al.* (2001) also facilitates the humanisation of antibody fragments by linking their respective variable regions to human constant regions, while avoiding loss of antigen binding activity of the variable regions.

The pComb3H (Rader and Barbas III, 1997) and pComb3X (Barbas *et al.*, 2001) were constructed to overcome a number of stability problems encountered with the original pComb3 vector (Barbas III *et al.*, 1991). The vectors allow for the directional cloning of antibody fragments in the correct orientation with the enzyme *Sfi*I and subsequent expression as fusions to the *omp*A leader sequence. The pComb3X vector has all the features of pComb3H, along with several additional ones (*Figure 1.8*). The pComb3X vector encodes both hexa-histidine (His_6) and influenza hemagglutinin (HA) peptide tags at the C-terminal of the antibody sequence, facilitating purification and detection of expressed antibody fragments. The vector also encodes an amber stop codon (TAG) at the junction of the regions encoding the HA tag and pIII protein, allowing for the expression of antibodies in their soluble form simply by changing the strain of *E. coli* used for expression. Phage propagation in suppressor strains (*e.g.* XL1-Blue) allow for the production of antibody fragments fused to the pIII protein (encoded by gene III) for phage display. However, in male non-suppressor strains (*e.g.* Top10F') this stop codon will be read and thus, soluble antibody (*i.e.* detached from pIII protein) will be produced.

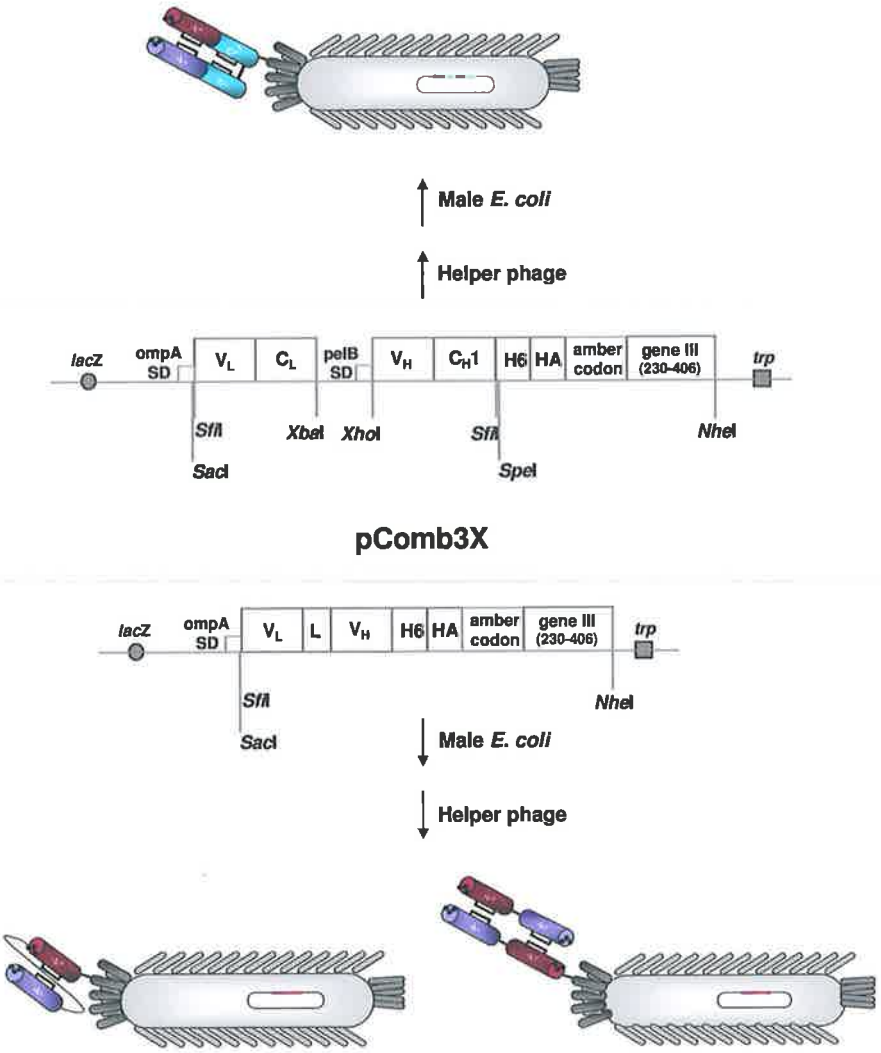


Figure 1.8. *Schematic representation of the pComb3X vector for phage display of scFv, diabody or Fab fragments. The vector is designed to express the antibody fragments on the surface of filamentous phage, where the antibody fragments are fused to the carboxy terminal domain of the minor coat protein pIII. The presence of two Shine-Dalgarno (SD) sequences facilitate ribosomal binding and subsequent translation of the heavy and light chains of the Fab fragment, which are directed to the periplasm for assembly by the pelB and ompA signal peptides, respectively. The amber codon allows for soluble antibody expression in non-suppressor strains of bacteria and the histidine (His_6) and hemaglutinin (HA) peptide tags facilitate purification and detection. Transcription is terminated via the transcription terminator (trp).*

1.5.3.5 Selection procedures for isolation of phage-displaying antibodies

Selection of phage-displaying antibody fragments involves the enrichment of specific binding phage from a large excess of non-binding clones. This selection process is generally performed on antigen-immobilised surfaces (*i.e.* 'biopanning') for the isolation of antibody fragments from phage display libraries. However, other methods such as selection on eukaryotic cells (Rader and Barbas, 1997) or tissue sections (Van Ewijk *et al.*, 1997), may be required.

Biopanning can be performed on antigen-coated immunotubes (Krebber *et al.*, 1997), single microtitre wells (Barbas *et al.*, 2001), immunoaffinity columns (Hoogenboom and Winter, 1992) or Biacore chip surfaces (Marks *et al.*, 1991, Malmbourg *et al.*, 1996). The phage repertoire are incubated with the immobilised antigen of interest and non-specific unbound phage are eliminated by stringent washing (*Figure 1.9*). Specific bound phage can be retrieved by elution with acidic (Krebber *et al.*, 1997) or basic (Marks *et al.*, 1991) solutions; with excess competing antigen (Clackson *et al.*, 1991; Burmester *et al.*, 2001); by passive bacterial elution (Wind *et al.*, 1997) or following enzymatic cleavage of a protease-sensitive site engineered between the antibody and gene III sequence (Ward *et al.*,1996; Barbas *et al.*, 2001). Eluted phage are infected into bacteria, which are amplified prior to phage rescue by coinfection with helper phage. The resulting phage particles are precipitated from the culture supernatant and are subjected to the next round of selection. Normally three to four rounds of selections are performed to enrich for specific phage (Kontermann *et al.*, 2001) and positive binders can be monitored throughout the process by ELISA.

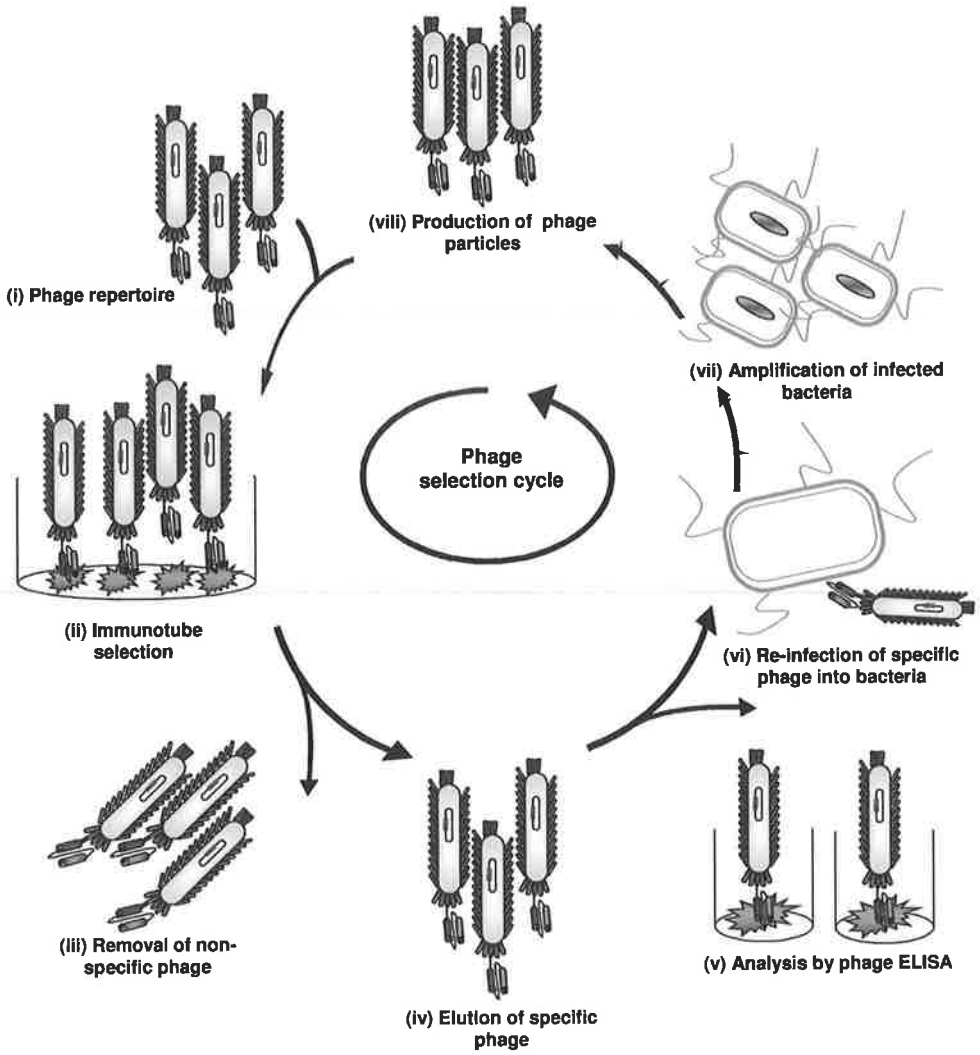


Figure 1.9. *Diagrammatic representation of steps involved in the 'biopanning' of combinatorial phage-displayed libraries.* ***(i)*** *The phage repertoire* ***(ii)*** *is incubated with the antigen of interest immobilised on a solid support.* ***(iii)*** *Non-specific phage are removed by washing and* ***(iv)*** *specifically bound phage are eluted using various conditions.* ***(iv)*** *Eluted phage can be monitored for specific binding to antigen by ELISA* ***(vi)*** *before they are reinfected into bacteria,* ***(vii)*** *which are amplified for further rounds of selection and enrichment.*

1.5.3.6 Antibody engineering

The major advantage of recombinant antibody technology is the possibility of improving the properties of existing antibody fragments. Once a specific antibody is isolated from a library, the domains of an antibody fragment can be further engineered in a number of ways to produce antibodies with lower immunogenicity (Thompson *et al.*, 1998), higher affinity (Marks *et al.*, 1992; Low *et al.*, 1996; Schier *et al.*, 1996[a]), altered antigenic specificity (Ohlin *et al.*, 1996, Parsons *et al.*, 1996) or enhanced stability (Reiter *et al.*, 1994; Martineau and Betton 1999, Worn and Pluckthun 1999). Several strategies have been applied to improve the affinity of antibody fragments by random means, including chain shuffling, DNA shuffling, error-prone PCR and mutator strains or by site-directed based mutagenesis.

The method of chain shuffling was originally proposed in 1989 by Huse and colleagues and involves the re-cloning of the gene for one of the variable domains (*e.g.* V_H) into a repertoire of genes for the other chain (*e.g.* V_L). The resulting mutant library contains the V_H domain gene of the parent antibody and random V_L chain genes. Following subsequent screening of the mutant library against the target of interest, isolated specific clones with improved binding characteristics are subject to heavy chain shuffling. In this case, the selected antibodies with new V_L domains are cloned into a repertoire of V_H chain genes. However, in order to maintain the specificity of the parent scFv, the CDR3 region of the V_H chain must be conserved. This method has been successfully applied and has resulted in antibodies displaying 20-fold (Marks *et al.*, 1992) improvement in affinity. Schier *et al.* (1996[a]) described a 6-fold increase in a human scFv specific for the tumour antigen c-*erb*B-2 by light chain shuffling and 5-fold by subsequent heavy chain shuffling. However, the technique is dependent on the availability of large highly diverse variable domain libraries, in order to supply sufficient sequence variation for improved affinity.

Random mutagenesis techniques, such as error-prone PCR, mimic the *in vivo* process of somatic hypermutation by providing variable gene diversification by randomly introducing mutations throughout an antibody sequence. Error-prone PCRs are performed using DNA polymerases with reduced fidelity, resulting in random insertions, deletions and substitutions in the nucleotide sequence. The technique has been successfully employed to generate antibodies with altered specificity (Korpimäki

et al., 2002) and increased affinity (Hawkins *et al.*, 1992). Although certain mutations may result in improved affinity, some mutations may be detrimental to the antibody function, and thus, the number of errors introduced should be kept to a minimum. DNA shuffling, which involves the recombination of mutations created by error-prone PCR (Stemmer *et al.*, 1994; Crameri *et al.*, 1996) and bacterial mutator strains (Low *et al.*, 1996) have also been used for affinity maturation of phage-displayed antibodies.

The random approaches discussed have been successfully used to mature some antibodies with relatively low starting affinity. However, little useful information is generated with respect to the location of mutations that modulate affinity. Directed modifications to defined residues or regions of the variable genes using codon-based mutagenesis, oligonucleotide-directed mutagenesis and PCR techniques (Glaser *et al.*, 1992; Virnekas *et al.*, 1994; Jackson *et al.*, 1995; Yang *et al.*, 1995; Schier *et al.*, 1996[b]) allow for the identification of conserved structural and functional residues as well as those that modulate affinity. The technique of site-directed mutagenesis involves the specific mutation of one or more of the amino acids in the antibody sequence. Directed mutations of the complementarity determining regions of an antibody have proven to be the most effective in regards to improvements in antibody affinity. The mutation of residues in CDRs may result in increased affinity by introducing new contact residues or by replacing low affinity or 'repulsive' contact residues (Kontermann, 2001) but mutations of CDRs (particularly in CDRL3 and CDRH3) can also abolish antigen binding. Yang *et al.* (1995) reported a 420-fold increase in affinity of an anti-gp120 Fab fragment following mutation of four CDRs. Schier *et al.*, (1996[b]) also described the mutation of CDR3 regions of variable light (V_L) and heavy (V_H) of a human scFv specific for the tumour antigen c-erbB-2, which yielded an scFv with a 1,230-fold increase in antibody affinity compared to the wildtype scFv.

With the advent of recombinant antibody technology and the development of new expression vectors, it is now also possible to link the antibody fragment to an enzyme allowing direct detection or to various peptide tags (*e.g.* histidine, hemagglutinin, biotin acceptor domain) to facilitate purification, detection and orientated immobilisation (Krebber *et al.*, 1997, Barbas *et al.*, 2001; Warren *et al.*, 2005).

Antibody engineering techniques have also been employed to manipulate the fragments to create dimeric, trimeric and multimeric scFvs by reducing or completely removing the peptide linker between the variable fragments (Holliger *et al.*, 1993[a]), yielding multivalent fragments with increased functional binding affinity (avidity) for antigen comparative to their monovalent counterparts. These multimeric molecules can be specific for one particular target antigen or have multiple specificities to different antigens, by associating different scFv molecules derived from different parental antibodies (Holliger *et al.*, 1993[b]). The range of antibody formats achieved by combining antibody fragments is virtually unlimited.

1.6 Enzyme-linked immunosorbent assays

Enzyme immunoassays exploit both the specific and sensitive interaction of an antibody to its corresponding antigen and the extremely catalytic power and specificity of enzymes. The technique has been commonly used in analytical biochemistry for the detection of a wide range of compounds and facilitates the detection of antibody-antigen complexes by labelling one of the reactants with an enzyme (*e.g.* horse radish peroxidase (HRP) or alkaline phosphatase (AP)).

Enzyme immunoassays can be divided into two categories, heterogenous or homogenous. Homogenous assays are carried out in solution and do not involve the separation of reactants, whereas heterogenous assays involve the immobilisation of one of the reagents on a solid phase and the removal of unreacted reagents prior to measurement (*e.g.* Enzyme-linked immunosorbent assays (ELISA); O'Kennedy, 1989). An ELISA involves the immobilisation of an antigen or antibody onto a solid support (*e.g.* microtitre plate), where it serves to capture the complementary enzyme-labelled reactant from the sample. By measuring the extent of enzymatic reaction the activity or concentration of immunoreactant in the test sample can be determined. The majority of the assays developed during the research presented in this thesis employed immobilised antigen and thus, this format is described within the following text.

Both competitive and non-competitive ELISA formats exist. Non-competitive formats are generally used to determine the concentration of antibody in a sample, as illustrated in *Figure 1.10 (A)*. Following binding of an antibody to its specific immobilised antigen, resulting antibody-antigen complexes can then be detected

directly by using a labelled antibody or indirectly using a species- or peptide tag-specific enzyme-labelled secondary antibody. In this format the rate of antigen-antibody complex formation is proportional to the concentration of the labelled reagents (Tijssen, 1985).

Competitive assays, as the name suggests, involve the competition of free antigen in solution with immobilised antigen for antibody binding sites (*Figure 1.10 (B)*). Binding of the enzyme-labelled antibody to the immobilised antigen is inhibited by addition of free antigen and thus, the resulting enzyme signal is inversely proportional to the concentration of free antigen in the sample solution. Absorbance values can be plotted against the logarithm of antigen concentration to create a dose-response curve, facilitating quantitative detection of the antigen with reference to this standard curve (Tijssen, 1985). In this format, assay sensitivity is a function of both antibody affinity and equilibrium between both immobilised antigen and free antigen in solution. Thus, higher concentrations of immobilised antigen can result in unfair binding bias towards the immobilised antigen and reduce sensitivity to free antigen in solution. Similarly, higher concentrations of antibody will require higher concentrations of free antigen in solution to inhibit its binding to immobilised antigen, resulting in decreased assay sensitivity. Thus, both antibody and immobilised antigen concentrations must be optimised accordingly.

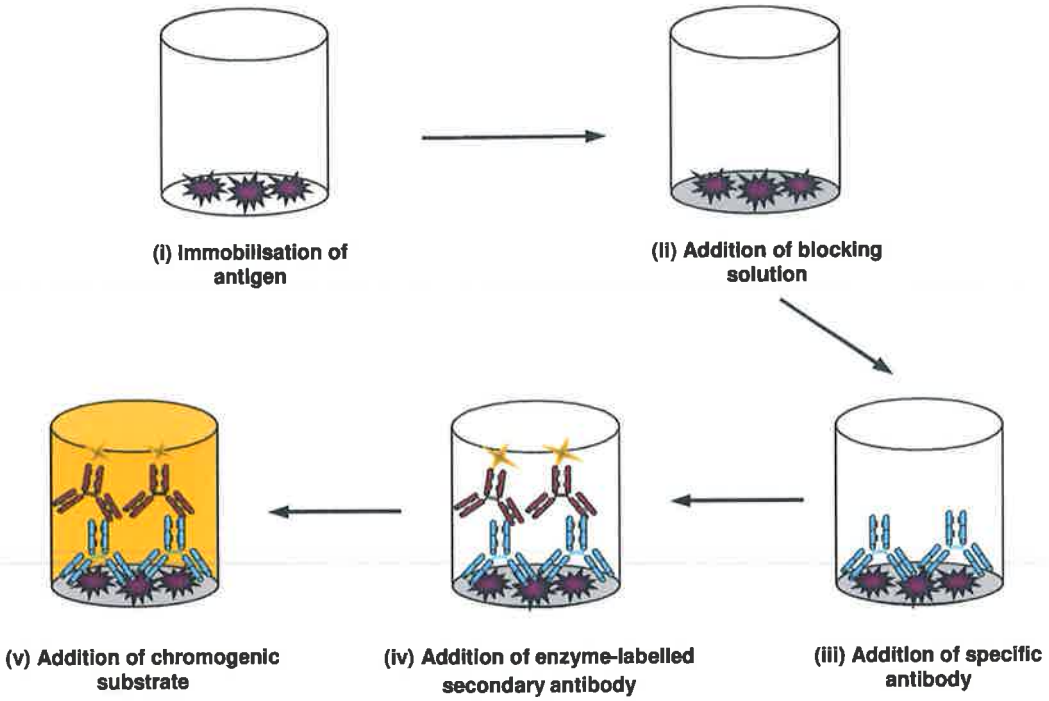


Figure 1.10. *(A) Schematic illustration of a typical non-competitive indirect ELISA.* ***(i)*** *The wells of an immunoplate are coated with the antigen of interest.* ***(ii)*** *Any remaining unreacted sites are then blocked by the addition of a blocking solution (generally a protein solution) to eliminate any non-specific binding.* ***(iii)*** *Specific antibody, at the required dilution is added to the plate and* ***(iv)*** *bound antibody is detected with the addition of a species- or peptide tag-specific enzyme-labelled secondary antibody.* ***(v)*** *Following addition of a chromogenic substrate, a colour develops, which is directly proportional to the amount of specific antibody present. Each step requires an incubation period (generally one hour at 37°C or overnight at 4°C), which is followed by washing procedures to remove any unbound reagents.*

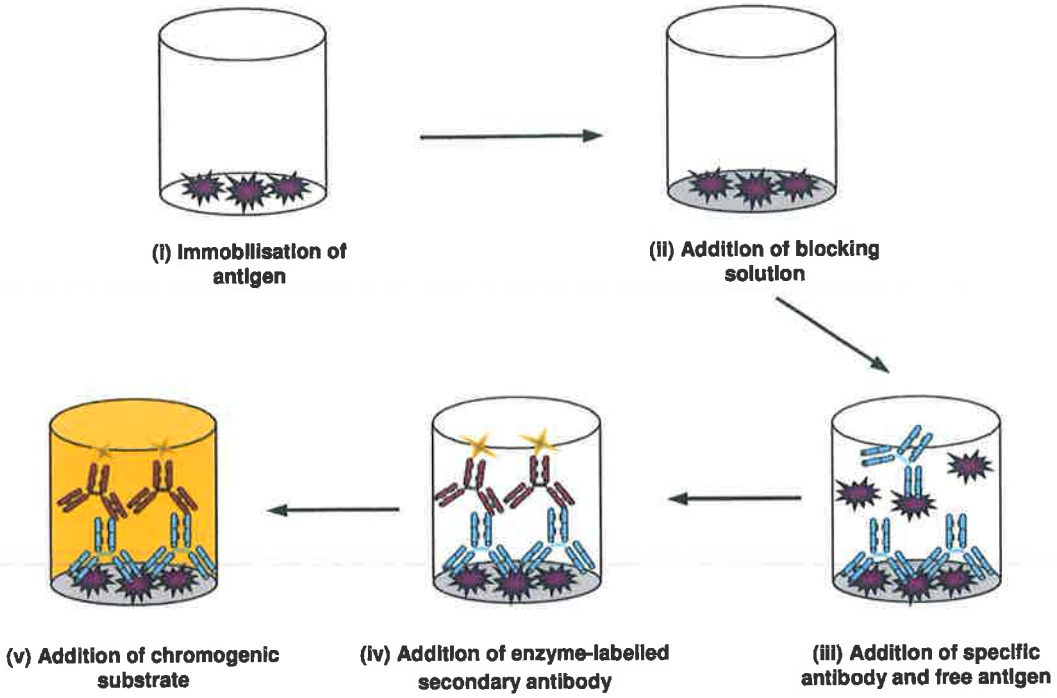


Figure 1.10. *(B) Schematic illustration of a typical competitive indirect ELISA.* ***(i)*** *The wells of an immunoplate are coated with the antigen of interest.* ***(ii)*** *Any remaining unreacted sites are then blocked by the addition of a blocking solution (generally a protein solution) to eliminate any non-specific binding.* ***(iii)*** *Specific antibody and free antigen are added to the plate and free and immobilised antigens compete for binding to the antibody.* ***(iv)*** *Bound antibody is detected with the addition of a species- or peptide tag-specific enzyme-labelled secondary antibody.* ***(v)*** *Following addition of a chromogenic substrate, a colour develops, which is inversely proportional to the amount of free antigen present. Each step requires an incubation period (generally one hour at 37°C or overnight at 4°C), which is followed by washing procedures to remove any unbound reagents.*

1.7 Biacore

The optical phenomenon of surface plasmon resonance (SPR) has been successfully applied to various biosensors with numerous applications. Although several commercial biosensors have been developed using SPR technology as their detection method, the Biacore™ system, first launched by PharmaciaAB (now GE Healthcare) in 1990, has proven to be an extremely valuable tool for antibody-antigen interaction analysis.

The phenomenon of SPR occurs as a result of total internal reflection (TIR) of light (Kretschmann and Raether, 1968; Liedberg *et al.*, 1983). When a plane-polarised light beam propagates in a medium of the higher refractive index (*e.g.* glass) and meets an interface with a medium of lower refractive index (*e.g.* sample solution), the light is totally internally refracted, above a certain angle (*Figure 1.11*). Under conditions of TIR an electromagnetic field component, referred to as the evanescent wave, penetrates into the medium of lower refractive index a short distance in order of one wavelength (Dillon *et al.*, 2003). As the evanescent wave moves further away from the interface into the lower dense medium the wave decays exponentially. However, if the interface between the media is coated with a thin layer of metal (*i.e.* gold in the case of the Biacore instrument), the evanescent wave interacts with electron clouds, referred to as plasmons, on the metal layer. This causes the plasmons to resonate which results in a quantum mechanical wave known as the surface plasmon wave. Some of the energy of the reflected light (incident light) is taken up by the surface plasmon wave resulting in a dip in the intensity of reflected light at a certain angle being observed (Panayotou *et al.*, 1993). The specific angle at which SPR occurs is known as the SPR angle (θ_{SPR}). A linear correlation exists between resonance angle shift and surface protein concentration allowing for the 'real-time' detection of mass changes without the need for labelling (Hutchinson, 1995). Thus, the interaction of biomolecules (*e.g.* antibody binding antigen) at the metal surface causes an increase in the mass at the surface which in turn causes a change in refractive index, which results in a shift in the SPR angle (*Figure 1.12*). These changes are monitored continuously by the Biacore instrument and presented in a sensogram, where the change in SPR angle (expressed as resonance units) is plotted as a function of time. A response of 1,000 RU is approximately equivalent to a change of 0.1°C in the SPR

angle (θ_{SPR}), which correlates to about 1ng/mm^2 in surface protein concentration (Stenberg *et al.*, 1991).

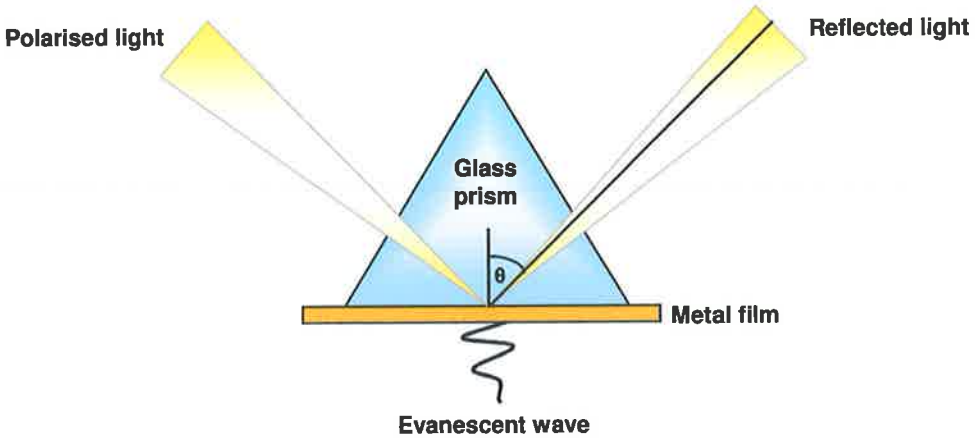


Figure 1.11. *Schematic representation of the phenomenon of SPR occurs at the interface of two media of different refractive index. When a plane-polarised light beam propagates in a medium of the higher refractive index (e.g. glass) and meets an interface with a medium of lower refractive index (e.g. sample solution), the light is totally internally refracted, above a certain angle. Under conditions of TIR an electromagnetic field component, referred to as the evanescent wave, penetrates into the medium of lower refractive index a short distance in order of one wavelength. When the interface between the media is coated with a thin layer of metal, the evanescent wave interacts with electron clouds, referred to as plasmons, on the metal layer. This causes the plasmons to resonate which results in a quantum mechanical wave known as the surface plasmon wave. Some of the energy of the reflected light (incident light) is taken up by the surface plasmon wave resulting in a dip in the intensity of reflected light at a certain angle being observed.*

The SPR-based Biacore™ instruments employ a sensor chips with integrated microfluidics, an autosampler and an optical detection unit with control software. There are a variety of Biacore™ sensor chips available (www.biacore.com) for various applications and the majority of these comprise of a carboxymethyl (CM) dextran modified gold surface deposited on a glass support. This CM dextran matrix layer of the sensor chip facilitates increased binding capacity and dynamic range of the biosensor (Löfås and Johnsson, 1990) and allows for the covalent immobilisation

of specific ligands to the surface (Jönsson *et al.*, 1991) via a reaction with *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*'-(dimethylaminopropyl) carbodiimide hydrochloride. Protein is passed over the surface in a solution of low ionic strength with a pH value below the isoelectric point of the protein (Johnsson *et al.*, 1991) and attached to surface by electrostatic attraction forces and a simultaneous reaction with the NHS active esters. The remaining unreacted active esters are transformed into amides via a reaction with ethanolamine. The amount of immobilised ligand can be controlled by varying the protein concentration, protein ionic strength, pH, reaction times and reagent concentration.

Over the past decade Biacore technology has facilitated the detection of a range of analytes including food contaminants (Haasnoot *et al.*, 2003; Daly *et al.*, 2000; van der Gaag 2003), illicit drugs (Brennan *et al.*, 2003; Dillon *et al.*, 2003) and the quantification of antibody concentrations in serum (Wong *et al.*, 1997; Ahmad *et al.*, 2004; Newcombe *et al*, 2006). The SPR-based biosensors are also routinely used to generate sensitive and reliable kinetic data from antibody/antigen interactions for both therapeutic and diagnostic applications. 'Real-time' biomolecular interaction analysis (BIA) has facilitated kinetic-based screening of both antibodies (Quinn *et al.*, 2001; Gomes and Andreau, 2002; Canziani *et al.*, 2004; Katsamba *et al.*, 2006) and antibody fragments (Rau *et al.*, 2002; Steukers *et al.*, 2006; Wassaf *et al.*, 2006; Leonard *et al.*, 2007).

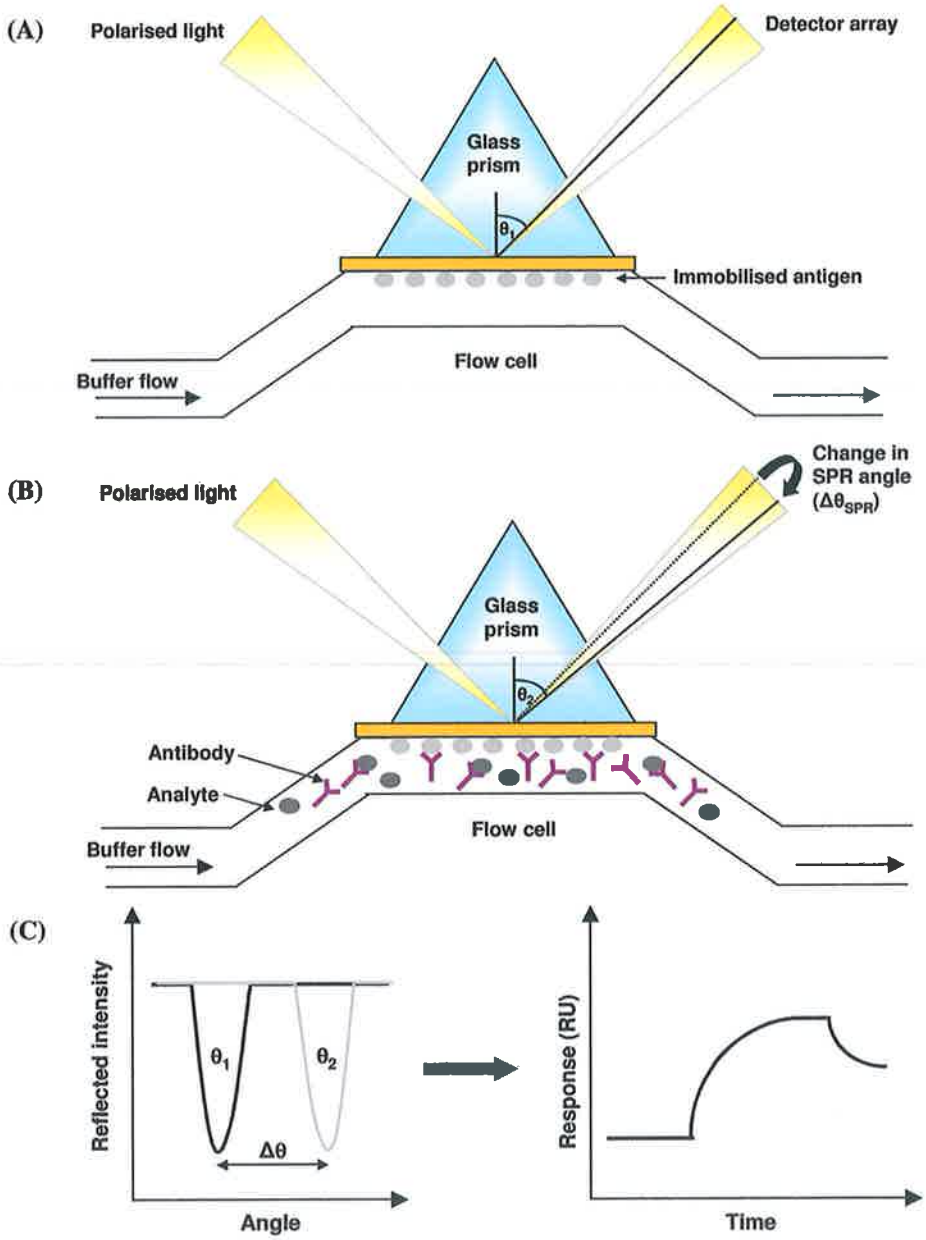


Figure 1.12. *Schematic representation of the detection principle employed by the Biacore™ instrument.* ***(A)*** *The antigen is immobilised on the sensor chip surface using EDC/NHS chemistry. Under conditions of total internal reflection an evanescent wave propagates into the medium of lower refractive index, resulting in a dip in the intensity of reflected light at a particular angle referred to as the SPR angle (θ_{SPR1}).* ***(B)*** *When specific antibody is passed over the surface, it binds to the antigen and causes an increase in the mass concentration at the surface of the sensor chip, which in turn causes a change in refractive index resulting in a shift in the SPR angle of the reflected light (θ_{SPR2}).* ***(C)*** *The difference in SPR ($\Delta\theta_{SPR}$) is recorded and plotted as a function of time and presented as a sensogram.*

1.8 Aims of research

The main aim of this research was the development of rapid immunoassays for the detection of the food contaminants, aflatoxin B_1 and ampicillin, and for the diagnosis of caseous lymphadenitis (CLA) disease in sheep.

Chapter 3 focuses on the production of a recombinant form of phospholipase D (PLD) and its subsequent use in an ELISA and Biacore-based assays to detect antibodies to *Corynebacterium pseudotuberculosis*, the causative agent of CLA in sheep. A number of positive and negative control sera were initially used to optimise all assay parameters influencing both sensitivity and specificity. Optimised assay formats were then applied to a range of clinical samples from Irish herds and the performance characteristics of the assays were determined.

Chapter 4 describes the conversion of a previously generated anti-AFB_1 genetically-derived scFv to a chimeric Fab fragment by the addition of human constant regions, using PCR assembly methods combined with phage display techniques. Two Fab clones were isolated and applied to the development of immunoassays and compared with regard to expression, stability, specificity and sensitivity to the parent scFv. Finally, the antibodies were subcloned into an expression vector encoding a biotin acceptor domain for subsequent expression of *in vivo* biotinylated recombinant fragments.

Chapter 5 describes the generation of ampicillin-specific polyclonal antibodies and their subsequent use in biosensor-based assays. Specific polyclonal antibodies, generated with an ampicillin-protein conjugate, were applied to an SPR-based biosensor for the detection of ampicillin in processed milk. The applicability of a novel biochip sensor system for the rapid detection of antibiotic residues was also investigated. Lastly, naïve and immune phage display libraries were also screened against a number of ampicillin conjugates for the selection of an ampicillin-specific single chain variable fragment (scFv) antibody derivative.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Reagents

All reagents were of analytical grade and purchased from Sigma-Aldrich Ireland Ltd. (Dublin, Ireland), unless otherwise specified.

Reagents	**Supplier/Distributor**
Pierce Bicinchoninic acid assay kit, Pierce Blue Ranger pre-stained molecular weight markers, Pierce EZ-link NHS-LC-Biotin, Promega Wizard Plus mini-prep kit, Promega Reverse Transcription kit.	Medical Supply Co. (MSC) Ltd., Damastown, Mulhuddart, Dublin 15, Ireland.
Tryptone, Agar technical, Yeast extract.	Oxoid Ireland c/o Fannin Healthcare, Blackthorn Road, Sandyford Industrial Estate, Foxrock, Dublin 18, Ireland.
Eppendorf Perfectprep Gel clean up kit.	Unitech Ltd., Magna Business Park, Magna Drive, Citywest Rd., Dublin 24, Ireland.
Trizol, T4 DNA Ligase.	Gibco BRL, Renfew Rd., Paisley PA49RF, Scotland.
Invitrogen PCR Optimiser Kit.	Bio Sciences Ltd., 3 Charlemont Terrace Crofton Road, Dun Laoghaire, Co. Dublin, Ireland.
New England Biolabs (NEB) M13 K07 helper phage, NEB *Sfi*I, *Bam*HI, *Eco*RI restriction enzymes and buffers.	ISIS Ltd., Unit 1 & 2, Ballywaltrim, Business Centre, Boghall Road, Bray Co. Wicklow, Ireland.

Reagents	Supplier
Stratagene VCSM13 Helper Phage.	Techno-Path, Rosse Centre, Holland Rd., National Technological Park, Plassey, Limerick, Ireland.
PCR primers.	MWG Biotech Ltd., Milton Keyes, MK12 5RD, UK.
Nitrocellulose membrane.	Schleicher and Schuell Bioscience GmbH, Hahnestrasse 3, D-37586, Dassel, Germany.
Horseradish peroxidase-labelled rabbit anti-sheep IgG.	Dako Diagnostics Ireland Ltd., 12 Camden Row, Dublin 8, Ireland.

2.1.2 Equipment

Class	Equipment	Supplier
Biacore	Biacore 3000™, Biacore 1000™.	Biacore AB, Sovereign Court, 230 Upper 5th Street, Central Milton Keynes, MK9 2HR, UK.
Blood Tube Rotator	SB 2 Blood Tube Rotator.	Stuart Scientific, London, England.
Centrifuges	Biofuge Pico microcentrifuge,	Heraeus Instruments Inc., 111-a Corporate Boulevard, South Plainfield, New Jersey, USA.
	Beckman J2-21 centrifuge,	Beckman-Coulter Inc., 4300 N Harbour Boulevard, Fullerton, CA 92834-3100, USA.
	Eppendorf centrifuge 5810R.	Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany.
Microtitre Plate Readers	Titerkek Multiscan Plate Reader,	Medical Supply Co. Ltd., Damastown, Mulhuddart, Dublin 15, Ireland.
	Tecan Safire^{2} ™.	Tecan UK Ltd., Theale Court, 11-13 High Street, Theale, Reading RG7 5AH, UK.

Class	Equipment	Supplier
pH Meter	3015 pH Meter.	Jenway Ltd., Gransmore Green, Felsted, Dunmow, Essex, UK.
Spectrophotometers	UV 160A Spectrophotometer, Nanodrop ND-1000.	Shimadzu Corp., Albert-Hahn-Str. 6-10, 47269 Duisburg, Germany. Nanodrop Technologies, 3411 Silverside Rd Bancroft Building Wilmington, DE 19810 USA.
Protein electrophoresis equipment	Atto dual minislab system AE-6450, Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer cell.	Medical Supply Company, Damastown, Mulhuddart, Dublin 15, Ireland. Alpha Technologies Ltd., The Leinster Technology Centre, Blessington Industrial Estate, Blessington, Co. Wicklow, Ireland.
Orbital Incubator	Gallenkamp Orbital Incubator.	Sanyo Gallenkamp plc., Monarch Way, Belton Park, Loughborough, Leicester, UK.
Waterbath	RM6 Lauda Waterbath.	AGB Scientific Ltd., Dublin Industrial Estate, Glasnevin, Dublin 9.

Class	Equipment	Supplier
Filtration apparatus	Millipore Filtration Apparatus.	AGB Scientific Ltd., Dublin Industrial Estate, Glasnevin, Dublin 9.
PCR machine	Biometra T-Gradient Uno II Thermocycler.	Anachem Ltd., Anachem House, Charles St., Luton, Bedfordshire, UK.
UV gel imager	UVP ImageStore 7500 gel documentation system.	Ultra Violet Products, Upland, CA, USA.

2.1.3 Consumables

Consumable	Supplier
Biacore CM5 research grade sensor chips.	Biacore AB, Sovereign Court, 230 Upper 5th Street, Central Milton Keynes, MK9 2HR, UK.
General plastic consumables *e.g.* microcentrifuge tubes, cuvettes, pipette tips etc.	Sarstedt, Drinagh, Co. Wexford.
Maxisorb 96 well plates, Maxisorb Immunotubes.	Nunc, Kamstrup DK, Roskilde, Denmark.
Purabind A-FP Nitrocellulose.	Whatman International Ltd., 20/20 Maidstone, Kent ME16 0LS, UK.

2.1.4 Bacterial Strains

Bacterial Strain	Source	Genotype
E. coli XL-1 Blue	Stratagene	*rec*A1 *end*A1 *gyr*A96 *thi-1* *hsd*R17 *sup*E44 *rel*A1 *lac*[F´ *pro*AB *lac*I ^{q} ZΔM15 *Tn*10(Tet ^{R})]
E. coli TG1	Stratagene	*sup*E *thi-1* Δ(*lac-pro*AB) Δ(*mcr*B-*hsd*SM)5(r_K^- m_k^-) [F´ *tra*D36 *pro*AB *lac*I ^{q} ZΔM15]
E. coli DH5α	Donated by Dr. John Prescott of the Ontario Veterinary College, University of Guelph, Canada.	F$^-$ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17 (r_K^-, m_K^+) *sup*E44 *thi-1* gyrA96 *rel*A1 *pho*A
E. coli XL-10 Gold	Stratagene	Tet^{R} Δ (*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi-1 rec*A1 *gyr*A96 *rel*A1 *lac* Hte [F´ *pro*AB, *lac*I ^{q}ZΔM15 *Tn*10(Tet ^{R}) Amy Cam ^{R}]*
E. coli JM83	Donated by the Technical Preparation Lab, DCU.	F$^-$ *ara* Δ(*lac-pro*AB) *rpsL* (Strr)[Φ*80 lac*ZΔM15] *thi*
E. coli Top10F'	Invitrogen	F´{*lac*I ^{q} *Tn*10 (Tet ^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str ^{R}) *end*A1 *nup*G

E. coli AVB100	Avidity Inc.	[MC1061 *araD139* Δ(*ara-leu*)*7696* Δ (*lac*)*l74 galU galK hsdR2*($r_K^- m_K^+$) *mcrB1 rpsL*(Str^r)] with a *birA* gene stably integrated into the chromosome.
E. coli ER2738	New England Biolabs (NEB)	F′*proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15 zzf::Tn*10(Tet^R)/ *fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5*

2.1.4.1 Maintenance of Bacterial Stocks

A working stock of bacteria was streaked on LB agar plates containing the appropriate antibiotic. Bacterial glycerol stocks were prepared by growing an overnight culture from a single bacterial colony. These were stored in 15% (v/v) glycerol and 1% (w/v) glucose at – 80°C.

2.2 General Formulations

2.2.1 Culture media formulations

Culture Media	Formulation	
2 x Tryptone and Yeast Extract (**2 x TY**) Medium	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
Terrific Broth (**TB**)	Tryptone	12 g/l
	Yeast Extract	24 g/l
	Glycerol	4 ml/l
	KH_2PO_4	17 mM
	K_2HPO_4	72 mM
Luria-Bertani (**LB**)	Tryptone	10 g/l
	Yeast Extract	5 g/l
	NaCl	10 g/l
Super Broth (**SB**)	Yeast Extract	20 g/l
	Tryptone	30 g/l
	MOPS	10 g/l
	pH 7.0	
Low Expression Medium (**LE**)	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
	Glucose	1% (w/v)
	Chloramphenicol	25 µg/ml
	IPTG	5 mM

Culture Media	**Formulation**	
Non–Expression Medium (**NE**)	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
	Glucose	1% (w/v)
	Chloramphenicol	25 μg/ml
Super Optimal Catabolites (**SOC**) Medium	Tryptone	20 g/l
	Yeast Extract	5 g/l
	NaCl	0.5 g/l
	KCl	2.5 mM
	$MgCl_2$	20 mM
	Glucose	20 mM
	pH	7.0
Solid medium was made up by adding 15g/l of bacteriological agar to the above media.		

2.2.2 Buffer formulations

2.2.2.1 Phosphate Buffered Saline (PBS)

Phosphate buffered saline, pH 7.4, containing 0.15M sodium chloride (NaCl), 2.5mM potassium chloride (KCl), 10mM disodium hydrogen phosphate ($NaHPO_4$) and 1.8mM sodium dihydrogen phosphate (KH_2PO_4), was prepared in distilled water.

2.2.2.2 Phosphate Bufferd Saline/Tween (PBST)

Tween 20 surfactant (Sigma) was added to phosphate buffered saline (PBS) (as described in *Section 2.2.1.2.1*) to a final concentration of 0.05% (v/v) and mixed.

2.2.2.3 Hepes Buffered Saline (HBS)

Hepes Buffered Saline, pH 7.4, containing 10mM Hepes, 150mM sodium chloride (NaCl), 3.4mM ethylenediaminetetraacetic acid (EDTA) and 0.05% (v/v) Tween 20 was prepared in ultra pure water. This was filtered (pore size 0.22μm) and degassed by filtration (Millipore sintered glass filtration unit) immediately before use.

2.2.2.4 Carbonate buffer

Carbonate buffer, pH 9.5, containing Na_2CO_3 and $NaHCO_3$, was prepared in distilled water to a final concentration of 0.05M.

2.2.2.5 Tris–Acetate–EDTA Buffer (TAE)

All agarose gels were run in TAE Buffer, pH 8.3, containing 40mM Tris acetate and 1mM EDTA, in ultra pure water.

2.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions according to Laemmli (1970) to assess protein purity. The method employs SDS (which denatures the proteins), β-mercaptoethanol (a reducing agent), and heat to dissociate proteins into individual polypeptides subunits before they are loaded onto the polyacrylamide gel. The polymerisation of acrylamide, driven by ammonium persulphate, gives rise to the cross-linked gel of

defined pore-size. The pore size of the gel, dependent on the amount of acrylamide and degree of crosslinking, facilitates protein separation on the basis of size. N,N,N',N'-tetramethylethylenediamine (TEMED) is also included to accelerate the polymerisation of acrylamide and bis-acrylamide by catalysing the formation of free radicals from ammonium persulphate.

The compositions of gels and buffers are shown in *Table 2.1*. Protein samples, mixed with sample loading buffer and boiled for 5 minutes, were run at 15mA per gel on an Atto AE-6450 minigel (MSC Ltd.) until samples had reached the base of the stacking gel, and then at 20mA per gel until the dye had reached the base of the separating gel. Proteins were then visualised by staining the gel with Coomassie blue, as per *Section 2.2.2.1* or the gels were used for subsequent Western blotting, as described in *Section 2.2.3*.

2.2.3.1 Coomassie blue staining for SDS-PAGE gel

Coomassie blue staining solution (0.2% (w/v) Coomassie blue R250 in 30:10:60 (v/v/v) methanol: acetic acid: water) was prepared and gels were stained for 30 minutes and destained overnight at 4°C (in destaining solution: 10:7:53 (v/v/v) methanol: acetic acid: water).

Table 2.1. *Composition of stacking gel (5%), separating gel (10%), electrophoresis buffer and sample loading buffer for SDS-PAGE.*

SDS-PAGE Buffers	**Composition**
Stacking gel	5% (w/v) acrylamide 0.13% (w/v) bis-acrylamide 125mM Tris (pH 6.8) 0.1% (w/v) SDS 0.15% (w/v) ammonium persulphate 0.25% (v/v) TEMED
Separating gel	10% (w/v) acrylamide 0.27% (w/v) bis-acrylamide 375mM Tris (pH 6.8) 0.1% (w/v) SDS 0.08% (w/v) ammonium persulphate 0.08% (v/v) TEMED
Electrophoresis buffer	25mM Tris (pH 8.8) 192mM glycine 0.1% (w/v) SDS 60mM Tris (pH 6.8)
Sample loading buffer	25% (v/v) glycerol 2% (w/v) SDS 14.4mM β-mercaptoethanol 0.1% (w/v) bromophenol blue

2.2.4 Western blotting*

Proteins are eluted from a polyacrylamide gel onto a nitrocellulose membrane by electrophoretic transfer. For semi-dry blotting, the gel and the membrane are sandwiched horizontally between two stacks of buffer-wetted filter papers that are in direct contact with two closely spaced solid-plate electrodes. The blot, *i.e.* the nitrocellulose membrane containing the transferred proteins, can be then be reacted with different probes, such as antibodies, for the identification of the corresponding antigen.

Bjerrum and Schafer-Nielson transfer buffer (48mM Tris, 39mM glycine, 10% (v/v) methanol, 0.0375% (w/v) SDS, pH 9.2) was prepared the day preceding the blotting experiments and stored at 4°C. Following separation of the proteins by SDS-PAGE, as described in *Section 2.2.2*, the polyacrylamide gel, filter paper and nitrocellulose membrane were soaked in transfer buffer for approx. 15 minutes. The proteins were then transferred to the membrane by electrophoresis using a Trans-Blot SD semi-dry cell (Bio-Rad) at 15V for 20 minutes. The membrane was blocked with PBS containing 5% (w/v) Marvel^{TM} dried skimmed milk powder (Premier Foods Ltd., UK) overnight at 4°C, washed three times with PBS for 10 minutes each time. 10ml of the appropriate antibody, at the required dilution, was prepared in PBST containing 2% (w/v) Marvel^{TM} and added to the nitrocellulose for 1.5 hours shaking at room temperature. The membrane was then washed thoroughly (as before) and a secondary antibody (if required), prepared in PBST containing 2% (w/v) Marvel, was added and incubated as before. Once the blot was washed, the appropriate substrate was added and the reaction was allowed to proceed until the bands could be clearly visualised.

** This method is generic. Any amendments or further details on reagents are clearly cited within the relevant results chapters.*

2.2.5 Agarose Gel Electrophoresis

DNA was analysed by electrophoresis on an agarose gel. Briefly, agarose was dissolved to the appropriate concentration (typically 0.7 – 1.2% (w/v)) in TAE buffer and boiled until the solution was clear. When cool, the intercalating dye ethidium bromide (EtBr) (CAUTION*) was added to the gel at a concentration of 0.5μg/ml. This allowed visualisation of DNA migration on the gel. The gel was then cast on a

horizontal gel apparatus (Atto AE–6100) and electrophoresed in 1 X TAE at 70V. Gels were visualised on a UV transluminator and photographed using a UV image analyser (UVP ImageStore 7500 gel documentation system).

** Ethidium bromide (EtBr) is a highly toxic chemical and potent mutagen. It can be absorbed through the skin and is an irritant to the eyes, mouth, skin and upper respiratory tract. Therefore, extreme care was taken to avoid any direct contact with the chemical. To prevent inhalation exposure, any work with EtBr was carried out in a fume hood and personal protective equipment (lab coat, safety glasses and gloves) was worn at all times. When transporting agarose gels, containing EtBr, clean gloves were worn to prevent cross-contamination of doorknobs and other surfaces and the gel was carried in a leak-proof secondary container. A UV light was used to check any area of suspected contamination and the area was promptly cleaned, as outlined in the MSDS. Contaminated waste was disposed of by incineration.*

2.3 Development of immunoassays for the detection of antibodies to *Corynebacterium pseudotuberculosis*

2.3.1 Expression of the recombinant protein phospholipase D (PLD)

2.3.1.1 Isolation of plasmid pJGS90

A single *E. coli* DH5α colony, containing the appropriate plasmid, was used to inoculate 5ml of LB containing 100μg/ml ampicillin and incubated at 37°C overnight with shaking at 250rpm. The following day the plasmid was isolated and purified using a Wizard Plus Miniprep kit (Promega, UK), according to the manufacturer's instructions.

The overnight culture was centrifuged at 3,000g for 10min (Eppendorf 5810R), the supernatant discarded and the pellet resuspended in 250μl of cell resuspension solution (50mM Tris-HCl, pH 7.5, 10mM EDTA, 100μg/ml RNase A). The suspension was subsequently transferred to a sterile 1.5ml microcentrifuge tube and 250μl of cell lysis solution (0.2M NaOH, 1% (w/v) SDS) was added. The solution was mixed by inversion four times and incubated for 1 - 5 minutes at room temperature until the suspension cleared. 10μl of alkaline protease solution was

added during the lysis step to inactivate endonuclease I and reduce the overall level of protein contamination in the final plasmid DNA sample. Following addition of the alkaline protease solution, the tube immediately inverted four times and incubated at room temperature for a maximum of 5 minutes. 350µl of neutralisation buffer (4.09M guanidine hydrochloride, 0.75M potassium acetate, 2.12M glacial acetic acid, pH 4.2) was added, the solution inverted four times and centrifuged at 14,000*g* for 10 minutes (Heraeus Biofuge Pico) at room temperature. The cleared lysate was then transferred to a spin column, re-centrifuged at 14,000*g* for 1 minute (Heraeus Biofuge Pico) and the flow through discarded. The column was washed with 750µl of wash solution (60% (v/v) ethanol, 60mM potassium acetate, 8.3mM Tris-HCl, pH 7.5, and 40mM EDTA), centrifuged at 14,000*g* for 1 minute (Heraeus Biofuge Pico) and the 'flow-through' discarded. The spin column was washed as before with 250µl of wash solution, transferred to a sterile eppendorf and 100µl of 'nuclease-free' water was added. The column was re-centrifuged at 14,000*g* for 1 minute (Heraeus Biofuge Pico) and the eluted plasmid DNA was stored at -20°C.

2.3.1.2 Preparation of $CaCl_2$ - competent *E. coli* XL10-Gold cells

A single colony of *E. coli* XL10-Gold was used to inoculate 5ml of 2xTY, containing 10µg/ml tetracycline and 25µg/ml chloramphenicol, and incubated at 37°C overnight with shaking. The overnight culture was then used to seed 100ml of 2xTY broth, containing the appropriate antibiotics, at a 1% (v/v) cell density. The cells were incubated at 37°C with shaking until the OD_{550} reached between 0.3 and 0.4. The cells were then cooled on ice for 15min and maintained at 4°C for the remainder of the method steps. Cells were collected by centrifugation at 3,000*g* for 20min at 4°C (Eppendorf 5810R), the supernatant discarded and the pellet resuspended in 20ml of cold 100mM $MgCl_2$. The cells were then centrifuged at 3,000g for 20min at 4°C (Eppendorf 5810R), the pellet resuspended in 20ml of cold 50mM $CaCl_2$ and incubated on ice for 30min. The cells were re-centrifuged again as before, the supernatant decanted and the bacterial cell pellet resuspended in 2ml 50mM $CaCl_2$. The competent cells were then dispensed, flash frozen in liquid nitrogen and stored at -80°C.

2.3.1.3 Transformation of *E. coli* XL-10 Gold with plasmid pJGS90

The $CaCl_2$-competent XL-10 Gold cells, prepared as per *Section 2.2.4.2*, were thawed on ice and 200µl transferred into a pre-chilled sterile tube. The pJGS90 plasmid was added to the cells and the mixture was incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 45 seconds and immediately put back on ice for 2 minutes. SOC media (800µl) was added and the culture was incubated at 37°C for one hour with gentle shaking. Following incubation, the cells were plated out on LB agar containing 10µg/ml tetracycline, 100µg/ml ampicillin and 25µg/ml chloramphenicol and incubated in an inverted position at 37°C overnight.

2.3.1.4 Restriction analysis on pJGS90 plasmid

Component	*Concentration per reaction*
Plasmid DNA (pJGS90)	1µg
*Eco*RI	12U
*Bam*HI	12U
NEB Buffer 2 (10X stock)[*a]	1X
NEB BSA (100X stock) [*b]	1X

Molecular grade water[*c] was added to a final volume of 20µl.

The digest was incubated for 2 hours at 37°C and then analysed on a 1% (w/v) agarose gel containing ethidium bromide (EtBr) for visualisation of DNA bands, as described in *Section 2.2.5*.

*[*a] New England Biolabs (NEB) Buffer 2 is supplied as a 10X stock, where 1X contains 50mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$ and 1mM dithiothreitol, pH 7.9.*

*[*b] NEB BSA is supplied as a 10mg/ml (100X) stock and is required at a final concentration of 100µg/ml for optimal activity of some restriction endonucleases.*

*[*c] Molecular grade water (Sigma-W4502) was used in all molecular biology experiments. The water is filtered (0.1µm) and free of nucleases and proteases.*

2.3.1.5 Small-scale protein expression

A single colony was used to inoculate 5 ml LB broth containing 10µg/ml tetracycline, 100µg/ml ampicillin and 25µg/ml chloramphenicol and grown overnight at 37°C with

shaking. The overnight culture was then used to inoculate 10ml of LB broth, containing the appropriate antibiotics, at 1% (v/v) cell density and the culture was incubated at 37°C with shaking, until the OD_{600} reached approximately 0.5. The culture was induced with 1mM IPTG, incubated for a further 4 hours at 37°C and then centrifuged at 3,000*g* for 20 minutes (Eppendorf 5810R). The bacterial cell pellet was resuspended in either 1ml of native buffer (distilled water) or urea denaturing buffer (8M urea, 100mM NaH_2PO_4, 10mM Tris, pH 8) and sonicated for 30sec with 2.5sec pulses at 220W. The samples were finally centrifuged to remove cell debris and the supernatants analysed by SDS-PAGE.

2.3.1.6 Optimisation of PLD expression conditions

2.3.1.6.1 *Determination of protein solubility*

A single colony was used to inoculate 5ml LB broth containing the appropriate antibiotics, previously defined, and grown overnight at 37°C with shaking. 200µl of this overnight culture was then used to inoculate 10ml of LB broth, containing the appropriate antibiotics, and the culture was incubated at 37°C with shaking, until the OD_{600} reached approximately 0.5. The culture was induced with 1mM IPTG, incubated for a further 4 hours at 37°C and then centrifuged at 3,000*g* for 20min (Eppendorf 5810R). The bacterial cell pellet was resuspended in either 1ml of distilled water (soluble protein) or urea denaturing buffer (insoluble protein) and sonicated for 30sec with 2.5sec pulses at 220W. Following centrifugation at 18,000*g* for 15min (Eppendorf 5810R), the supernatants were analysed by SDS-PAGE.

2.3.1.6.2 *Optimisation of IPTG concentration*

Bacterial cultures (n=6) were prepared and incubated, as described in *Section 2.3.1.5*, with the exception that protein expression was induced with various IPTG concentrations, ranging from 0 to 1mM.

2.3.1.6.3 *Optimisation of sonication condition*

Bacterial cultures (n=7) were prepared and incubated, as described in *Section 2.3.1.5*, with the exception that various sonication times, ranging from 0 to 60 seconds, were used to determine optimal sonication conditions.

2.3.1.6.4 *Time-course analysis*

Bacterial cultures (n=7) were prepared and incubated, as described in *Section 2.3.1.5.* The only exception was that 1ml samples were taken at hourly intervals following induction with IPTG for up to five hours and then following overnight induction. A non-induced sample was also taken before induction to act as a control.

2.3.1.7 Expression and purification of PLD under denaturing conditions

2.3.1.7.1 *Large-scale protein expression*

Starter cultures (10ml LB media, containing 10μg/ml tetracycline, 100μg/ml ampicillin and 25μg/ml chloramphenicol) were inoculated with a single colony of XL10-Gold *E. coli* cells (containing the pJGS90 plasmid bearing the PLD-encoding gene) and grown overnight at 37°C, with shaking at 250rpm. The following morning 500ml LB, containing the appropriate antibiotics, as before, was inoculated with 5ml of the overnight culture and incubated at 37°C with shaking, until the OD_{600} reached approximately 0.5. Protein expression was then induced upon addition of 0.05mM IPTG. Following incubation for 4 hours at 37°C, the culture was centrifuged at 3,000*g* for 20 minutes (Eppendorf 5810R) and the supernatant discarded. The bacterial cell pellet was resuspended in 20ml of denaturing buffer and vortexed gently. The culture was finally centrifuged at 3,000*g* for 20min at 4°C (Eppendorf 5810R) to remove cell debris and the cytoplasmic lysate was purified, as per *Section 2.3.1.7.2.*

2.3.1.7.2 *Purification of recombinant protein by immobilised metal affinity chromatography (IMAC)*

1ml of Ni-NTA resin (Qiagen Ltd.) was equilibrated with 10 ml of running buffer (8 M urea, 100mM NaH_2PO_4, 10 mM Tris, pH 8.0) in a 15ml polypropylene tube (Sarstedt Ltd.). The solution was then centrifuged, the supernatant decanted and the resin incubated with 4ml of the cytoplasmic lysate for 90 min at room temperature on an end-over-end rotator. This was then centrifuged and the supernatant ('flow-through') retained for analysis. The resin was then washed three times with 10ml of wash buffer (8M urea, 100mM NaH_2PO_4, 10mM Tris, pH 6.3) and wash fractions were also retained for analysis. The immobilised recombinant protein was finally eluted in four 1ml fractions with elution buffer (8M urea, 100mM NaH_2PO_4, 10mM Tris, pH 4.5). All fractions were analysed by SDS-PAGE, as described in *Section 2.2.3.*

2.3.1.8 Expression and purification of PLD under native conditions

2.3.1.8.1 *Large-scale protein expression*

Starter cultures (10ml SB media, containing 1% (w/v) glucose, 10μg/ml tetracycline, 100μg/ml ampicillin and 25μg/ml chloramphenicol) were inoculated with a single colony of XL10-Gold *E. coli* cells containing pJGS90, and grown overnight at 37°C, with shaking at 250rpm. The following morning, 500ml SB, containing 1% (w/v) glucose and the appropriate antibiotics, was inoculated with 5ml of the overnight culture and grown at 37°C for 8 hours with shaking at 200rpm. The culture was then centrifuged at 3,000*g* for 20min at 4°C (Eppendorf 5810R) and the supernatant was discarded. The pellet was resuspended in 500ml of fresh SB media, containing appropriate antibiotics (but no glucose) and the cells were incubated at 30°C with shaking at 200rpm. The culture was then induced with 0.05mM IPTG and incubated overnight at 30°C with shaking at 200rpm. The following morning the culture was centrifuged at 3,000*g* for 30min at 4°C (Eppendorf 5810R) and the supernatant was discarded. The pellet was resuspended in 20ml of column loading buffer (50mM NaH_2PO_4, pH8.0; 300mM NaCl, 10mM imidazole). The cells were sonicated on ice for 90sec min at 50% output (x2), followed by 15sec at 70% output (x2). The cells were then centrifuged in Oakridge tubes at 48,000*g* at 4°C for 30min (Beckman centrifuge) and the supernatant (lysate) was syringe filtered and purified, as per *Section 2.3.1.8.2.*

2.3.1.8.2 *Purification of recombinant protein by immobilised metal affinity chromatography (IMAC)*

The following purification method was carried out at 4°C and the column was never left to run completely dry. An Econo-Pac™ chromatography column (BioRad) was prepared by loading 2ml of resuspended Ni-NTA resin (Qiagen Ltd.) and left to settle to form a 1ml packed resin. The column was equilibrated with 10ml running buffer (50mM NaH_2PO_4, pH8.0; 300mM NaCl, 20mM imidazole). The lysate (20ml) was applied to the column 3 times and the flow-through collected. The column was then washed twice with 5ml wash buffer (50mM NaH_2PO_4, pH 8.0; 300mM NaCl, 20mM imidazole) to remove any non-specifically bound contaminating proteins. The protein was finally eluted with 5ml of elution buffer (50mM NaH_2PO_4, pH 8.0; 300mM NaCl, 250mM imidazole). The eluted PLD protein was buffer exchanged into PBS by

ultrafiltration (Vivaspin, Sartorius) and the performance of the purification process was evaluated using SDS-PAGE.

2.3.2 Development of an ELISA for the detection of CLA in sheep

All serum samples used in the development of the diagnostic assays for the detection of caseous lymphadenitis were kindly donated by Bernard Bradshaw of the Central Veterinary Research Laboratory (CVRL), Abbotstown, Dublin. A set of nine control serum samples, which had been taken from sheep 'on-site' were provided. These included five serum samples collected from sheep experimentally infected with *C. pseudotuberculosis* (GB04-013280, -013281, -013282, -013283, -013284) and four samples taken from sheep with no prior history of infection (GB04-013276, -013277, -013278, -013279). A panel of sera (n=92), which had been previously tested by a method described by ter Laak *et al.* (1992), were also obtained from Abbotstown.

2.3.2.1 Indirect ELISA for the detection of CLA in serum samples

Nunc Maxisorb™ plates was coated with 100µl of PLD, at a concentration of 10µg/ml in PBS (pH 7.4), and incubated for 1 hour at 37°C. Plates were washed three times with PBS and then blocked with 200µl PBS, pH 7.4, containing 4% (w/v) Marvel™ for 1 hour at 37°C. The plates were then washed three times with PBS and three times with PBST. CLA-positive and negative serum were diluted 1 in 50 in PBS, pH 7.4, containing 4% (w/v) Marvel™ and pre-incubated at 37°C for 1 hour. Following incubation, the immunoplate was washed as before and 100µl of each diluted serum sample was added to the wells. The plate was incubated again at 37°C for 1 hour and washed as before. This was followed by addition 100µl of a 1 in 4,000 dilution of HRP-labelled rabbit anti-sheep antibody (Dako) in PBS containing 4% (w/v) Marvel™ and incubated for 1 hour at 37°C. Plates were again washed and a chromogenic substrate, *o*-PD (0.4mg/ml *o*-phenylenediamine in 0.05 M phosphate citrate buffer, pH 5.0, and 0.4 mg/ml of urea hydrogen peroxidase) (Sigma-Aldrich Ireland Ltd.), was added and incubated for 30 minutes at 37°C. Absorbance was read at 450nm on a Titertek Reader.

2.3.3 Development of a Biacore assay for the detection of CLA in sheep

Analysis was performed using a Biacore 3000™ instrument operated with the Biacore 3000 Control Software package version 3.1.1. All data analysis was performed using

BIAevaluation 4.0.1. Research grade CM5 (carboxymethylated 5) sensor chips were employed and Hepes Buffered Saline (HBS), pH 7.4, containing 10mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), 150mM NaCl, 3.4mM EDTA and 0.05% (v/v) Tween 20, was used as the running buffer. This was freshly prepared, filtered (pore size 0.22μm) and degassed using a vacuum filtration apparatus (Millipore sintered glass filtration unit) before use. All samples were syringe filtered (0.2μm pore size) to remove any particulate matter.

2.3.3.1 Preconcentration studies

PLD (~10μg/ml) was prepared in 10mM sodium acetate buffer that had been adjusted with 10% (v/v) acetic acid to a range of pH values. The protein sample, at the respective pH, was passed over an underivatised chip surface, at a flow rate of 10μl/min for 1 minute, and the amount of electrostatic interaction monitored. The pH, which gave the highest degree of electrostatic interaction, *i.e.* maximum response, was chosen as the buffer for subsequent immobilisations.

2.3.3.2 Immobilisation of PLD protein onto sensor surface

Immobilisation of proteins was carried out according to standard amine coupling chemistry. Briefly, the carboxymethylated dextran surface was activated by mixing equal volumes of 100mM NHS (N–hydroxysuccinimide) and 400mM EDC (N-ethyl–N-(dimethyl–aminopropyl) carbodiimide hydrochloride) and injecting the mixture over the sensor chip surface for 8 minutes at a flow rate of 10μl/min. Protein in 10mM sodium acetate buffer, at pH 4.4, was injected over the activated surface for 40 minutes at a flow rate of 5μl/min. Unreacted NHS groups were capped by an injection of 1M ethanolamine, pH 8.5, for 7 minutes. Loosely bound protein was removed using three 30sec pulse of 5mM NaOH prior to use.

2.3.3.3 Non-specific binding studies*

Antibody solutions (or serum-containing antibody) at the optimal assay dilution were simultaneously injected over both the blank CM-dextran surface (reference flow cell) and a dextran surface with the analyte of interest immobilised. The binding response following each injection was monitored and used to determine the degree of non-specific binding of the antibodies to the dextran matrix.

2.3.3.4 Regeneration studies*

A known concentration of antibody (or serum-containing antibody) was passed over the chip surface, at the optimised flowrate, and the surface regenerated by passing over various concentrations of NaOH ranging from 1-100mM to assess the stability of the immobilised protein or hapten surface. This cycle of binding and regeneration was usually completed for greater than 50 cycles, and the binding signal measured to assess the stability and suitability of the immobilised surface for assay purposes.

** These methods are generic. Any amendments that have been made are clearly cited within the relevant results chapters within this thesis.*

2.4 Production and characterisation of genetically derived Fab fragments directed against aflatoxin B_1

2.4.1 Aflatoxin B_1 safety precautions

Aflatoxin B_1 (AFB_1) is a potent carcinogen that can cause heritable genetic damage. It is very toxic by inhalation, when in contact with skin or if swallowed. Therefore, all aflatoxin-related work was conducted in a fumehood and personal protective clothing was worn at all times. Spillages were cleaned up immediately using 12% (v/v) hypochlorite. All aflatoxin B_1-contaminated waste was decontaminated by treating waste with an equal volume of 12% (v/v) hypochlorite and incubation for 30 minutes at room temperature. The pH of the waste was then adjusted to 8.0 and left standing for 30 minutes. Finally, 5% (v/v) acetone was added and following incubation for 30 minutes the decontaminated waste was disposed of.

2.4.2 Construction of a chimeric mouse/human Fab library to AFB_1

The chimeric mouse/human Fab library was constructed using PCR assembly methods as described by Barbas *et al.* (2001). Variable regions were obtained from a murine AFB_1-specific scFv (Dunne *et al.*, 2005) and light- and heavy-chain constant regions were amplified from the pComb3X vector series, which contain a previously cloned human Fab. These pComb3X vectors were kindly provided by Prof. Carlos Barbas of the Scripps Research Institute, La Jolla, California 92037, USA.

2.4.2.1 Amplification of murine antibody variable regions

The primers listed below were obtained from MWG Biotech Ltd. and were used for the subsequent amplification of the murine variable antibody regions from a pAK100 vector containing the anti-AFB_1 scFv (kindly donated by Dr. Lynsey Dunne). The V-specific primers were adapted for the pComb3 vectors (Barbas *et al.*, 2001) from Krebber *et al.* (1997). Primers were mixed according to the degree of degeneration. MSCVL-1 and MHCL5-B primers were used for variable lambda (V_λ) light chain amplification and the MhyVH1 primer and MHyIgGCH1-B primer mix (reverse; MHyIgGCH1B1-B4) were used for V_H1 amplification. The following sequences are given using the standard IUPAC nomenclature of mixed bases where: R = A or G; M = A or C.

2.4.2.1.1 *Primers for amplification of variable heavy and light chain genes*

Variable light lambda (V_λ) forward primer

MSCVL-1
5′ GGGCCCAGGCGGCCGAGCTCGATGCTGTTGTGACTCAGGAAT 3′

Variable light lambda (V_λ) reverse primer

MHCL5-B
5′ CGAGGGGGCAGCCTTGGGCTGACCTAGGACAGTCAGTTTGG 3′

Variable heavy (V_H1) forward primer

MhyVH1
5′ GCTGCCCAACCAGCCATGGCCCTCGAGGTRMAGCTTCAGGAGTC 3′

Variable heavy (V_H) reverse primers

MHyIgGCH1-B1
5′ CGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGACCGTGGT 3′ 1

MHyIgGCH1-B2
5′ CGATGGGCCCTTGGTGGAGGCTGAGGAGACTGTGAGAGTGGT 3′ 1

MHyIgGCH1-B3
5′ CGATGGGCCCTTGGTGGAGGCTGCAGAGACAGTGACCAGAGT 3′ 1

MHyIgGCH1-B4
5′ CGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGACTGAGGT 3′ 1

2.4.2.1.2 *PCR components for amplification of variable chain genes of scFv*

Component	*Concentration per reaction (1X)*
Forward Primer Mix	25pmol
Reverse Primer Mix	25pmol
PCR Buffer (10X)	1X
$MgCl_2$	1.5mM
dNTP's	200µM
Plasmid DNA	10ng
Taq polymerase	1.25U

Molecular grade water was added to a final volume of 50µl.

2.4.2.1.3 *PCR conditions for amplification of variable chain genes of scFv*

30 cycles of :

94°C x 15 sec

56°C x 30 sec

72°C x 90 sec

followed by 72°C x 10 min

Five microlitres of each reaction was anlaysed on a 2% (w/v) agarose gel, as described in *Section 2.2.5.* The reaction was scaled up (5X), the PCR products pooled and ethanol-precipitated, as described in *Section 2.4.2.1.4.* The PCR products were purified from a 1.5% (w/v) agaorse gel, as described in *Section 2.4.2.1.5.* DNA was quantified by meauring the absorbance at 260nm on a Nanodrop spectrophotometer (ND-1000).

2.4.2.1.4 Ethanol Precipitation of DNA

One-tenth the volume of 3M sodium acetate, pH ~5.2-6, was added to a solution of DNA. The solution was mixed and two volumes of absolute ethanol was added, mixed and incubated at –20°C for 2 hours or overnight, depending on the concentration of DNA*. Following incubation, DNA was pelleted by centrifugation at 20,000*g* for 15-30 minutes at 4°C (Eppendorf 5810R). The supernatant was removed carefully without disrupting the pellet and the tube was centrifuged at 20,000*g* for 1

minute at 4°C (Eppendorf 5810R) to remove any residual liquid. The pellet was washed to remove excess contaminants (especially salts and chelators) in 1ml 70% (v/v) ethanol and centrifuged at 20,000*g* for 2 minutes at 4°C (Eppendorf 5810R). The supernatant was removed carefully and the tube re-centrifuged briefly to remove any remaining liquid. The pellet was air-dried and resuspended in the required amount of molecular grade water.

**More concentrated DNA solutions (>100ng/ml) required less incubation time.*

2.4.2.1.5 Purification of PCR reaction products

Purification of PCR reaction products was performed using an Eppendorf Perfectprep® Gel Cleanup Kit. The PCR products were separated by agarose gel electrophoresis, as described in *Section 2.2.4*. The appropriate bands were excised using a clean scalpel, transferred to a clean collection tube and weighed. Binding buffer was added to a volume of three times the weight of the agarose slice. The samples were inverted three times and incubated at 50°C for approx. 10 minutes, until the agarose had melted. Isopropanol was added at a volume equal to that of the weight of the agarose slice. The mixture was poured into a spin column and centrifuged for 2 minutes at 14,000*g* (Hereaus Biofuge Pico). The 'flow-through' was discarded and the spin column washed with 750µl of wash buffer. The column was centrifuged for 1 minute at 14,000*g* (Hereaus Biofuge Pico) and the 'flow-through' discarded. The column was centrifuged as before to remove any residual wash buffer. The spin column was transferred to a clean tube, 30µl of molecular grade water was added and the column centrifuged at 14,000*g* for 1 minute (Hereaus Biofuge Pico) to elute DNA. Purified PCR products were stored at –20°C until required.

2.4.2.2 Amplification of human antibody constant regions

Human C_λ region-pelB leader sequences and C_H1 chain human were amplified from pComb3Xλ and pComb3XTT, respectively. These pComb3 vectors contain standard human Fab inserts (Barbas *et al.*, 2001).

2.4.2.2.1 *Primers for amplification of constant heavy and light chain genes*

Constant light lambda (C_λ) primers

HLC-F (forward)
5′ GGTCAGCCCAAGGCTGCCCCC 3′

Lead-B (reverse)
5′ GGCCATGGCTGGTTGGGCAGC 3′

Constant heavy (C_H1) primers

HIgGCH1-F (forward)
5′ GCCTCCACCAAGGGCCCATCGGTC 3′

dpseq (reverse)
5′ AGAAGCGTAGTCCGGAACGTC 3′

2.4.2.2.2 *PCR components for amplification of constant heavy and light chain genes*

Component	*Concentration per reaction (1X)*
Forward Primer Mix	25pmol
Reverse Primer Mix	25pmol
PCR Buffer (10X)	1 X
$MgCl_2$	1.5mM
dNTP's	200µM
Plasmid DNA (pComb3XTT or pComb3Xλ)	10ng
Taq polymerase	1.25U

Molecular grade water was added to a final volume of 50µl.

2.4.2.2.3 *PCR conditions for amplification of constant heavy and light chain genes*

20 cycles of :

94°C x 15 sec

56°C x 30 sec

72°C x 90 sec

followed by 72°C x 10 min

A five microlitre volume from each reaction was anlaysed on a 2% (w/v) agarose gel, as described in *Section 2.2.5.* The reaction was scaled up (5X), the PCR products pooled and ethanol-precipitated (*Section 2.4.2.1.4*). The PCR products were then purified from a 1.5% (w/v) agarose gel, as described in *Section 2.4.2.1.5.* DNA was quantified by meauring the absorbance at 260nm on a Nanodrop spectrophotometer (ND-1000).

2.4.2.3 Assembly of mouse variable regions with human constant regions

Mouse variable region PCR products were combined with the human constant region PCR products by overlap extension PCR to generate chimeric light-chain and heavy-chain fragments.

2.4.2.3.1 *Primers for PCR assembly of mouse V_L sequences with human C_λ PCR product*

RSC-F (forward)
5′ GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC 3′

Lead-B (reverse)
5′ GGCCATGGCTGGTTGGGCAGC 3′

2.4.2.3.2 *PCR components for assembly of mouse V_L sequences with human C_λ PCR product*

Component	*Concentration per reaction (1X)*
Forward primer (RSC-F)	25pmol
Reverse primer (Lead-B)	25pmol
PCR Buffer (10X)	1X
$MgCl_2$	1.5mM
dNTP's	200µM
Purified V_L product	50ng
Purified C_λ product	50ng
Taq polymerase	1.25U

Molecular grade water was added to a final volume of 50µl.

2.4.2.3.3 *Primers for PCR assembly of mouse V_H sequences with human C_H1 PCR product*

leadVH (forward)
5′ GCTGCCCAACCAGCCATGGCC 3′

dpseq (reverse)
5′ AGAAGCGTAGTCCGGAACGTC 3′

2.4.2.3.4 *PCR components for assembly of mouse V_H sequences with human C_H1 PCR product*

Component	*Concentration per reaction (1X)*
Forward primer (leadVH)	25pmol
Reverse primer (dpseq)	25pmol
PCR Buffer (10X)	1 X
$MgCl_2$	1.5mM
dNTP's	200μM
Purified V_L product	50ng
Purified C_λ-pelB product	50ng
Taq polymerase	1.25U

Molecular grade water was added to a final volume of 50μl.

2.4.2.3.5 *PCR conditions for assembly of mouse variable regions with human constant regions*

15 cycles of :
94°C for 15 seconds
56°C for 30 seconds
72°C for 2 minutes
followed by 72°C for 10 minutes

Five microlitres of each reaction was anlaysed on a 1.5% (w/v) agarose gel, as described in *Section 2.2.5.* The reaction was scaled up (5X), the PCR products pooled

and ethanol- precipitated, as described in *Section 2.4.2.1.4.* The PCR products were purified from a 1% (w/v) agaorse gel, as described in *Section 2.4.2.1.5.* DNA was quantified by measuring the absorbance at 260nm on a Nanodrop spectrophotometer (ND-1000).

2.4.2.4 Assembly of chimeric Fab fragment

Chimeric light-chain fragments were combined with the chimeric Fd fragments by overlap extension PCR to generate chimeric Fab fragments.

2.4.2.4.1 *Primers for PCR assembly of chimeric light-chain sequences with chimeric heavy-chain (Fd) sequences*

RSC-F (forward)
5′ GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC 3′

dp-EX (reverse)
5′ GAGGAGGAGGAGGAGGAGAGAAGCGTAGTCCGGAACGTC 3′

2.4.2.4.2 *PCR components for assembly of chimeric light-chain sequences with chimeric heavy-chain (Fd) sequences*

Component	*Concentration per reaction (1X)*
Forward primer (RSC-F)	25pmol
Reverse primer (dp-EX)	25pmol
PCR Buffer (10X)	1 X
$MgSO_4$	4µl
dNTP mix	200µM
Purified chimeric light-chain product	100ng
Purified chimeric heavy-chain (Fd) product	100ng
HiFi *Taq* polymerase*	0.4µl

Molecular grade water was added to bring the final reaction volume to 100µl.

** High fidelity (HiFi) polymerase (Invitrogen) was used to improve efficiency of generating Fab overlap PCR product. The unique enzyme mix contains thermostable Taq and Pwo DNA polymerases and is designed to give high fidelity, high yield and high specificity PCR products.*

2.4.2.4.3 *PCR conditions for assembly of chimeric light-chain sequences with chimeric heavy-chain (Fd) sequences*

94°c for 2 minutes

12 cycles of :

94°C for 15 seconds

56°C for 20 seconds

72°C for 3 minutes

followed by 72°C for 10 minutes

Five microlitres of each reaction was anlaysed on a 1.5% (w/v) agarose gel (*Section 2.2.5*). The reaction was scaled up (5X), the PCR products pooled and ethanol-precipitated (*Section 2.4.2.1.4*). The PCR products were purified from a 1% (w/v) agarose gel, as described in *Section 2.4.2.1.5*. DNA was quantified by meauring the absorbance at 260nm on a Nanodrop spectrophotometer (ND-1000).

2.4.2.5 *Restriction-digest of the purified overlap PCR product and vector DNA*

Restriction-digest of the purified overlap PCR product:

Component	*Concentration per reaction (1X)*
Purified overlap PCR product	8µg
*Sfi*I (16U per µg of DNA)	130U
NEB Buffer 2 (10X)	1X
BSA (100X)	1X

Molecular grade water was added to a final volume of 200µl.

Restriction-digest of the purified vector DNA:

Component	*Concentration per reaction (1X)*
Vector DNA (pComb3XSS)	28µg
*Sfi*I (7U per µg of DNA)	200U
NEB Buffer 2 (10X)	1X
BSA (100X)	1X

Molecular grade water was added to a total volume of 200µl.

The digests were incubated for 5 hours at 50°C. PCR products were pooled and ethanol-precipitated. The digested PCR fragment and the pComb3XSS vector and stuffer fragment were purified on a 1% (w/v) agarose gel and 0.6%(w/v) agarose gel, respectively, by electroelution. DNA was quantified by meauring the absorbance at 260nm on a Nanodrop spectrophotometer (ND-1000).

2.4.2.6 Ligation of the digested overlap PCR product with vector DNA

A small-scale ligation was initially performed to assess the efficiency of ligation and transformation. Two control ligations were also performed. The first control ligation was to assess the ligation efficiency of the vector DNA by ligating it with the purified stuffer fragment. The second control ligation was performed, containing only vector DNA, to ensure the vector did not self-ligate and to estimate the amount of uncut and partially cut vector DNA.

2.4.2.6.1 Test ligations

Small-scale test ligation:

Component	*Concentration per reaction (1X)*
pComb3XSS (digested and purified)	140ng
Overlap PCR product (digested and purified)	140ng
T4 DNA ligase buffer	1 X
T4 DNA ligase	1µl

Molecular grade water was added to a total volume of 20µl.

Control ligation 1 (control with stuffer fragment):

Component	*Concentration per reaction (1X)*
pComb3XSS (digested and purified)	140ng
Stuffer fragment (purified)	140ng
T4 DNA ligase buffer	1 X
T4 DNA ligase	1µl

Molecular grade water was added to a total volume of 20µl.

Control ligation 2 (test for vector self-ligation):

Component	*Concentration per reaction (1X)*
pComb3XSS (digested and purified)	140ng
T4 DNA ligase buffer	1 X
T4 DNA ligase	1µl

Molecular grade water was added to a total volume of 20µl.

Ligations were incubated at 16°C overnight. One microlitre of each ligation was transformed into *E. coli* ER2537 by electroporation, as described by *Section 2.4.2.8.* The transformed cultures were then diluted 10- and 100-fold with prewarmed (37°C) SOC and 100µl of each dilution plated out on LB plates containing 100µg/ml carbenicillin.

2.4.2.6.2 Library ligation

Component	*Concentration per reaction (20X)*
pComb3XSS (digested and purified)	2.8µg
Overlap PCR product (digested and purified)	2.8µg
T4 DNA ligase buffer	1 X
T4 DNA Ligase	10µl

Molecular grade water was added to a total volume of 400µl.

Ligations were incubated at 16°C overnight. One microlitre of glycogen was added to the ligation and it was ethanol-precipitated as described in *Section 2.4.2.1.4.* The DNA pellet was resuspended in 20µl of molecular grade water and transformed into *E. coli* ER2738 electrocompetent cells by electroporation (*Section 2.4.2.7* and *2.4.2.8*).

2.4.2.7 Production of electrocompetent *E. coli* ER2738 cells

All work surfaces, culture flasks and centrifuge containers were bleached prior to cell preparation to ensure they were free of phage contamination. Prewarmed SB (15ml) was inoculated with a single colony of *E. coli* ER2738 and incubated at 37°C overnight with shaking at 250rpm. Six 2L culture flasks, containing 500ml of SB broth, 0.4% (w/v) glucose and 10mM $MgCl_2$, were each inoculated with 2.5ml of the

overnight culture and incubated at 37°C with shaking at 250prm until OD_{600} reached 0.8-0.9. The cells and 500ml centrifuge tubes (Sorvall) were incubated on ice for 15 minutes. From this stage onwards everything was kept on ice and done as quickly as possible. Cells were then centrifuged at 3,000*g* for 20 minutes at 4°C (Eppendorf 5810R). The supernatant was decanted and each of the six pellets resuspended in 25ml of ice-cold 10% (v/v) glycerol and then two of each of the resuspended pellets combined in one pre-chilled centrifuge tube (Sorvall) and pre-chilled 10% (v/v) glycerol added to a final volume of 500ml. The cells were pelleted by centrifugation at 3,000*g* for 20 minutes at 4°C (Eppendorf 5810R). Each of the three pellets was resuspended in 500ml of pre-chilled 10% (v/v) glycerol and centrifuged as before. The supernatant was decanted and each pellet resuspended in 25ml of ice-cold 10% (v/v) glycerol and transferred to a prechilled 50ml polypropylene tube. The cells were re-centrifuged at 2,500*g* for 15 minutes at 4°C (Eppendorf 5810R). The supernatant was carefully decanted and each pellet resuspended in 5ml of the pre-chilled 10% (v/v) glycerol and combined. Cells were aliqouted into 1.5ml sterile microcentrifuge tubes (300µl/tube) and flash frozen in a liquid nitrogen bath and transferred to a -80°C freezer until required. Cells were tested for competency and potential contamination with phagemids, helper and lytic phage prior to use.

2.4.2.8 Library transformation

Ligations and electroporation cuvettes (0.2cm, Bio-Rad) were incubated on ice for 10 minutes. At the same time the electrocompetent ER2738 cells were thawed on ice. Each ligation was added to the cells (300µl), mixed gently, transferred to a chilled cuvette and incubated in ice for 1 minute. The sample was the electroporated at 2.5kV, 25µF, 200Ω ($\tau \approx$ 4msec) (Bio-Rad Gene Pulser Xcell^{TM}). The cuvette was immediately flushed with 1ml SOC, followed by 2ml SOC (x2) at room temperature and transferred to 50ml polypropylene tube. The cells were incubated at 37°C for one hour with shaking at 250rpm. Following incubation 10ml of pre-warmed (37°C) SB media containing 20µg/ml carbenicillin was added and the culture was incubated for a further hour at 37°C with shaking (2µl of the culture was taken to estimate titre of transformed bacteria before incubation*).

**For estimation of transformation titre, 2µl of culture was diluted in 200µl SB medium and plated out (10µl and 100µl) on LB agar containing 100µg/ml carbenicillin. The plates were incubated overnight at 37°C and the total number of transformants calculated.*

2.4.3 Selection and characterisation of specific Fab fragments from a library

2.4.3.1 Rescue of Fab-displaying phage

Following incubation of the culture (*Section 2.3.1.6.2*), carbenicillin was added to a final concentration of 30μg/ml and the culture incubated for an additional hour at 37°C with shaking. 5ml of M13K07 helper phage (10^{11}pfu/ml) and 180ml of prewarmed SB, containing 50μg/ml carbenicillin, was then added and the 200ml culture incubated for 2 hours at 37°C with shaking at 300rpm. Kanamycin, at a concentration of 70μg/ml, was then added and the culture was incubated overnight at 37°C with shaking at 300prm.

2.4.3.1.1 Preparation of M13K07 helper phage

SB (2ml) was inoculated with 2μl of an *E. coli* ER2738 culture and incubated at 37°C for one hour with shaking at 250rpm. Dilutions (10^{-6}, 10^{-7} and 10^{-8}) of M13K07 helper phage ($1x10^{11}$pfu/ml), purchased from NEB, were prepared in SB medium. 1μl of each dilution was added to 50μl of the ER2738 culture and incubated for 15 minutes at room temperature. Cooled liquefied LB top agar (3ml) was then added to the culture and poured onto prewarmed LB agar plates. The plates were incubated at 37°C overnight.

Pre-warmed SB (10ml) was inoculated with 10μl of ER2738 and incubated at 37°C for one hour with shaking at 250rpm. The culture was then inoculated with a single M13K07 plaque and incubated at 37°C for 2 hours with shaking at 250rpm. The infected culture was transferred to a 2L flask containing 500ml SB, followed by the addition of kanamycin, at a final concentration of 70μg/ml. The culture was then incubated at 37°C overnight with shaking at 250rpm. The following morning the culture was centrifuged at 2,500*g* for 15 minutes (Eppendorf 5810R). The supernatants were incubated in a water bath at 70°C for 20 minutes and centrifuged at 2,500*g* for 15 minutes (Eppendorf 5810R). The supernatants were transferred to sterile 50ml tubes, the phage titre* estimated and the phage stored at 4°C.

** For estimation of helper phage titre, 2ml SB medium was inoculated with 2μl of ER2738 and incubated for 1 hour at 37°C. Dilutions (10^{-7}, 10^{-8} and 10^{-9}) of M13K07 helper phage preparation were prepared in SB medium.1μl of each dilution was used to infect 50μl of the ER2738 culture and the culture was incubated for 15 minutes at room temperature. Cooled*

liquefied LB top agar (3ml) was the added to the culture and poured onto prewarmed LB agar plates. The plates were incubated at 37°C overnight and the M13K07 titre calculated.

2.4.3.2 Concentration of phage by PEG/NaCl precipitation

The following morning, the culture was centrifuged at 3,000g for 15 minutes at 4°C (Eppendorf 5810R). The supernatant was transferred to a sterile 500ml centrifuge tube (Sorvall) and 4%(w/v) of PEG-8000 and 3%(w/v) of NaCl were added and dissolved by incubation for 5 minutes at 37°C with shaking at 300rpm. Cultures were incubated on ice for 30 minutes and centrifuged at 15,000*g* for 15 minutes at 4°C (Eppendorf 5810R). The supernatant was discarded and any residual liquid was removed. The pellet was resuspended in 2ml PBS containing 1% (w/v) BSA. The phage preparation was centrifuged at 20,000*g* for 5 minutes at 4°C and the supernatant filtered (0.2μm). Sodium azide was added to a final concentration of 0.02% (v/v) and the phage stored at 4°C until required (*Section 2.4.3.3*).

2.4.3.3 Selection of antigen binders by biopanning

2.4.3.3.1 Selection of antigen binders on immunotubes

A Nunc Maxisorb™ immunotube were coated with 5ml of AFB_1-BSA, at a concentration of 10μg/ml, and incubated overnight at 4°C. The immunotube was washed twice with sterile filtered PBS, pH 7.4, and blocked with PBS containing 3% (w/v) BSA for 1 hour at 37°C. One millilitre of PBS containing 1% (w/v) BSA was mixed with 250μl of freshly prepared phage library (prepared as per *Section 2.4.3.2*) and added to the immunotubes. Following incubation for 1 hour at room temperature with mixing by rotation, non-specific phage was removed by washing the immunotube ten times with PBST and ten times with PBS. Bound phage was eluted with 1μg/ml of AFB_1 in PBS containing 5% (v/v) methanol.

2.4.3.3.2 Selection of antigen binders on microtitre wells

Nunc Maxisorb™ plates were coated with 100μl of AFB_1-BSA, at a concentration of 10μg/ml and incubated overnight at 4°C. Wells were washed twice with PBS and blocked with 300μl of PBS containing 3% (w/v) BSA for 1 hour at 37°C. The plates were then washed three times with PBS and three times with PBST. Fresh prepared phage library was then added (100μl) and the plate was incubated at 37°C for 2 hours. Non-specific phage was removed by washing the plate five times with PBST and then

five times with PBS. Bound phage was eluted with the addition of 100µl/well of freshly prepared trypsin, at a concentration of 10mg/ml in PBS, and incubation for 30 minutes at 37°C.

2.4.3.4 Re-infection of *E. coli* ER2738 cells with eluted phage

Eluted phage (from *Section 2.4.3.3*) was added to an overnight culture of *E. coli* ER2738 (2ml) and incubated for 15 minutes at room temperature. Pre-warmed SB (6ml), containing 20µg/ml carbenicillin was then added and the culture incubated at 37°C for one hour (2µl of this culture was taken for estimation of output titre*). Following incubation, carbenicillin was added to a final concentration of 30µg/ml and the culture incubated for an hour at 37°C with shaking. M13 helper phage (2ml at 10^{11}pfu/ml) and prewarmed SB (39ml), containing 50µg/ml of carbenicillin, were added and the culture incubated for approx. 2 hours at 37°C with shaking at 300rpm. Kanamycin was then added, at a final concentration of 70µg/ml, and the culture incubated overnight at 37°C with shaking at 300rpm. The culture was centrifuged at 3,000*g* for 15 minutes at 4°C (Eppendorf 5810R). The supernatant was transferred to a sterile 500ml centrifuge tube (Sorvall) and the phage concentrated by PEG/NaCl precipitation as described in *Section 2.4.3.2*. Freshly prepared phage was then used in the next round of panning (*Section 2.4.3.3.2*).

**For estimation of output titre, infected eluted phage (2µl) was diluted in 200µl of SB media and plated out (in 10µl and 100µl volumes) on LB agar containing 100µg/ml carbenicillin. The plates were incubated overnight at 37°C and the total number of transformants calculated.*

2.4.3.5 Polyclonal phage pool ELISA

A Nunc Maxisorb™ plate was coated with 100µl per well of 10µg/ml of AFB_1-BSA (18 wells) and 10µg/ml of BSA (18 wells) and incubated overnight at 4°C. The plate was washed three times with deionised water and then blocked with 300µl PBS containing 5% (w/v) Marvel™ for 1 hour at 37°C. Phage (from each round of panning), diluted 3-fold in PBS containing 5% (w/v) Marvel™, was added to the plate in triplicate at 100µl per well. Positive (anti-AFB_1 monoclonal antibody) and negative (M13 helper phage) controls were also added to the plate in triplicate. The plate was incubated for 2 hour at 37°C and then washed 10 times with deionised water. Bound

antibodies were detected with the addition of 100µl of a 1 in 1,000 dilution of HRP-labelled anti-HA antibody (Roche) in PBS containing 5% (w/v) Marvel™. The plate was incubated for 1 hour at 37°C, washed as before and *o*-PD (Sigma) was added (100µl/well). Following incubation for 30 minutes at 37°C, the absorbance was read at 450nm on a Tecan Safire^{2} ™.

2.4.3.6 Master plate construction

A 96 well sterile culture plate was filled with 100µl per well of SB containing 50µg/ml carbenicillin. Each well was inoculated with a single colony from the phage titre plates (*n*=96). A negative (non-inoculated well) and positive (inoculated with just *E. coli* ER2738) control was also included. The plate was incubated at 37°C overnight while shaking at 150rpm. This was known as the master plate and was stored at -80°C with 15% (v/v) glycerol. Another plate was prepared with 180µl per well SB containing 50µg/ml carbenicillin. A few microlitres from each well of the master plate was transferred to the corresponding well on the second plate and incubated at 37°C with shaking at 150rpm until $OD_{600} \approx 0.5$. SB (5ml) containing M13 helper phage (500µl at 10^{11}pfu/ml) was added to each well (25µl/well) and the plate was incubated for 1½ hours at 37°C with shaking at 150rpm. SB containing 70µg/ml of kanamycin was then added to each well (25µl/well) and the plate was incubated at 37°C overnight. The following morning the plate was centrifuged at 2,500*g* for 10 minutes (Eppendorf 5810R) and the supernatant analysed by ELISA (as per *Section 2.4.3.7*).

2.4.3.7 Monoclonal Phage ELISA

A Nunc Maxisorb™ plate was coated with 100µl of 10µg/ml of AFB_1-BSA and incubated overnight at 4°C. The plate was washed once with PBS and blocked with 200µl per well of PBS containing 5% (w/v) Marvel™ for 1 hour at 37°C. Phage supernatant (50µl/well) and PBS containing 5% (w/v) Marvel™ (50µl/well) were added to the corresponding well in each ELISA plate, mixed gently and incubated for 2 hours at 37°C. The plates were washed three times with PBS and three times with PBST, followed by the addition of 100µl per well of HRP-labelled anti-HA antibody, at a 1 in 1,000 dilution in PBST containing 5% (w/v) Marvel™. The plate was incubated for 1 hour at 37°C, washed as before and *o*-PD (Sigma) was added

(100µl/well). Following incubation for 30 minutes at 37°C, the absorbance was read at 450nm on a Tecan Safire$^{2\,TM}$.

2.4.3.8 Production of soluble Fab fragments

Plasmids (containing the antibodies of interest) were isolated from *E. coli* ER2738 as described in *Section 2.3.1.1* and transformed into a nonsuppressor strain of *E. coli* (*i.e.* Top10F′, Invitrogen) by electroporation as per *Section 2.4.2.8*. Single colonies were used to inoculate 5 ml SB broth containing 50µg/ml carbenicillin and incubated at 37°C with shaking until $OD_{600} \approx 0.5$ (Stocks were made of each culture by pipetting 10µl of each culture onto LB agar containing 100µg/ml carbenicillin, which were incubated overnight at 37°C). The cultures were induced with 2mM IPTG and incubated overnight at 37°C with shaking at 250rpm. The induced cultures were centrifuged at 2,800*g* for 15 minutes at 4°C (Eppendorf 5810R) and the pellets resuspended in 300µl PBS. The cell suspension was transferred to a 1.5ml microcentrifuge tube and cells lysed by freezing them in a –80°C freezer for 3-5 minutes followed by thawing in a 37°C water bath. This freeze/thaw process was repeated three times and the cell debris pelleted by centrifugation at 20,000*g* for 15 minutes (Eppendorf 5810R). The supernatants (containing antibody fragments) were then transferred to a sterile 1.5ml microcentrifuge tube and analysed by ELISA (*Section 2.4.3.11*).

2.4.3.9 Sequencing of the anti-AFB_1 antibody sequences

2.4.3.9.1 Preparation of stab cultures

Liquid LB agar (1ml), containing the appropriate antibiotics, was poured into a 2ml screw-cap vial under sterile conditions and left to solidify. A sterile inoculation loop was used to pick a single colony of *E. coli,* containing the plasmid bearing the anti-AFB_1 antibody gene* and stabbed into the agar several times. The vials (with the caps slightly loose) were incubated at 37°C overnight. The following day the caps were sealed tightly and stored at 4°C until required.

** E. coli Top10F′ containing the plasmid pComb3X bearing either the G6 or D11 Fab insert or E. coli JM83 containing the plasmid pAK400 bearing the scFv insert were used.*

2.4.3.9.2 Sequence analysis

Plasmid DNA (prepared as in *Section 2.3.1.1*) or stab cultures (prepared as in *Section 2.4.3.9.1*) containing either the anti-AFB_1 G6 Fab, D11 Fab or scFv gene sequence were sent to Qiagen (UK) Ltd, for sequencing. Single reads were performed in both directions using the primers listed in *Table 2.2*. Sequence analysis of the scFv was performed with the oligonucleotide primers, pAK400for and pAK400back. Sequence analysis of the Fab fragments (heavy and light chains) was performed with the oligonucleotide primers, ompseq and pelseq. Sequence analysis of the Fab heavy chain was performed with the oligonucleotide primers, pelseq (primes from pelB leader sequence forward through heavy chain) and dpseq (primes from HA tag back through heavy chain). While sequence analysis of the Fab light chain was performed with the oligonucleotide primers, ompseq (primes from ompA leader region forward through light chain) and kpel (primes from pelB leader sequence back through light chain).

Sequencing results, received as linear nucleotide sequences, were subsequently translated into amino acid sequences using the Expasy translate tool. Protein and nucleotide sequences were aligned using the PBIL protein alignment and MultiAlin nucleotide sequence alignment programs, respectively (*Table 2.3*).

Table 2.2. *Primers for sequencing scFv or Fab fragments in pAK400 or pComb3X vectors*

Name	Nucleotide Sequence
pAKfor	5′ CGGCAGCCGCTGGATTGTTATTAC 3′
pAKback	5′ CATTTTTCACTTCACAGGTCAAGC 3′
ompseq	5′ AAGACAGCTATCGCGATTGCAG 3′
pelseq	5′ ACCTATTGCCTACGGCAGCCG 3′
kpel	5′ CGGCTGCCGTAGGCAATAGGT 3′
dpseq	5′ AGAAGCGTAGTCCGGAACGTC 3′

Table 2.3. Web-based proteomics and sequence analysis tools

Bioinformatics Tool	Website address
Expasy translate tool	http://expasy.org/tools/dna.html
MultiAlin nucleotide sequence alignment	http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html
PBIL protein alignment tool	http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin.html
Kabat Rules	http://acrmwww.biochem.ucl.ac.uk/abs/

2.4.3.10 Antibody expression and purification

2.4.3.10.1 *Shaker culture production of Fab fragments*

A single colony of Top10F′ *E. coli* cells containing pComb3X (bearing the G6 or D11 antibody encoding gene) was inoculated into 10ml SB media, containing 1% (w/v) glucose, 50μg/ml carbenicillin (SB_{GC}) and grown overnight at 37°C, with shaking at 250rpm. The overnight cultures were then used to seed 500ml SB_{GC} at a cell density of 1% and grown at 37°C for 8 hours with shaking at 220rpm. The culture was then centrifuged at 3,200*g* for 20min at 4°C (Eppendorf 5810R) and the supernatant was discarded. The pellet was resuspended in 500ml of fresh SB_C media (no glucose) and the cells were incubated for one hour at 25°C with shaking at 200rpm. The culture was then induced with 1mM IPTG and incubated overnight at 25°C with shaking at 220rpm. The following morning the culture was centrifuged at 3,000*g* for 30min at 4°C (Eppendorf 5810R) and the supernatant was discarded. The pellet was resuspended in 20ml of column loading buffer (50mM NaH_2PO_4, pH 8.0; 300mM NaCl, 10mM imidazole). The cells were sonicated on ice for 90sec min at 50% output (x2), followed by 15sec at 70% output (x2). The cells were then centrifuged in Oakridge tubes at 48,000*g* at 4°C for 30min (Beckman centrifuge) and the supernatant (crude periplasmic lysate) was syringe filtered (0.2μm) and purified, as per *Section 2.4.3.10.4.*

2.4.3.10.2 *Shaker culture production of scFv fragments*

Soluble expression of the AFB_1-specific scFv was carried out as described in *Section 2.4.3.10.1* but with the following exceptions. Cultures of *E. coli* JM83 containing the pAK400 vector (bearing the scFv gene) were prepared in TB supplemented with 1% (w/v) glucose, 25μg/ml chloramphenicol and 25μg/ml streptomycin sulphate (TB_{GCS}) and TB_{CS} (no glucose) for protein expression.

2.4.3.10.3 *Production of Fab fragments by fermentation*

Starter cultures (50ml SB media, containing 100μg/ml ampicillin) were inoculated with a single colony of Top10F′ *E. coli* cells containing pComb3XSS bearing the G6 antibody encoding gene, and grown overnight at 37°C, with orbital shaking at 250rpm. The following morning, 6L of SB was added to the fermenter and sterilised. Ampicillin (100μg/ml) and the 50ml overnight culture were then added through the side port of the fermenter unit under sterile conditions. The culture was grown for 6 hours at 37°C with shaking at 100rpm and an airflow rate of 1wm. Antibody expression was then induced with the addition of IPTG, to a final concentration of 0.5mM, and the culture was incubated overnight at 26°C with shaking at 100rpm. The following morning the culture was decanted into 250ml centrifuge bottles (Sorvall) and centrifuged at 3,000*g* for 30min at 4°C (Eppendorf 5810R) and the supernatant was discarded. Pellets were resuspended in a total volume of 80ml of column loading buffer (50mM NaH_2PO_4, pH 8.0; 300mM NaCl, 10mM imidazole). The 80ml cell suspension was then sonicated on ice for 3 minutes at 50% output (x2), followed by 10 seconds at 80% output (x2). The cells were then centrifuged in Oakridge tubes at 48,000*g* at 4°C for 30min (Beckman centrifuge) and the supernatant (lysate) was sterile filtered (0.2μm) to remove small particulates. The filtered supernatants were transferred to prepared IMAC columns (2ml supernatant per 1ml packed resin) and purified as described in *Section 2.4.3.10.4*.

2.4.3.10.4 *Purification of anti-AFB_1 recombinant antibodies by immobilised metal affinity chromatography (IMAC)*

The following purification method was carried out at 4°C and the column was never left to run completely dry. An Econo-Pac™ chromatography column (BioRad) was prepared by loading 2ml of resuspended Ni-NTA resin (Qiagen Ltd.) and left to settle

to form a 1ml packed resin. The column was equilibrated with 10ml running buffer (50mM NaH_2PO_4, pH 8.0; 300mM NaCl, 10mM imidazole). The lysate (20ml) was applied to the column 3 times and the 'flow-through' collected. The column was then washed twice with 5ml wash buffer (50mM NaH_2PO_4, pH 8.0; 300mM NaCl, 40mM imidazole) to remove any non-specifically bound contaminating proteins. The antibody was finally eluted with 5ml of elution buffer (50mM NaH_2PO_4, pH 8.0; 300mM NaCl, 250mM imidazole). The eluted antibody was buffer exchanged into PBS and concentrated by ultrafiltration (Vivaspin, Sartorius). The resultant antibody solution was dispensed in 20µl volumes and stored at –20°C until required. The performance of the purification process was evaluated using SDS-PAGE, as described in *Section 2.2.3.*

2.4.3.11 Enzyme-linked immunosorbent assay (ELISA)

2.4.3.11.1 *Checkerboard ELISA for determination of optimal Fab or scFv antibody dilution and optimal coating concentration of AFB_1-BSA conjugate*

Nunc Maxisorb™ plates was coated with 100µl of varying concentrations (0-50µg/ml) of AFB_1-BSA prepared in PBS for 1 hour at 37°C or overnight at 4°C. Plates were washed three times with PBS and blocked with 200µl of PBS containing 4% (w/v) Marvel™ for 1 hour at 37°C. The plates were then washed three times with PBST and three times with PBS. Serial dilutions of AFB_1-specific Fab or scFv antibody were prepared in PBST containing 2% (w/v) Marvel™ and 100µl of each dilution added to the plates in triplicate. The plates were then incubated at 37°C for 1 hour and washed as before. This was followed by the addition of 100µl of a 1 in 1,000 dilution of HRP-labelled anti-HA (Roche) or 1 in 1,000 dilution of HRP-labelled anti-His antibodies (Sigma) in PBST containing 2% (w/v) Marvel™ for detection of Fab and scFv fragments, respectively. Plates were incubated for 1 hour at 37°C, washed as before and the substrate TMB was added. The reaction was allowed to proceed until a blue colour developed and was then stopped using 100µl/well of 1M HCl. Absorbance was read at 450-650nm on a Tecan Safire^{2}™.

2.4.3.11.2 *Inhibition ELISA for Fab or scFv antibody*

Nunc Maxisorb™ plates were coated with the appropriate concentration of AFB_1-BSA and blocked, as described in *Section 2.4.3.11.1. A* 1mg/ml stock solution of AFB_1 was prepared in 100% methanol. Standards of decreasing concentrations of

analyte were prepared in PBS containing 5% (v/v) methanol. Each standard was added to an equal volume (50µl) of AFB_1-specific antibody at the optimal concentration in PBST containing 2% (w/v) Marvel™. The samples were incubated for 30 minutes at room temperature and added to the microtitre plate in triplicate. Bound antibody was detected following the addition of HRP-labelled anti-HA (Roche) or 1 in 1,000 dilution of HRP-labelled anti-His antibodies (Sigma) in PBST containing 1% (w/v) Marvel™ for detection of Fab and scFv, respectively, as described in *Section 2.4.3.11.1.*

2.4.3.11.3 *Cross-reactivity studies*

The Fab and scFv antibodies were assayed against a range of standards of aflatoxins B_1, B_2, M_1, G_1 and G_2. Stock solutions of the aflatoxins were prepared in methanol and diluted using PBS containing 5% (v/v) methanol. The assays were carried out as per *Sections 2.4.3.11.2*, except the aflatoxin standards were added to the immunoplate with the anti-AFB_1 antibody fragments.

2.4.3.12 Biacore analysis

Analysis was performed using a Biacore 3000™ instrument operated with the Biacore 3000 Control Software package version 3.1.1. All data analysis was performed using BIAevaluation 4.0.1. Assay development was carried out on a CM5 (carboxymethylated 5) sensor chip immobilised with an AFB_1 derivative, which was kindly donated by Claire Jones of Xenosense Ltd., Queens University, Belfast. Hepes Buffered Saline (HBS), pH 7.4, as prepared in *Section 2.3.3* was used as the running buffer and was freshly prepared, filtered and degassed before use.

2.4.3.12.1 *Biacore inhibition immunoassay*

Standards of free AFB_1 were prepared at varying concentration ranges in PBS containing 5% (v/v) methanol. Each standard was incubated with an equal volume of antibody at the required dilution and allowed equilibrate for 30 minutes at room temperature. Each sample was then injected over the immobilised sensor surface in triplicate and the surface regenerated with the appropriate concentration of sodium hydroxide. A calibration curve was then constructed by plotting the normalised response (RU/RU_0) against the log of AFB_1 concentration and a four-parameter equation fitted to the data using BIAevaluation software 4.0.1.

2.4.4 *In vivo* biotinylation of AFB_1-specific recombinant antibodies

2.4.4.1 Restriction-digest of pAK400 and pAK400Bio vectors

Component	*Concentration per reaction (1X)*
Purified vector DNA (pAK400 or pAK400Bio)	5µg
*Sfi*I (12U per µg of DNA)	60U
NEB Buffer 2 (10X)	1 X
NEB BSA (100X)	1 X

Molecular grade water was added to a total volume of 50µl.

The digests were incubated for 5 hours at 50°C. PCR products were ethanol-precipitated (*Section 2.4.2.1.4*) and the pAK400Bio and scFv fragment were purified on a 0.8% (w/v) agarose gel as per *Section 2.4.2.1.5*. DNA was quantified by meauring the absorbance at 260nm on a Nanodrop spectrophotometer (ND-1000).

2.4.4.2 Amplification of Fab with pAK400Bio compatible *Sfi*I sites

2.4.4.2.1 *Primers for amplification of Fab with pAK400Bio compatible SfiI sites*

FabBio-F (forward)
5′ TCTATGGGCCCAGCCGGCCGAGCTCGATGCT 3′

FabBio-B (reverse)
5′ AATTCGCGGGCCCCCGAGGCCACTAGTTTTGTC3′

2.4.4.2.2 *PCR amplification of Fab with pAK400Bio compatible SfiI sites*

Component	*Concentration per reaction (1X)*
Forward primer (FabBio-F)	50pmol
Reverse primer (FabBio-B)	50pmol
PCR Buffer (10X)	1 X
$MgCl_2$	3mM
dNTP's	200µM
Plasmid DNA (pComb3X)	10ng
Taq polymerase	1.25U

Molecular grade water was added to a final volume of 50µl.

2.4.4.2.3 *PCR conditions for amplification of Fab with pAK400Bio compatible SfiI sites*

94°C for 2 minutes

30 cycles of :

94°C for 15 seconds

43°C for 30 seconds

72°C for 2 minutes

followed by 72°C for 10 minutes

Five microlitres of each reaction was anlaysed on a 1.5% (w/v) agarose gel, as described in *Section 2.2.5.* The reaction was scaled up (6X), the PCR products pooled and ethanol-precipitated (*Section 2.4.2.1.4*). The PCR products were purified from a 1% (w/v) agaorse gel, as described in *Section 2.4.2.1.5.* DNA was quantified by meauring the absorbance at 260nm on a Nanodrop spectrophotometer (ND-1000).

2.4.4.3 Ligation of scFv and Fab fragments into pAK400Bio

Fab fragments and pAK400Bio were *Sfi*I deigested and purified from a 0.8% (v/v) agarose gel as described in *Section 2.4.4.5.* Purified digested scFv and Fab fragments and pAK400Bio were ligated at a ratio of 3 :1 (insert :vector) using T4 DNA ligase as described in *Section 2.4.2.6.*

2.4.4.4 Transformation of *E. coli* AVB100 cells with pAK400Bio by electroporation

Ligations were transformed into electrocompetent AVB100 cells (Avidity Inc.) by electroporation as outlined in *Section 2.4.2.8* (approx. 25ng DNA per 100µl cells with the total volume of DNA not exceeding 5% of the cell volume). Transformants were plated out on LB containing 25µg/ml chloramphenicol and incubated overnight at 37°C.

2.4.4.5 Purification of *in vivo* biotinylated anti-AFB_1 recombinant antibodies by monovalent avidin chromatography

A chromatography column (Bio-Rad) was prepared by loading 4ml of resuspended immobilised monomeric avidin resin (Pierce Biotechnology Inc.) and left to settle to

form a 2ml packed resin. The column was equilibrated with 8ml PBS, which was followed by 6ml of PBS containing 2mM D-Biotin to block any non-reversible biotin sites. Biotin was removed with the addition of 12ml of regeneration buffer (0.1M glycine, pH 2.8). The column was washed with 8ml of PBS and the lysate (containing the biotinylated antibody fragment) was applied to the column three times. The 'flow-through' was collected and the column was washed six times with 2ml of PBS and non-bound protein was monitored by measuring the absorbance of each fraction at 280nm. Biotinylated antibody was eluted with 6ml PBS containing 2mM D-biotin and collected in 1ml fractions. The column was regenerated by washing twice with 4ml regeneration buffer (0.1M glycine, pH 2.8) and stored upright at 4°C in PBS containing 0.01% (v/v) sodium azide.

2.5 Production and characterisation of polyclonal antibodies to ampicillin

2.5.1 Production and characterisation of hapten-carrier conjugates

2.5.1.1 Carbodiimide protocol for reacting the amine group on ampicillin with a carboxylic acid on a carrier protein

Ampicillin was coupled to BSA (bovine serum albumin), OVA (ovalbumin) or thyroglobulin (THY) using standard carbodiimide coupling chemistry. The required protein was dissolved in 0.05M MES, 0.5M sodium chloride, pH 6 at a concentration of 10mg/ml. NHS (*N*-hydroxysuccinimide) and EDC (*N*-ethyl–*N*-(dimethyl–aminopropyl) carbodiimide hydrochloride) were added to give a final concentration of 50mM and 20mM, respectively. The solution was incubated at room temperature for 15 minutes without agitation. Ampicillin was prepared in 0.1M sodium phosphate, pH 7.5, to give a molar excess of 100 times that of the protein concentration. The activated protein was then added slowly to the drug with gentle stirring. The solution was incubated at room temperature, under gentle rotation for at least 2 hours, and dialysed against 100 volumes of PBS overnight at 4°C and further dialysed against 100 volumes of PBS for 4 hours at 4°C.

2.5.1.2 Carbodiimide protocol for reacting carboxylic acid on ampicillin with amines on a carrier protein

Ampicillin was dissolved in 0.2M borate buffer, pH 8.5 at a concentration of 10mg/ml. NHS and EDC were added to give a final concentration of 400mM and 100mM, respectively. The solution was incubated at room temperature for 10 min without agitation. Protein (BSA, OVA or THY) was prepared in 0.2M borate buffer, pH 8.5 to give a molar ratio of 1:100 (protein:hapten) and was added drop wise to the ampicillin solution with gentle stirring. The solution was incubated at room temperature, with gentle strring for at least 2 hours, and dialysed against 100 volumes of PBS overnight at 4°C and further dialysed against 100 volumes of PBS for 4 hours at 4°C.

2.5.1.3 Bicinchoninic Acid Assay (BCA)

Standard protein solutions with concentrations ranging from 0 to 2 mg/ml were prepared in PBS. BCA reagent was prepared by adding 1 part reagent B (4% (w/v) cupric sulphate) to 50 parts reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartate in 0.01 M sodium hydroxide). 10 µl of conjugate dilution or protein standard was added to microtitre wells. Prepared BCA reagent (200 µl) was then added to each well. The plate was incubated at 37°C for 30 min and absorbance was read at 562nm. A standard curve of known protein concentration versus absorbance was constructed from which the unknown sample protein concentrations were determined.

2.5.2 Production and characterisation of mouse antiserum

2.5.2.1 Immunisation schedule for the production of mouse antiserum to ampicillin

All processes involving the use of live animals were licensed by the Department of Health and Children. Extreme care was taken to minimise stress to the animals involved.

6 week old Balb/c female mice were immunised by sub–cutaneous injection with an emulsion (250µl) consisting of a 1mg/ml solution of AMP-BSA conjugate mixed 1:1 with Freund's Complete Adjuvant, which had been previously vortexed until a stable

emulsion had formed. Mice were re-immunised intraperitoneally 21 days later with an emulsion (250µl) consisting of a 1mg/ml solution of conjugate mixed 1:1 with Freund's Incomplete Adjuvant. A blood sample was collected from the tail of the mouse (the least invasive method) after 7 days and the antibody titre was determined against the respective antigen.

The cycle of re-immunisation and bleeding procedure was repeated until an acceptable titre was obtained. One week after the final immunisation, the animal was sacrificed by cervical dislocation and the spleen was removed.

2.5.2.2 Preparation of mouse serum for estimation of titre

For titre estimation the blood collected was allowed to clot at 4°C overnight. It was then centrifuged at 20,000*g* for 15 minutes (Eppendorf 5810R) and the supernatant removed for titre determination by ELISA, as described in *Section 2.5.2.3*.

2.5.2.3 Non-competitive ELISA for estimation of mouse serum antibody titre

Nunc Maxisorb™ immunoplates were coated with 100µl per well of the appropriate ampicillin-protein conjugate at a concentration of 10µg/ml and incubated at 37°C for 1 hour. Plates were washed three times with PBS. The plates were then blocked with PBS containing 4% (w/v) Marvel™ for 1 hour at 37°C. Serial dilutions of mouse antiserum in PBS containing 2% (w/v) Marvel™ were added to the wells and allowed to bind at 37°C for 1 hour. This was followed by addition of a 1 in 2,000 dilution of HRP-labelled goat anti-mouse antibody (Sigma) in PBS containing 2% (w/v) Marvel™ and incubated for 1 hour at 37°C. Plates were again washed and the chromogenic substrate *o*-PD was added and incubated for 30 minutes at 37°C. Absorbance was read at 450nm on a Titertek Reader.

2.5.2.4 Competitive ELISA for mouse antiserum

Nunc Maxisorb™ plates were coated with the appropriate concentration of conjugate and blocked as described in *Section 2.5.2.3*. From a 5mg/ml stock solution of ampicillin, standards with decreasing concentrations of free analyte were prepared in PBS. Each standard was added to the microtitre plate with an equal volume (50µl) of mouse antiserum, prepared in PBST containing 2% (w/v) Marvel™, at the optimal

dilution. Bound serum antibodies were detected following the addition of HRP-labelled goat anti-mouse antibody and *o*-PD substrate, as described in *Section 2.5.2.3.*

2.5.2.5 Enzymatic pre-treatment of ampicillin

Penicillinase, a mixture of β-lactamase I and II, prepared from *Bacillus cereus* was purchased from Sigma-Aldrich Ireland Ltd. A solution of penicillinase 10 UB/ml was prepared in water and added to a 1mg/ml solution of ampicillin in PBS to a final concentration of 1UB/ml. The solution was incubated for 1 hour at 37°C in a water bath to allow hydrolysis of the β-lactam ring.

2.5.3 Production of ampicillin-specific polyclonal antibodies

2.5.3.1 Immunisation schedule for the production of rabbit antiserum to ampicillin

A New Zealand white female rabbit was immunised by sub-cutaneous injection with an emulsion (1ml) consisting of a 1mg/ml solution of ampicillin-BSA conjugate mixed with an equal volume with Freund's Complete adjuvant, which had been previously vortexed until a stable emulsion had formed. The rabbit was re-immunised 21 days later intraperitoneally with an emulsion (1ml) consisting of a 1mg/ml solution of ampicillin-BSA conjugate mixed with an equal volume of Freund's Incomplete adjuvant. A blood sample was then collected from the marginal ear vein of the animal between 10 and 15 days after immunisation and the antibody titre determined against the respective antigen.

The cycle of re-immunisation and bleeding procedure was repeated until the titre (the highest dilution of antisera that will still give a positive reaction against the antigen) was sufficient. Once an acceptable titre (in excess of 1/100,000) was obtained the animal was anaesthetised and sacrificed by cardiac puncture. Blood was removed from the heart with a syringe and serum was prepared as per *Section 2.5.3.2.*

2.5.3.2 Preparation of rabbit serum

1ml blood samples from the marginal ear vein were collected for estimation of specific antibody titre by ELISA, as described in *Section 2.5.3.3.* Blood was allowed to clot for at 4°C overnight. It was then centrifuged at 3,000*g* for 20 minutes (Eppendorf 5810R) and the serum removed and stored at –20°C until further analysis.

2.5.3.3 Non-competitive ELISA for estimation of rabbit serum antibody titre

The serum antibody titre was estimated as described in *Section 2.5.2.3*. However, the secondary antibody was HRP-labelled goat anti-rabbit antibody, prepared in PBS containing 2% (w/v) Marvel™, at a dilution of 1 in 5,000.

2.5.4 Purification of polyclonal antibody from whole serum

Purification of polyclonal antibody from the rabbit serum was initially carried out by precipitation with saturated ammonium sulphate. This was followed by protein G-affinity chromatography and then subtractive immunoaffinity chromatography.

2.5.4.1 Saturated ammonium sulphate precipitation

The ammonium sulphate precipitation of antibody from rabbit serum was carried out according to the method of Hudson & Hay (1980). 100% (w/v) ammonium sulphate was prepared in distilled water at 50°C, the pH was adjusted to 7.2 and the solution was incubated at 4°C overnight. 10ml of the cold saturated ammonium sulphate was then added dropwise to an equal volume of rabbit serum on ice with stirring. Once all the ammonium sulphate was added, the mixture was left on ice for a further 30 minutes with stirring. The solution was then centrifuged at 3,000*g* for 20 minutes (Eppendorf 5810R) and the supernatant discarded. The remaining pellet was washed twice in 10ml of 45 % (w/v) ammonium sulphate, then dissolved in 5ml of PBS and dialysed against 5L of PBS overnight at 4°C to remove any residual ammonium sulphate.

2.5.4.2 Protein G affinity chromatography for purification of polyclonal antibodies

A 2ml protein G-sepharose column was poured and equilibrated with 50ml PBS, pH 7.4. The dialysed ammonium sulphate rabbit immunoglobulin fraction (2ml) was applied to the column and the flow rate adjusted to 1ml/min. The sample was re-applied to the column twice and the column was washed with 20ml PBST. Bound antibody was eluted with 0.1M glycine-HCl, pH 2.2 and fractions were neutralised with by addition of 2M Tris-HCl, pH 8.6. The optical density of each fraction was recorded at 280nm and fractions containing protein ($OD_{280} > 0.05$) were pooled and dialysed against 5L PBS overnight at 4°C and for a further four hours at 4°C in fresh PBS buffer. Dialysed fractions were aliquoted and stored at –20°C.

2.5.4.3 Immunoaffinity Chromatography

Subtractive immunoaffinity chromatography was carried out on protein G affinity-purified anti-ampicillin polyclonal antibodies to remove all BSA-binding antibodies from the antibody population. The matrix was prepared by chemical coupling of BSA to sepharose-4B, as described in *Section 2.5.4.3.1.*

2.5.4.3.1 *Preparation of immunoaffinity matrix*

Cyanogen bromide-activated sepharose was swollen with 1mM HCl for 20 minutes and dried under vacuum (Millipore sintered glass filtration unit with 0.22μm pore size filter). 5-10mg/ml of hapten–carrier conjugate per millitre of wet gel was dissolved in coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3) and added dropwise to the gel with stirring. This was incubated overnight with stirring, washed with coupling buffer and incubated with 1M ethanolamine, pH 8, for 2 hours with gentle rotation to block unreacted sites. The gel was washed six times alternating between 0.1M acetate buffer, 0.5M NaCl, pH 4 and coupling buffer. The gel was then washed with PBS containing 0.02% (w/v) azide and stored in this solution at 4°C.

2.5.4.3.2 *Purification using BSA-sepharose affinity column*

A 2ml affinity column was equilibrated with PBS and one column volume of protein G-purified polyclonal antibody was loaded onto the gel. Contaminants were removed by washing with four column volumes of PBST and four column volumes of PBS. Bound antibodies were eluted using 0.1M glycine/HCl, pH 2.2. Fractions were immediately neutralised with 2M Tris, pH 8.6, pooled and dialysed against 5L PBS at 4°C overnight and for a further four hours at 4°C in fresh PBS buffer.

2.5.5 Antibody characterisation

2.5.5.1 Enzyme-linked immunosorbent assay (ELISA)

2.5.5.1.1 *Checkerboard ELISA for determination of optimal polyclonal antibody dilution and optimal coating concentration of protein conjugates*

Nunc Maxisorb™ plates was coated with 100µl of varying concentrations (0-50µg/ml) of AMP-OVA prepared in PBS for 1 hour at 37°C or overnight at 4°C. Plates were washed three times with PBS and blocked with 200µl of PBS containing 4% (w/v) Marvel™ for 1 hour at 37°C. The plates were then washed three times with PBST and three times with PBS. Serial dilutions of polyclonal antibody were prepared in PBST containing 1% (w/v) Marvel™ and 100µl of each dilution added to the plates in triplicate. Then plates were then incubated at 37°C for 1 hour and washed as before. This was followed by the addition of 100µl of a 1 in 5,000 dilution of HRP-labelled goat anti-rabbit antibody (Sigma) in PBST containing 1% (w/v) Marvel™ and incubation for 1 hour at 37°C. Plates were again washed and a chromogenic substrate *o*-PD was added and incubated for 30 minutes at 37°C. Absorbance was read at 450nm on a Titertek Reader.

2.5.5.1.2 *Competitive ELISA for polyclonal antibody*

Nunc Maxisorb™ plates were coated with the appropriate concentration of conjugate and blocked, as described in *Section 2.5.5.1.1.* From a 5mg/ml stock solution of ampicillin, standards of decreasing concentrations of analyte were prepared in PBS. Each standard was added to the microtitre plate with an equal volume (50µl) of polyclonal antibody at the optimal concentration in PBST containing 2% (w/v) Marvel™. Bound antibody was detected following the addition of HRP-labelled antibody and *o*-PD substrate, as described in *Section 2.5.5.1.1.*

2.5.5.2 Biacore analysis

2.5.5.2.1 Immobilisation of ampicillin onto sensor chip surface

Immobilisation of ampicillin was carried out according to a method described by Gaudin *et al.* (2001). All injections were made at a constant flow rate of 5µl/min. The carboxymethylated dextran surface was activated by mixing equal volumes of 100mM NHS (N–hydroxysuccinimide) and 400mM EDC (N-ethyl–N-(dimethyl–aminopropyl) carbodiimide hydrochloride) and injecting the mixture over the sensor

chip surface for 20 minutes. A 10mM solution of ampicillin in 50 mM borate buffer, pH 8.5, was freshly prepared prior to use and injected over the activated surface for 7 minutes. This was followed by an injection of 1M ethanolamine, pH 8.5, for 7 minutes. Immediately after immobilisation, the surface was conditioned by injecting 10µl of 100 mM NaOH containing 20% (v/v) DMF and 10µl of 100 mM HCl containing 20% (v/v) DMF prior to use.

2.5.5.2.2 *Biacore inhibition immunoassay on immobilised sensor chip surface*

Standards of free analyte were prepared at varying concentration ranges. Each standard was incubated with an equal volume of antibody and allowed equilibrate for 30 minutes at room temperature. Each sample was then injected over the immobilised sensor surface in triplicate and the surface regenerated with the appropriate concentration of sodium hydroxide. A calibration curve was then constructed by plotting the change in response (RU) for each standard against the log of concentration and a four-parameter equation fitted to the data using BIAevaluation software 4.0.1.

2.5.5.2.3 *Ampicillin spiked whole milk preparation*

5mg of ampicillin was dissolved in 1ml of whole milk (Avonmore pasteurised and homogenised milk with a 3.5% fat content, purchased locally). Serial dilutions were prepared in whole milk and each sample defatted by centrifugation at 14,000*g* for 15 minutes at 4°C (Hereaus Biofuge Pico). The aqueous phase was carefully removed using a pipette and mixed 1:1 with the appropriate antibody dilution. The samples were incubated at room temperature for 30 minutes before analysis.

2.5.5.3 Fluorescence Immunoassay (FIA)

2.5.5.3.1 *Production of biotinylated ampicillin*

A fivefold molar excess of EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce) and ampicillin, was dissolved in 0.1 M sodium phosphate buffer, pH 7.2. The mixture was incubated in a microcentrifuge tube with gentle agitation for 1 hour at room temperature. The reaction was stopped by the addition of a cross-linked agarose gel with a primary amine, *i.e.* Affi-Gel 102 (Bio-Rad Laboratories) and incubation for an additional 30 min with gentle agitation at room temperature. The biotinylated ampicillin (AMP-

BIO) was separated from the gel by centrifugation. BIO-AMP was always freshly prepared before use.

2.5.5.3.2 *Checkerboard FIA for determination of optimal polyclonal antibody dilution and optimal BIO-AMP dilution*

A black Nunc Maxisorb™ plate (provide minimum background and back light scatter in fluorescence assays) was coated with 100µl of varying concentrations (0-140µg/ml) of anti-ampicillin polyclonal antibody in 0.05M carbonate buffer, pH 9.5, overnight at 4°C. Plates were washed three times with PBS and blocked with 200µl of PBS containing 0.4% (w/v) Tween 20 (Sigma) for 1 hour at 37°C. The plates were then washed three times with PBS. Serial dilutions of BIO-AMP were prepared in PBS and 100µl of each dilution added to the plates in triplicate. The plates were then incubated at 37°C for 1 hour and washed as before. This was followed by the addition of 100µl of a 1 in 1,000 dilution of Streptavidin-Cy5 (Amersham Biosciences) in PBS and incubation for 1 hour at 37°C. Plates were again washed and fluorescence was read at 649nm (excitation) and 670nm (emission) on a Tecan Safire^{2} ™.

2.5.5.3.3 *Checkerboard FIA for determination of optimal BIO-AMP dilution and optimal Streptavidin-Cy5 dilution*

Black Nunc Maxisorb™ plates was coated with 100µl of anti-ampicillin polyclonal antibody, at a concentration of 4.38µg/ml in carbonate buffer, pH 9.5, overnight at 4°C. Plates were washed three times with PBS and blocked with 200µl of PBS containing 0.4% (w/v) Tween 20 (Sigma) for 1 hour at 37°C. The plates were then washed three times with PBS. Serial dilutions of BIO-AMP were prepared in PBS and 100µl of each dilution added to the plates in triplicate. The plates were then incubated at 37°C for 1 hour and washed as before. Serial dilutions of Strep-Cy5 were prepared in PBS and 100µl of each dilution added to the plates in triplicate and they were incubated for 1 hour at 37°C. Plates were again washed and fluorescence was read at 649nm (excitation) and 670nm (emission) on a Tecan Safire^{2} ™.

2.5.5.3.4 Competitive FIA

Black Nunc Maxisorb™ plates were coated with ampicillin-specific polyclonal antibody at a concentration of 4.38µg/ml and blocked with 200µl of PBS containing 0.4% (w/v) Tween 20 (Sigma) for 1 hour at 37°C. From a 5mg/ml stock solution of

ampicillin, standards with decreasing concentrations of free analyte, were prepared in PBS. Each standard was added to the microtitre plate with an equal volume (50µl) of BIO-AMP prepared in PBS at the optimal concentration. Bound antibody was detected following the addition of Streptavidin-Cy5 and the fluorescence read on a Tecan Safire$^{2\,TM}$.

2.5.5.4 Bio-chip application

Prior to use chips were cleaned with isopropanol, rinsed thoroughly with distilled water and dried under a stream of nitrogen. Anti-ampicillin polyclonal antibody, at a concentration of 14µg/ml, was prepared in carbonate buffer, pH 9.5. 14ng (1µl) of the solution was spotted onto the surface of each cone of the disposable bio-chip using a pipette and incubated in a humidifier for 1 ½ hours at room temperature with 80% humidity. The surface of the chip was then blocked by filling the chambers with a solution of PBS containing 0.4% (w/v) Tween 20 and incubated for 1½ hours at room temperature with gentle agitation. The chips were then washed three times with PBS and the chambers filled with a solution of BIO-AMP, diluted 20-fold and incubated 1½ hours at room temperature. The chips were washed three times with PBST and three times with PBS. Strep-Cy5, at a dilution of 1/400 in PBS, was added to the chambers and the chips were incubated for 1 hour at 37°C. Bio-chips were washed three times with distilled water and fluorescence was read using the bio-chip detection system (a customised system developed in the School of Physical Sciences & National Centre for Sensor Research (NCSR), DCU, by the research group of Prof. Brian MacCraith).

2.5.6 Production of a murine scFv antibody library to ampicillin

2.5.6.1 Extraction of RNA from the spleen of a mouse

Total RNA was removed from the spleen of an immunised mouse using TRIzol reagent. The spleen from a mouse immunised with AMP-BSA was removed aseptically, weighed and homogenised in 1ml of Trizol reagent per 50-100 mg tissue. The homogenate was centrifuged 12,000*g* for 10 minutes at 4°C (Eppendorf 5810R) to remove insoluble material such as extracellular membranes, polysaccharides and high molecular weight DNA. The supernatant was removed and left at room temperature for 5 minutes before being supplemented with 200µl of chloroform per

ml of Trizol reagent used. Samples were shaken vigorously for 15 seconds and stored at room temperature for 15 minutes. The mixture was centrifuged at 12,000*g* for 15 minutes at 4°C (Eppendorf 5810R). Following centrifugation, three layers were observed, a lower red phenol/chloroform phase, a protein interphase and a colourless upper aqueous phase. The upper aqueous layer, containing RNA, was transferred to a clean tube and supplemented with 500µl of isopropanol per ml TRIzol reagent. The sample was incubated at room temperature for 10 minutes and centrifuged at 12,000*g* for 8 minutes at 4°C (Eppendorf 5810R). The supernatant was removed; the pellet washed with 1ml of 75% (v/v) ethanol and the solution was centrifuged at 8,000 *g* for 5 minutes at 4°C (Eppendorf 5810R). The ethanol was decanted, the pellet air–dried at room temperature for 5 minutes and re-dissolved in 250µl of molecular grade water.

2.5.6.2 Complementary DNA synthesis

Complementary DNA (cDNA) was synthesised by reverse transcription using a Promega reverse transcription system (MSC Ltd.) plus random primers. Random primers were used in preference to oligo dT's to compensate for any mRNA strands that may have been sheared of their poly A tails during the extraction process.

Component	*Concentration per reaction*
$MgCl_2$	5mM
10 X PCR Buffer	1X
dNTP mix	1mM
RNasin	1U per µl
Random Primers	200ng
AMV RT	15 U
RNA	7–10 µg

Molecular grade water was added to a final volume of 20µl.

The mixture was incubated at room temperature for 10 minutes to allow annealing of primers. The reaction was then incubated at 42°C for 1 hour and DNA analysed by agarose gel electrophoresis, as described in *Section 2.2.5*.

2.5.6.3 Amplification of antibody variable heavy and light chain genes

The primers listed below were obtained from MWG Biotech Ltd. and were used for assembling mouse scFv fragment in the orientation V_L-$(G_4S)_4$-V_H, which is compatible with the pAK vector system as described by Krebber *et al.* (1997). The sequences are given using the standard IUPAC nomenclature of mixed bases where: R=A or G; Y=C or T; M=A or C; K= G or T; S=C or G; W=A or T; H=A or C or T; B=C or G or T; V=A or C or G; D=A or G or T.

Primers, at a working concentration of 10nM, were mixed according to the degree of degeneration. Working primers were denoted LB (light chain back) and LF (light chain forward), for variable light chain gene (V_L) amplification. For heavy chain amplification, working primers were denoted HB (heavy chain back) and HF (heavy chain forward). For all non–degenerate primers (d=1), 1µl was included in the mix; for d=2–4, 2µl was included; for d=6–9, 3µl was included and for d=12–16, 4µl was included.

2.5.6.3.1 *Primers for amplification of variable heavy and light chain genes*

Variable light chain reverse primers		*Degeneracy*
LB1	5′gccatggcggactacaaaGAYATCCAGCTGACTCAGCC3′	2
LB2	5′gccatggcggactacaaaGAYATTGTTCTCWCCCAGTC3′	4
LB3	5′gccatggcggactacaaaGAYATTGTGMTMACTCAGTC3′	8
LB4	5′gccatggcggactacaaaGAYATTGTGYTRACACAGTC3′	8
LB5	5′gccatggcggactacaaaGAYATTGTRATGACMCAGTC3′	8
LB6	5′gccatggcggactacaaaGAYATTMAGATRAMCCAGTC3′	16
LB7	5′gccatggcggactacaaaGAYATTCAGATGAYDCAGTC3′	12
LB8	5′gccatggcggactacaaaGAYATYCAGATGACACAGAC3′	4
LB9	5′gccatggcggactacaaaGAYATTGTTCTCAWCCAGTC3′	4
LB10	5′gccatggcggactacaaaGAYATTGWGCTSACCCAATC3′	8
LB11	5′gccatggcggactacaaaGAYATTSTRATGACCCARTC3′	16
LB12	5′gccatggcggactacaaaGAYRTTKTGATGACCCARAC3′	16
LB13	5′gccatggcggactacaaaGAYATTGTGATGACBCAGKC3′	12
LB14	5′gccatggcggactacaaaGAYATTGTGATAACYCAGGA3′	4

LB15	5′gccatggcggactacaaaGAYATTGTGATGACCCAGWT3′	4
LB16	5′gccatggcggactacaaaGAYATTGTGATGACACAACC3′	2
LB17	5′gccatggcggactacaaaGAYATTTTGCTGACTCAGTC3′	2
LBλ	5′gccatggcggactacaaaGATGCTGTTGTGACTCAGGAATC3′	1

Variable light chain forward primers		*Degeneracy*
LF1	5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTKATTTCCAGCTTGG3′	1
LF4	5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTTATTTCCAACTTTG3′	1
LF5	5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTCAGCTCCAGCTTGG3′	1
LFλ	5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACCTAGGACAGTCAGTTTGG3′	1

Variable heavy chain reverse primers		*Degeneracy*
HB1	5′ggcggcggcggctccggtggtggtggatccGAKGTRMAGCTTCAGGAGTC3′	8
HB2	5′ggcggcggcggctccggtggtggtggatccGAGGTBCAGCTBCAGCAGTC3′	9
HB3	5′ggcggcggcggctccggtggtggtggatccCAGGTGCAGCTGAAGSARTC3′	4
HB4	5′ggcggcggcggctccggtggtggtggatccGAGGTCCARCTGCAACARTC3′	4
HB5	5′ggcggcggcggctccggtggtggtggatccCAGGTYCAGCTBCAGCARTC3′	12
HB6	5′ggcggcggcggctccggtggtggtggatccCAGGTYCARCTGCAGCARTC3′	4
HB7	5′ggcggcggcggctccggtggtggtggatccCAGGTCCACGTGAAGCARTC3′	1
HB8	5′ggcggcggcggctccggtggtggtggatccGAGGTGAASSTGGTGGARTC3′	4
HB9	5′ggcggcggcggctccggtggtggtggatccGAVGTGAWGSTGGTGGAGTC3′	12
HB10	5′ggcggcggcggctccggtggtggtggatccGAGGTGCAGSTGGTGGARTC3′	4
HB11	5′ggcggcggcggctccggtggtggtggatccGAKGTGCAMCTGGTGGARTC3′	4
HB12	5′ggcggcggcggctccggtggtggtggatccGAGGTGAAGCTGATGGARTC3′	2
HB13	5′ggcggcggcggctccggtggtggtggatccGAGGTGCARCTTGTTGARTC3′	2
HB14	5′ggcggcggcggctccggtggtggtggatccGARGTRAAGCTTCTCGARTC3′	4
HB15	5′ggcggcggcggctccggtggtggtggatccGAAGTGAARSTTGAGGARTC3′	4
HB16	5′ggcggcggcggctccggtggtggtggatccCAGGTTACTCTRAAASARTC3′	8
HB17	5′ggcggcggcggctccggtggtggtggatccCAGGTCCAACTVCAGCARCC3′	6
HB18	5′ggcggcggcggctccggtggtggtggatccGATGTGAACTTGGAASARTC3′	1
HB19	5′ggcggcggcggctccggtggtggtggatccGAGGTGAAGGTCATCGARTC3′	1

Variable heavy chain forward primers		*Degeneracy*
HF1	5'ggaattcggcccccgaggcCGAGGAAACGGTGACCGTGGT3'	1
HF2	5'ggaattcggcccccgaggcCGAGGAGACTGTGAGAGTGGT3'	1
HF3	5'ggaattcggcccccgaggcCGCAGAGACAGTGACCAGAGT3'	1
HF4	5'ggaattcggcccccgaggcCGAGGAGACGGTGACTGAGGT3'	1

2.5.6.3.2 ***PCR components for amplification of variable heavy and light chain genes***

Component	*Concentration per reaction*
Forward Primer Mix	50pmol
Reverse Primer Mix	50pmol
10 X Buffer	1 X
$MgCl_2$	1.5mM
dNTP's	200µM
cDNA	0.5µg
Taq polymerase	1.25U

Molecular grade water was added to a final volume of 50µl.

2.5.6.3.3 ***PCR conditions for amplification of variable chain genes***

94°C x 5 min

7 cycles of :

94°C x 1 min

63°C x 30 sec

58°C x 50 sec

72°C x 1 min

24 cycles of :

94°C x 1 min

63°C x 30 sec

72°C x 1 min

followed by 72°C x 10 minutes.

2.5.6.4 Splice by overlap extension (SOE) PCR

Variable heavy and light chains were annealed and amplified using a splice by overlap extension (SOE) to produce an 800bp fragment.

2.5.6.4.1 *Primers for SOE PCR*

scfor 5′ ttactcgcggcccagccggccatggcggactaccccg 3′

scback 5′ ggaattcggcccccgag 3′

2.5.6.4.2 *SOE PCR components for amplification of joined antibody light and heavy chain genes*

Component	*Concentration per reaction*
10 X buffer	1 X
$MgCl_2$	1mM
dNTPs	200μM
V_H	50ng
V_L	50ng
scfor	50pmol
scback	50pmol
Taq polymerase	1.25U

Molecular grade water was added to a final volume of 50μl.

2.5.6.4.3 *SOE PCR conditions for amplification of joined antibody light and heavy chain genes*

5 cycles of :

94°C x 1 minute

45°C x 50 minutes

72°C x 1 minute

(addition of scfor and scback primers and *Taq* polymerase)

25 cycles of :

94°C x 1 minute

68°C x 30 seconds

72°C x 1 minute

2.5.6.5 Isolation of pAK100 vector

A single colony of *E. coli* XL1-Blue containing the pAK100 vector (all pAK vectors were kindly donated by Prof. A. Plückthun of the Universität Zürich, Switzerland) was used to inoculate 10ml of LB media, containing 30μg/ml tetracycline, at 37°C with shaking at 250rpm. The plasmid was isolated and purified using a Wizard Plus miniprep kit (Promega, UK), according to the manufacturer's instructions, as described in *Section 2.3.1.1.*

2.5.6.6 *Sfi*I restriction-digest of scFv insert and pAK100 vector

Component	*Concentration per reaction*
SOE Product	5μg
*Sfi*I (16U per μg)	80U
NEB Buffer 2 (10X)	1X
BSA (100X)	1X

Molecular grade water was added to a final volume of 100μl.

Component	*Concentration per reaction*
Plasmid DNA (pAK100)	10μg
*Sfi*I (6U per μg)	60U
NEB Buffer 2	1X
BSA	1X

Molecular grade water was added to a final volume of 100μl.

The restriction was incubated at 50°C for 5 hours. The digested scFv fragment and vector were purified (*Section 2.4.2.1.5*) and quantified by agarose gel electrophoresis prior to ligation.

2.5.6.7 Library ligation

Purified digests of SOE product and pAK100 vector were ligated using T4 DNA ligase at 16°C overnight.

Component	*Concentration per reaction (1X)*
pAK100 (digested and purified)	1.4μg
SOE PCR product (digested and purified)	1.4μg
T4 DNA ligase buffer	1 X
T4 DNA ligase	10μl

Molecular grade water was added to a total volume of 200μl.

Ligations were incubated at 16°C overnight. One microlitre of glycogen was added to the ligation and it was ethanol-precipitated as described in *Section 2.4.2.1.4*. The DNA pellet was resuspended in molecular grade water and transformed into *E. coli* XL1-Blue electrocompetent cells (prepared as in *Section 2.4.2.7*) by electroporation, as described by *Section 2.5.6.8*.

2.5.6.8 Library transformation

Ligations were transformed into electrocompetent XL1-Blue cells by electroporation as outlined in *Section 2.4.2.8*. Following incubation cells were plated out on NE agar plates and incubated at 37°C overnight. Transformants were also titred, using 2xTY as a diluent, and each dilution was plated on NE plates. Plates were scraped using 2xTY, from which glycerol library stocks were prepared. Library stocks were flash frozen in liquid nitrogen and stored at -80°C.

2.5.7 Selection of specific scFv fragments

2.5.7.1 Rescue of scFv-displaying phage

50 ml of NE media was inoculated with 10^9 cells from glycerol library stock. The culture was then shaken at 37°C at 225-250rpm until OD_{600} of 0.5-0.6 was reached. 10^{11} pfu/ml VCSM13 helper phage and 0.5mM IPTG solution was added and left for 30 minutes at 37°C without agitation. The culture was then diluted in 100 ml of LE media in a 2 L baffle flask. The culture was shaken at 200rpm for 2 hours at 26°C for phage production, and 30μg/ml kanamycin was then added. Phage production was allowed to proceed at 26°C overnight, with vigorous aeration.

2.5.7.2 Concentration of phage by PEG/ NaCl precipitation

150 ml of overnight culture was transferred to a sterile sorvall tube and centrifuged at 10,000 rpm, 4°C for 10 minutes. One-fifth the volume (30 ml) of PEG/NaCl (20%

(w/v) polyethylene glycol 10000, 0.25 M NaCl prepared in ultra pure water) was added to the supernatant, mixed and incubated on ice for at least 1 hour. The mixture was centrifuged at 10,000 rpm, 4°C for 30 minutes. The supernatant was discarded and the pellet resuspended in 40 ml ultra pure water and 8 ml PEG/NaCl. This was incubated on ice for 20 minutes and centrifuged at 10,000 rpm at 4°C for 20 minutes. The supernatant was removed and the pellet was re-centrifuged at 7,000 rpm at 4°C for 10 minutes. Any remaining supernatant was removed and the pellet was resuspended in 2 ml sterile filtered PBS. Phage were stored at 4°C (short-term) or at -80°C (long- term) in 15% (v/v) glycerol.

2.5.7.3 Phage titre

4 ml of 2 x TY, 30μg/ml tetracycline was inoculated with a single colony of XL-1 blue and incubated overnight at 37°C with shaking at 225-250rpm. This culture used to seed 5 ml of 2 x TY, 30μg/ml tetracycline at a 1% (v/v) cell density. The culture was kept at 37°C while shaking at 225-250rpm until the OD_{600nm} = 0.5. Serial dilutions (10^{-1}-10^{-11}) of phage were prepared in the bacterial culture and allowed to stand at 37°C for 30 minutes without agitation. 100 μl of each dilution was spread on NE agar plates and incubated overnight at 37°C.

2.5.7.4 Selection of antigen binders by panning

An immunotube was coated overnight at 4°C with 4ml of the antigen of interest, at a concentration of 1 to 10μg/ml. The immunotube was washed three times with PBS and blocked with PBS containing 4% (w/v) milk powder, for 2 hours at room temperature. The tube was washed three times with PBST and three times with PBS. 1 ml of phage from precipitation were pre-blocked in 3ml of PBS containing 4% (w/v) milk marvel and this was added to the blocked immunotube. Phage were allowed to bind for two hours at room temperature under gentle agitation. The tube was washed as before and bound phage were eluted by the addition of 800μl of 0.1M glycine/HCl, pH 2.2, for ten minutes. The solution was immediately neutralised with 48μl of 1M Tris–HCl, pH 8.6. The phage were then titred and used to re-infect XL–1 Blue cells to generate library stocks for subsequent rounds of panning.

2.5.7.5 Re-infection of *E. coli* XL-1 Blue cells with eluted phage

5 ml 2xTY, 30μg/ml tetracycline was inoculated with a single colony of XL-1 Blue and grown overnight with shaking (200-250rpm) at 37°C. 5ml 2xTY, 30μg/ml tetracycline was inoculated with 1% (v/v) of the overnight culture and grown with shaking at 37°C until OD $_{6000nm}$ = 0.4-0.5. The culture was then incubated at 37°C without agitation for 10 minutes. 700μl of phage rescued from panning step were added to the culture and allowed to stand at 37°C for 30 minutes without agitation. The culture was then centrifuged at 3,000*g* for 2 minutes (Eppendorf 5810R) and resuspended in 600μl 2xTY. This was plated out on three NE agar plates and incubated overnight at 37°C. The following day infected cells were scraped from the solid media using 2xTY, concentrated by centrifugation at 3,000*g* for 2 minutes (Eppendorf 5810R) and resuspended in 2 ml 2xTY with 15% (v/v) glycerol. Stocks were aliquoted into 500 μl volumes, flash frozen and stored at – 80°C

2.5.7.6 Master plate construction

A 96 well sterile culture plate was filled with 200μl of 2 x TY, 1% (w/v) glucose, 25 μg/ml chloramphenicol and 30μg/ml tetracycline. Each well was inoculated with a single colony from the phage titre plates, leaving one or two wells as non-inoculated controls. The plate was incubated at 37°C overnight while shaking at 150rpm. This was known as the master plate and was frozen at -20°C with 15% (v/v) glycerol. Another plate was prepared with 200μl/well of 2xTY containing 1% (w/v) glucose and 25μg/ml chloramphenicol. 2μl from each well on the master plate was transferred to the corresponding well on the second plate and incubated at 37°C with shaking at 150rpm until OD_{600} ~ 0.4. Following incubation, 25μl of 2xTY containing 1% (w/v) glucose, 25μg/ml chloramphenicol, 9.5mM IPTG and 5 x 10^9 VCS M13 helper phage/ml was added to each well. The culture was left to stand for 30 minutes at 37°C followed by shaking at 150rpm for 2 hours at 26°C. The plate was centrifuged at 2,500*g* for 10 minutes (Eppendorf 5810R). The supernatant was removed by inversion and the pellets resuspended in 200μl of 2xTY containing 1% (w/v) glucose, 25μg/ml chloramphenicol, 1mM IPTG and 30μg/ml kanamycin. The plate was incubated at 26°C overnight while shaking at 150rpm. The plate was centrifuged at 2,500*g* for 10 minutes (Eppenforf 5810R and 75μl of the supernatant used for analysis in phage ELISA.

2.5.7.7 Phage ELISA

A microtitre plate was coated with 100µl of 1-10µg/ml of appropriate conjugate and incubated overnight at 4°C. The plate was washed three times with PBST and three times with PBS. The plate was then blocked with 150µl PBS containing 4% (w/v) Marvel™ for 1 hour at 37°C. 75µl of supernatant from the working phage plate and 25µl of PBS containing 4% (w/v) was added to the corresponding well in each ELISA plate, mixed gently and incubated for 2 hours at 37°C. The plates were washed as before and 100µl of anti-Fd bacteriophage antibody, at a 1 in 1000 dilution in PBS containing 1% (w/v) Marvel™, was added to each well. This was incubated for 1 hour at 37°C. The plate was washed as before and 100µl of peroxidase-labelled anti-rabbit antibody (at a 1 in 5000 dilution in PBS containing 1% (w/v) Marvel™) was added and incubated for 1 hour at 37°C. The plates were washed and 100µl of *o*-phenylenediamine (*o*-PD) substrate was added and incubated for 30 minutes at 37°C. Absorbance was read at 450nm on a Titertek Reader. The steps from phage rescue (*Section 2.5.7.1*) to phage ELISA (*Section 2.5.7.7*) are repeated until positive clones were identified.

Chapter 3

Development of an ELISA and Biacore-based assay for the detection of antibodies to Corynebacterium pseudotuberculosis in sheep

3.1 Introduction to Caseous Lymphadenitis

3.1.1 Corynebacterium pseudotuberculosis

Corynebacterium pseudotuberculosis is the causative agent of caseous lymphadenitis, an infectious disease affecting sheep and goats. These gram-positive facultative intracellular pathogenic bacteria, formerly known as *Corynebacterium ovis,* are strongly catalase- and urease-positive and can ferment glucose, galactose, maltose and mannose (Muckle and Gyles, 1982). They are sensitive to, but not killed by ampicillin, ceftiofur, cloxacillin, oxytetracycline, penicillin G, and tetracycline (Olson *et al.*, 2002). Phenotypical and genotypical investigations examining possible strains of *C. pseudotuberculosis* have revealed uniform biochemical and cultural characterisations of the bacterium (Barksdale *et al.*, 1981; Muckle and Gyles, 1982; Sutherland *et al.*, 1993; Literák *et al.*, 1999), although two distinct biotypes based on their nitrate-reducing ability have been confirmed (Biberstein *et al.*, 1971; Muckle and Gyles, 1982). The biotype that infects sheep and goats does not reduce nitrate to nitrite, whereas the equine and bovine isolates do reduce nitrate (Biberstein *et al.*, 1971; Songer *et al.*, 1990).

3.1.2 Caseous lymphadenitis

Caseous lymphadenitis (CLA) is a disease affecting sheep and goats caused by *C. pseudotuberculosis* infection (Brown and Olander, 1987) and is characterised by the enlargement and suppuration of one or more lymph nodes (Schreuder *et al.*, 1994). The bacteria can infect other animal species, such as horses, causing ulcers and abscesses (Knight, 1969) and cows, causing mastitis and visceral lesions (Watson and Preece, 2001). Human infection has also been reported, although it is usually associated with those occupationally exposed to farm animals (farmers, abattoir workers, etc.) (Peel *et al.*, 1997).

In sheep and goats infection can result in both an external and an internal form of the disease. External CLA causes abscesses in the superficial lymph nodes and subcutaneous tissues, which can be well tolerated by individual animals. However, an internal form of CLA results in abscesses located internally in organs and lymph nodes such as the lungs, liver, kidneys and mediastinal, bronchial and lumbar lymph nodes (Piontowski and Shivvers, 1998). This internal form of CLA can lead to

chronic disease with a range of ill effects including emaciation, reproductive complications and rarely, death. Both forms may develop simultaneously, and the clinical signs and lesions of either form may not become apparent until days (Kuria *et al.*, 2001) or even months (Ashfaq and Campbell, 1980) after initial infection. The abscesses develop a thick wall allowing the bacteria inside to survive while producing few obvious clinical signs in the animal (Batey, 1986). Ayers (1977) suggested that the precrural and prescapular lymph nodes are most likely involved in infection in sheep, whereas in goats the parotid node is more commonly affected. An abscess in the brain caused by *C. pseudotuberculosis* infection in a young goat has also been reported (Glass *et al.*, 1993).

The pathogenesis of caseous lymphadenitis usually involves entry of *C. pseudo-tuberculosis* through broken skin, where the bacteria are carried to the local lymph node and multiply causing inflammation, necrosis, and abcessation of the node (Hodgson *et al.*, 1990). Two factors are thought to play vital roles in the development of the disease. The first is the high lipid content of the bacteria, which allows the organism to resist digestion by cellular enzymes and so persist as a facultative intracellular parasite. The second factor is the production of an exotoxin, which has proved to be highly toxic *in vivo* in the presence of a lowered pH or a partial haemolysin, causing degradation of red cell membranes by the reticuloendothelial system (Hsu *et al.*, 1985).

Both cell-mediated and humoral immunity play an important role in the protection of an animal against *C. pseudotuberculosis* infection (Lan *et al.*, 1999). It was found that the primary infiltrating cells in abscess walls were macrophages expressing high levels of major histocompatibility complex (MHC) class II molecules (Ellis, 1988). Lan *et al.* (1999) detected significant amounts of tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) in immunised mice with a secondary infection of *C. pseudotuberculosis*, compared to levels during primary infection. When administered with anti-TNF-α and anti-IFN-γ monoclonal antibodies, bacterial proliferation in the immune mouse model increased, suggesting the importance of these cytokines in the host resistance to *C. pseudotuberculosis.* However, the way in which TNF-α and anti-IFN-γ induce enhanced resistance during secondary infection is unknown. Both CD4

and CD8 T cells have also been shown to play a vital role in the establishment of protective immunity against *C. pseudotuberculosis* infection. Lan *et al.* (1998) found that administration of anti-CD4 monoclonal antibodies caused increased mortality, suppression of bacterial elimination and a reduction in cytokine production in mice, suggesting CD4 cells are involved in acquired resistance to secondary *C. pseudotuberculosis* infection. Immunisation of mice with anti-CD8 monoclonal antibodies was not shown to enhance mortality and proliferation of the bacteria and interleukin-4 (IL-4) was undetected, suggesting that Th1 cells play an important role in the host immune response against secondary *C. pseudotuberculosis* infection (Lan *et al.*, 1999).

3.1.3 Phospholipase D

The most significant component of *C. pseudotuberculosis* in relation to disease is its exotoxin, a phospholipase D (PLD). Structurally PLD is a glycoprotein with an amino acid composition similar to that of collagen (Brown and Olander, 1987), a molecular weight of approximately 31 to 35kDa (Ellis *et al.*, 1991; Hodgson *et al.*, 1990; Hsu *et al.*, 1985; Menzies *et al.*, 1994; Menzies *et al.*, 2004; Muckle *et al.*, 1992; Songer *et al.*, 1990;) and an isoelectric point (pI) of 9.2 to 9.6 (Hsu *et al.*, 1985; Hodgson *et al.*, 1990). It can be found in the cytoplasm and in smaller amounts in the cell wall. *Corynebacterium ulcerans* is the only other member of the *Corynebacterium* genus that also features phospholipase D (Barksdale *et al.*, 1981), although the molecule is known to be very similar to the toxic enzyme found in the brown recluse spider, *Loxosceles reclusa* (Bernheimer *et al.*, 1985).

Various antigenic proteins have been isolated from *Corynebacterium pseudotuberculosis* all of which have elicited an immune response in test animals (Ellis *et al.*, 1991). However, three various antigenic proteins (at 31.5, 68 and 120kDa), one of which was PLD, were consistently detected in sera from infected animals (Muckle *et al.*, 1992), suggesting these immunodominant antigens may be potentially useful in vaccine or diagnostic development. Although the exact role of the PLD toxin in disease or immunity has not been very well defined to date, studies have indicated that the exotoxin causes increased vascular permeability and, thus, is thought to play a vital role in the spread of the bacterium from the site of infection to regional lymph nodes (Piontkowski *et al.*, 1998; Songer, 1997). It has also been

shown that antibodies to the exotoxin may limit dissemination in the infected host (Brown and Olander, 1987).

3.1.4 Transmission of CLA

CLA is most commonly introduced into a herd via an asymptomatic carrier animal originating from an infected herd (Ayers, 1977; Holstad, 1986). *C. pseudotuberculosis* can survive for prolonged periods in soil contaminated by pus (Knight, 1969), making it a difficult organism to control. It has been shown experimentally that the bacterium can survive for at least 24 hours in prepared commercial sheep dips without a significant decrease in viability (Nairn and Robertson, 1974) and for up to eight months in soil samples inoculated with pus (Augustine and Renshaw, 1982). It is widely believed that infection is caused largely through wounds in the skin or the mucous membrane and it has also been found that lesions typical of CLA can be induced after application of cultures and pus to intact skin (Nairn and Robertson, 1974; Batey, 1986). In sheep and goats with the internal form of CLA, a high prevalence of thoracic lesions indicated a possible role for airborne transmission (Brown and Olander, 1987). Contamination of shearing wounds is believed to be of prime importance in the transmission of CLA (Al-Rawashdeh, 2000), reinforcing the need for routine disinfection of shearing equipment. In Australia, infection is thought to be transmitted in sheep through contaminated dips (Paton *et al.*, 1996). However, in the UK and Saudi Arabia, transmission is thought to be through head or neck lesions, as a result of close contact at shared troughs (Binns *et al.*, 2002).

3.1.5 Economic Importance

CLA is considered to be one of the most economically important diseases of sheep and goats in the U.S., Canada and Australia, causing an annual loss of $17 million in wool production alone in Australia and being the number one cause for condemnation of carcasses (Paton *et al.*, 1994). Significant losses can occur when the internal lesions go undetected causing decreased milk and wool production and reproductive performance (Stoops *et al.*, 1984). Other losses may result from the disruption of shearing time to treat a ruptured lesion or disinfect shearing equipment.

3.1.6 Prevalence

The disease is endemic in many countries worldwide, being most prevalent in nations where intensive husbandry is practiced. It is also emerging in European countries previously 'CLA-free' as border controls become less stringent and the movement of animals becomes less restricted. However, the actual prevalence of the disease is thought to be underestimated; primarily because the disease is often subclinical in nature, it can go unnoticed and secondly, CLA is not a notifiable disease in many countries and therefore, will go unreported. Finally, many animal owners are unaware of the economic impact of the disease and are unlikely to consult a veterinary practitioner for superficial abscesses.

In 1952 it was recorded that CLA was the third most important disease of sheep in the U.S., after emaciation and pleuritis (Maddy, 1953). Stoops *et al.* (1984) found a prevalence rate of 42.4% in sheep in five regions of western U.S. In Australia, it is the most prevalent disease of sheep and is regarded as the leading cause of loss of income to the sheep industry (Brown and Olander, 1987). In Europe the disease has been reported in France, Spain, Italy, Norway, Switzerland, Romania, Germany, the Netherlands and the United Kingdom (Schreuder *et al.*, 1994). Although the exact figures for the prevalence of CLA in the Netherlands are unknown, it was estimated that approximately 5% of goat herds are affected by the disease (Dercksen *et al.*, 2000). CLA was first reported in goats in the UK in 1990, which was traced back to infected Boer goats from Germany imported into the UK in 1986 (Lloyd *et al.*, 1990; (Lloyd *et al.*, 1999). It was later diagnosed in sheep in 1991 (Robins, 1991). Since that time, various studies have revealed how quickly the disease can spread throughout flocks in a short space of time. As the result of a postal survey of ovine CLA carried out in the UK, Binns *et al.* (2002) reported that between 1990 and 1999, 45% of farmers that responded had seen abscesses in their flocks. Of these farmers, only 14% had sent samples to a laboratory and of these 75% were confirmed as CLA. However, only a small proportion of farmer's questionnaires were returned, suggesting that there may be a reluctance to respond to a questionnaire about the disease. CLA was first recorded in Northern Ireland in 1999, where the source of infection was traced back to imported Scottish sheep (Malone *et al.*, 2006). O'Doherty *et al.* (2000) isolated *C. pseudotuberculosis* from superficial abscesses of a two-year old Suffolk ewe that had been imported into the Republic of Ireland from England. This was the

first reported case in the Republic of Ireland that *C. pseudotuberculosis* had been isolated from an abscess. Since then there have been a number of cases of the disease reported but it is possible, due to its insidious nature, that many other cases have gone undetected.

3.1.7 Diagnosis of CLA

The diagnosis of the CLA is problematic due to the insidious and subclinical nature of disease. Diagnosis of clinically unapparent infections would allow an appropriate culling protocol to be established and would curb transmission of the disease into a clean flock (Brown *et al.*, 1986).

A variety of diagnostic tests have been described in the literature: an anti-beta-haemolysin test (Zaki, 1968); a synergistic haemolysis test (Knight, 1978; Burrell, 1980[2]; Brown *et al.*, 1986); an immunodiffusion test (Burrell, 1980[1]); a complement fixation assay (Shigidi, 1979); an indirect haemagglutination test (Shigidi, 1979); a microagglutination assay (Menzies and Muckle, 1989); and diagnosis using polymerase chain reaction (PCR) (Çetinkaya *et al.*, 2002). The anti-beta-haemolysis (AHI or BHI) test exploited the fact that the PLD exotoxin of *C. pseudotuberculosis* inhibits red blood cell lysis by staphylococcal β-lysin (Zaki, 1968). Test serum was incubated with exotoxin and bovine erythrocytes and the staphylococcal haemolysin was added. The presence of antibodies against the exotoxin prevent it from occupying the receptor and the beta-lysin can exert its haemolytic effect. Shigidi (1979) compared five of these serological tests for their capacity to diagnose experimentally induced CLA in sheep. The tube agglutination, complement fixation, gel-diffusion, anti-haemolysin and the indirect haemagglutination tests were all examined. A group of test animals, comprising of 12 sheep, were inoculated with a broth serum culture of *C. pseudotuberculosis*. No single test correctly identified all infected sheep, however, the indirect haemagglutination test was determined to be the most reliable. The gel-diffusion test gave a number of false-positives and the complement fixation test did not identify inoculated animals. Menzies and Muckle (1989) developed a microagglutination assay for the detection of CLA, which was reported as rapid and easy to use. However, the results showed a lack of specificity. This was explained by possible cross-reactions with related bacteria such as *Mycobacterium paratuberculosis.* PCR was recently outlined as a tool that would aid in diagnosis of

CLA (Çetinkaya *et al.*, 2002). One of the factors responsible for many of the false-negative and false-positive results in serological tests is the antigenic similarities between the species within the *Corynebacterium* genus. PCR analysis would overcome this problem by allowing the definite identity of the organism causing infection.

However, although useful, the tests discussed can be laborious and time-consuming, and are unsuitable for testing large numbers of serum samples at one time. To overcome these problems, a number of enzyme-linked immunosorbent assays (ELISA) have been developed for the detection of antibodies to *C. pseudotuberculosis* (Maki *et al.*, 1985; Sutherland *et al.*, 1987; ter Laak *et al.*, 1992; Menzies *et al.*, 1994; Dercksen *et al.*, 2000; Kaba *et al.*, 2001). ELISAs detect humoral immunity in CLA, as with the other serological tests discussed. This type of method suffers from one major disadvantage, which is that antibodies to the toxin of *C. pseudotuberculosis* have only been detected in sheep 30 to 60 days after infection (Maki *et al.*, 1985). However, although it may not be able to detect early infection, ELISAs are rapid, cheap and standardisation is relatively straightforward. A number of different forms of antigen have been utilized in immunoassays. Maki *et al.* (1985) used the exotoxin and other *C. pseudotuberculosis* antigens in the development of ELISAs for the detection of CLA in sheep. The detection of anti-toxin antibodies proved to be a more sensitive measure of infection than detection of antibodies against cell wall components. Kaba *et al.* (2001) employed bacterial whole cell extract as the antigen in an ELISA for the detection of CLA in 142 goats. Specifically bound sera antibodies were detected using a biotinylated polyclonal anti-goat IgG and the optical density (OD) monitored following addition of a streptavidin peroxidase conjugate and the substrate ABTS (2,2'-azino-di [3-ethylbenzthiazoline-6-sulfonic acid]). The ELISA was found to have a diagnostic sensitivity of 85% and specificity of 96%.

A double-antibody sandwich ELISA was developed by ter Laak *et al.* (1992) for the detection of antibodies directed against the exotoxin of *C. pseudotuberculosis* and was subsequently used as part of a successful nationwide eradication program of CLA from goats in the Netherlands (Schreuder *et al.*, 1994; Dercksen *et al.*, 1996). The assay employed rabbit anti-crude exotoxin polyclonal antibodies as the capture agent to immobilize crude exotoxin, derived from whole cells. Serum samples were then

added to the plate, followed by addition of polyclonal anti-goat or anti-sheep horseradish peroxidase (HRP)-labelled antibodies for the detection of bound specific antibodies. OD values were recorded at 450nm following addition of the substrate 5-aminosalicyclic acid. Although the test recorded a specificity of 99.9% and sensitivity of 100%, there were a number of inconclusive samples taken from CLA-positive flocks.

Dercksen *et al.* (2000) compared the indirect double antibody sandwich ELISA (denoted ELISA A), previously developed by ter Laak *et al.* (1992), to a modified version of the assay (denoted ELISA B). Modifications included incubation times and temperatures, diluent preparation, the use of a monoclonal anti-bovine IgG_1 HRP-labelled antibody in place of the polyclonal anti-goat HRP-labelled antibody and, finally, the use of 3,3´,5,5´-tetra-methylbenzidine (TMB) chromogen instead of 5-aminosalicyclic acid. All of these amendments were made in an attempt to reduce the non-specific interactions previously observed using the method developed by ter Laak *et al.* (1992). ELISA A and B were also compared to modified ELISAs C and D, previously developed in Australia and described by Sutherland *et al.* (1987). These indirect ELISAs incorporated either a cell wall preparation (ELISA C) or crude exotoxin preparation (ELISA D) as the coating antigen. Specifically bound antibodies from sera were detected using an anti-sheep HRP-labelled serum, following the addition of TMB substrate. In total, sera from 118 sheep and 114 goats that had no record of CLA infection, and 65 sheep and 72 goats with clinical abscesses, were tested. Although the specificity of ELISA A was not improved by the modifications included in ELISA B, the sensitivity increased from 72±5% to 94±3% in goats and from 51±6 to 79±5 in sheep. Of all four assays, ELISA B was found to be the most efficient with the highest sensitivity (94±3% in goats and 79±5% in sheep) and specificity (98±1 in goats and 99±1 in sheep). Although the use of toxin-specific serum as the coating antigen has the advantage of recognising several antigenic proteins of *C. pseudotuberculosis*, it is also thought to be responsible for the number of false-positive results obtained. Dercksen *et al.* (2000) suggested that this could be due to possible cross-reactivity with other bacteria including other *Corynebacterium* species, *Listeria monocytogenes* or *Mycobacterium avium* subsp. *paratuberculosis*.

The double antibody sandwich ELISA B modified by Dercksen *et al.* (2000) was further investigated in 2006 by Malone and colleagues. 329 sheep (63 male and 266 female) of which 133 had developed typical CLA lesions were tested. However, the sensitivity and specificity of the ELISA was found to be 88% and 55%, respectively. These figures differ greatly from the previously determined diagnostic sensitivity and specificity of 79±5% and 99±1%, respectively, reported by Dercksen and colleagues (2000). Although Dercksen *et al.* (2000) had suggested that false-positive results could be due to possible cross-reactivity with other bacteria, Malone *et al.* (2006) found no evidence of these bacterial infections in any of the flocks examined.

To overcome the problem of cross-reactivity and false-positive results, purified or recombinant antigens have been employed in a number of serological assays. Menzies *et al.* (1994) devised an indirect ELISA utilising a recombinant form of the PLD antigen (Songer *et al.*, 1990) to detect specific antibodies in serum from goats infected with the disease. Crude cell extract, containing other *E. coli* proteins, was pre-blocked with sera from 'pathogen-free' sheep. Antibodies bound to any non-specific bacterial proteins were removed by centrifugation resulting in reduced levels of *E. coli* proteins that might contribute to non-specific reactions. A diagnostic specificity of 82.1% and a sensitivity of 86.3% were reported. A modified version of this assay was later investigated by Menzies *et al.* (2004). The ELISA employed immobilized metal affinity chromatography (IMAC) to purify a recombinant form of PLD, incorporating a $(His)_6$ tag. The purified antigen was coated on immunoplates at a concentration of 0.5μg per well and used to detect PLD-specific antibodies in sheep sera. Bound antibodies were detected using a polyclonal anti-goat alkaline phosphatase (AP)-labelled IgG and the OD recorded 10 minutes after addition of *p*-nitrophenyl phosphate (pNPP). Six goats (three with CLA infection and three free of infection) were tested repeatedly over a one year period and the assay was found to have a sensitivity and specificity of 81 and 97%, respectively.

As discussed, the majority of diagnostic assays measure the humoral rather than cell-mediated response to CLA. For this reason, Prescott and colleagues (2002) developed a whole blood assay for the detection of an interferon-gamma (IFN-γ) response to whole cell *C. pseudotuberculosis* antigens. This could be useful for testing animals only recently infected with CLA because IFN-γ production is prior to antibody

production. The assay employed a commercially available monoclonal antibody to bovine IFN-γ for the detection of cell-mediated immunity to *C. pseudotuberculosis*-infected sheep. The test detected experimentally infected sheep with a sensitivity of 95.7% and non-infected sheep with a specificity of 95.5%. A measure of reliability was reported as opposed to sensitivity or specificity because samples were taken from repeatedly infected animals rather than from one large group of infected sheep. Menzies *et al.* (2004) compared this assay to the PLD ELISA (described previously). The IFN-γ ELISA was found to have a sensitivity of 89.2% and a specificity of 97.1% when six (three infected and three non-infected) goats were tested repeatedly over a 363 day period. However, results implied that there was no relation between IFN-γ response and degree of infection in goats, contrary to what had been previously found in sheep (Prescott *et al.*, 2002).

3.1.8 Control of CLA

CLA is a difficult disease to control within a flock due to a number of factors. The subclinical nature of the infection, the long incubation period and recurring nature of the disease makes it extremely difficult to distinguish animals that carry the disease from those that do not. Once *C. pseudotuberculosis* has established itself within a flock, eradication is problematic, as the thick walls of the abscesses characteristic of the bacterial infection are virtually impossible to penetrate with medication (Maddy, 1953; Baird, 1997).

Vaccination has been suggested as a possible means of controlling the spread of the disease (Paton *et al.*, 1995), as a result of these challenges. Vaccines have been developed in the past but have proved to be problematic, often causing abscesses at the site of vaccination (Brogden *et al.*, 1984; Menzies *et al.*, 1991). The components of these vaccines have included whole cells (Menzies *et al.*, 1991), cell wall components (Brogden *et al.*, 1984) and inactivated whole cells and detoxified exotoxin in the same vaccine (Piontowski and Shivvers, 1998). Hodgson *et al.* (1999) produced a vaccine prepared using a genetically inactive form of PLD, an inactive His20→Ser PLD analogue. His20 of the protein has been identified as the enzyme active site, where site-specific acid substitution for His20 removed enzymatic activity (Hodgson *et al.*, 1994). By inactivating the protein biologically rather than chemically, the researchers hoped to remove the toxic residual activity that can be left

with chemically prepared vaccines (approx 1% of active PLD). However, although the vaccine produced less abscess formation at the vaccination site, compared to a chemically prepared vaccine, CSL Glanvac™6, the immune response induced was poor. Of sheep vaccinated with CSL Glanvac™6, 95% were protected against infection compared to 44% of sheep vaccinated with the recombinant vaccine.

One reason that may explain why a successful vaccine has not yet been generated is that the protective antigen of *C. pseudotuberculosis* is attached to the cell wall of the bacteria. Therefore, any chemical or enzymatic processes that alter the chemical configuration of the cell wall, will ultimately destroy the antigen(s) that are capable of evoking protective immunity in the host (Piontowski and Shivvers, 1998). Vaccination is used in both Australia and the U.S. as a method of disease control but commercial vaccines are not licensed in the UK and Ireland (Malone *et al.*, 2004). However, vaccination would not essentially eradicate CLA from a flock, it would merely reduce the prevalence and severity of the disease. The optimal method of control of CLA is eradication of infection by identification and removal of infected carrier animals (Prescott *et al.*, 2002). An eradication program that was carried out in the Netherlands successfully eliminated CLA from goats over a six-year period employing the ELISA format developed by ter Laak *et al.* (1992). Buildings and bedding materials used to house the goats were disinfected, the animals were tested for CLA and those tested positive were subsequently culled, resulting in eradication of the disease from the Netherlands between the years of 1988 and 1996 (Dercksen *et al.*, 1996, Schreuder *et al.*, 1994). A similar program eradicated CLA from a goat herd, comprising of 100 animals, in Norway. An ELISA method was also used as the diagnostic tool and infected individuals were subsequently culled (Nord, 1998). However, such an eradication program may not be financially viable in other countries where herds are larger or more economically significant. Also, the economical losses due to CLA are largely seen in sheep herds, which to date have not been the subject of a recorded CLA eradication program. The success of such an eradication project would rely largely on a rapid, economically viable diagnostic test that has adequate sensitivity and specificity to detect infected animals. Essentially, to control the spread of the disease, good herd management and an awareness of the disease by herd owners and veterinary practitioners is vital.

3.1.9 Chapter outline

This chapter focuses on the expression of a recombinant form of phospholipase D (PLD) and its subsequent use in an indirect ELISA and Biacore assay for the detection of caseous lymphadenitis (CLA) in sheep. The plasmid bearing the *C. pseudotuberculosis* PLD gene (denoted pJGS90), cloned by Dr. Glenn Songer of the Department of Veterinary Science and Microbiology, University of Arizona, Tuscon, USA, was kindly donated by Dr. John Prescott of the Department of Pathobiology, Ontario Veterinary College, University of Guelph, Ontario, Canada.

The pJGS90 plasmid was transformed into XL-10 Gold *E. coli* cells for high-level expression of the recombinant PLD. The expressed protein was then purified using metal chelate affinity chromatography (IMAC) and employed in the ELISA and Biacore-based assays to detect antibodies to *C. pseudotuberculosis*, the causative agent of CLA in sheep. Assay development and validation was carried out according to Jacobson (1998) and all reagents and protocols were optimised. Finally, the performance characteristics of the assays were determined using a number of positive and negative controls, which were applied to a range of clinical samples from Irish herds. These results were compared to results previously obtained for the same panel of sera by a double sandwich ELISA developed in Lelystad (ter Laak *et al.*, 1992; Dercksen *et al.*, 2000).

3.2 Results

3.2.1 Expression and purification of the recombinant protein phospholipase D

The PLD gene from *Corynebacterium pseudotuberculosis* had been previously cloned into the pTrcHisB vector, illustrated in *Figure 3.1*, which expresses the mature PLD amino acid sequence fused to a 34 amino acid tag encoded by the vector (Songer *et al.*, 1990; Menzies *et al.*, 2004). Cloning and subsequent expression of a recombinant protein offers numerous advantages over isolation of native proteins. It eliminates the need for growing large-scale cultures of the pathogen, provides a potentially unlimited supply of antigen and an affinity tag can be incorporated enabling one-step purification of the protein.

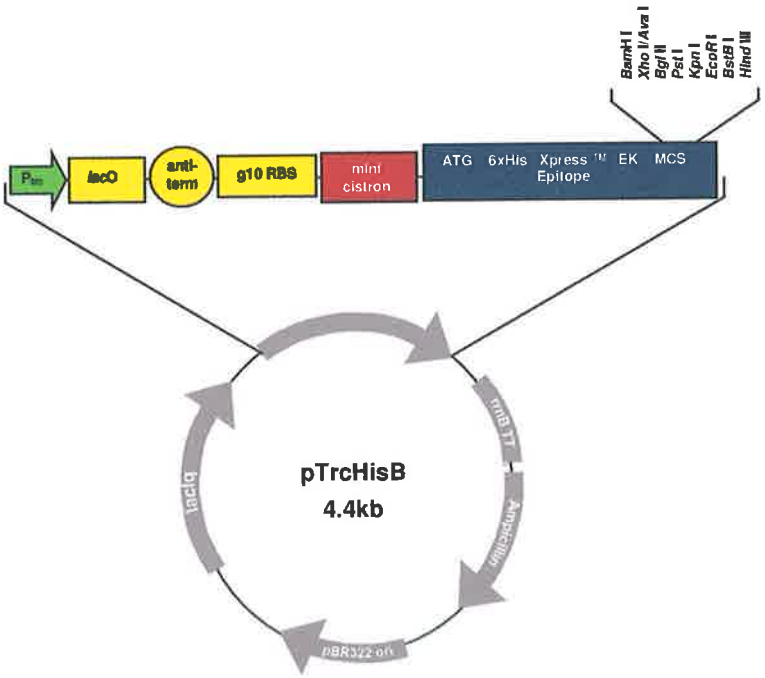


Figure 3.1. *Map of the pTrcHisB vector, encoding an ampicillin resistance gene. The vector offers a number of features including high-level regulated transcription from the trc promoter, enhanced translation efficiency of eukaryotic genes in E. coli and the lacO operator and* $lacI^q$ *repressor gene for transcriptional regulation in any E. coli strain. The vector also encodes an N-terminal polyhistidine* $(His)_6$ *tag for rapid purification with nickel-chelating resin and detection with an anti-His antibody.*

The plasmid bearing the PLD gene was isolated from *E. coli* DH5α cells and subsequently transformed into $CaCl_2$-competent XL10-Gold *E. coli* cells, as described in *Section 2.3.1.1, 2.3.1.2 and 2.3.1.3*. These cells are frequently used for high levels of protein expression and have a number of advantages over other *E. coli* strains. XL10-Gold cells are derivatives of Stratagene's highest-efficiency competent cell line XL2-Blue MRF'. The cells harbour the *lacI*^{q} mutation, so therefore produce enough *lac* repressor to efficiently regulate and repress protein expression and contain the *Hte* phenotype, which increases transformation efficiency of ligated DNA molecules. They also grow faster than XL-2 Blue cells, resulting in larger colonies. The cells are endonuclease deficient (*endA1*), which improves quality of plasmid miniprep DNA and recombination deficient (*recA*), which ensures insert stability. The *mcrA*, *mcrCB* and *mrr* mutations prevent cleavage of the cloned DNA that carries cytosine and/or adenine methylation, which is often present in eukaryotic DNA and cDNA. The *hsdR* mutation prevents the cleavage of the cloned DNA by the EcoK endonuclease system.

3.2.1.1 Restriction analysis on pJGS90 plasmid

Plasmid DNA was linearised using *Eco*RI and *Bam*HI restriction enzymes (New England Biolabs), as described in *Section 2.3.1.4*, to confirm the presence of the PLD gene insert. The digest was electrophoresed on an agarose gel and bands representing the linearised plasmid (~4.4kb), with the PLD gene removed (~ 850bp), can be clearly seen in *Figure 3.2*.

3.2.1.2 Initial small-scale expression

Following successful transformation, screening for positive transformants was performed on LB agar plates containing the appropriate antibiotics. Initial small-scale expression, as described in *Section 2.3.1.5*, was carried out on five different colonies to verify presence of the PLD protein. A number of different transformants were chosen as expression levels can vary between freshly transformed bacterial cells. It can be seen from the gel pictures in *Figure 3.3 (A)* and *(B)* that bands are evident at approximately 35kDa, which corresponds with the molecular weight of PLD found in previously published research (Menzies *et al.*, 2004).

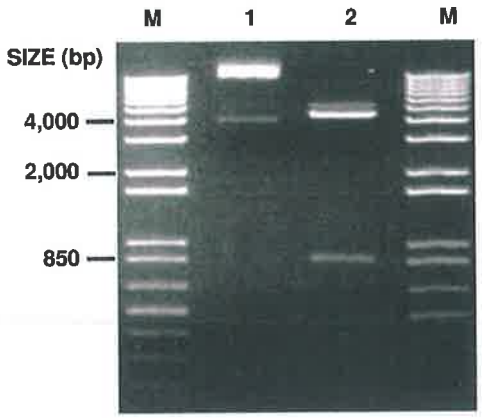


Figure 3.2. *Agarose gel illustrating EcoRI and BamHI restriction analysis of pJGS90 from one of the selected transformants. Lanes:* ***(M)*** *1kb plus DNA ladder;* ***(1)*** *Uncut pJGS90 plasmid;* ***(2)*** *Restricted plasmid (~4,400bp) with the PLD gene removed (~850bp).*

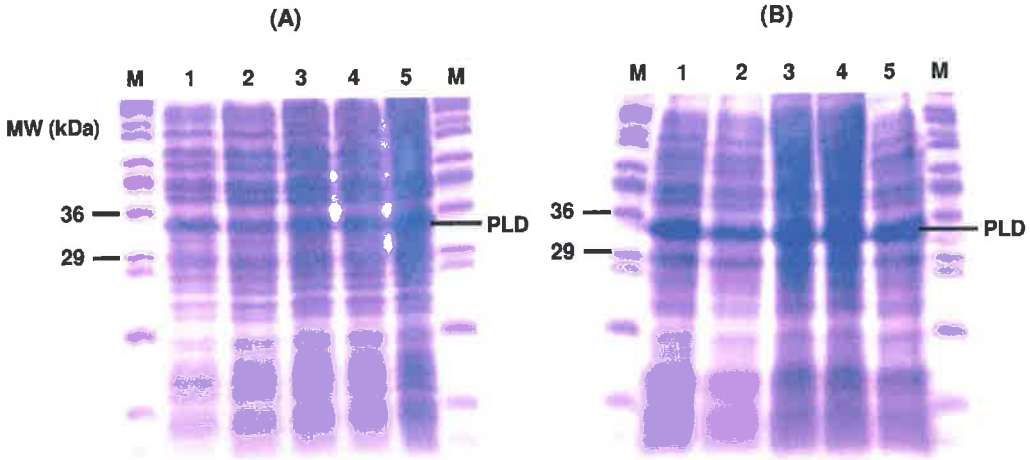


Figure 3.3. *SDS-PAGE analysis on the expression of PLD under* ***(A)*** *native conditions and* ***(B)*** *denaturing conditions. Lanes:* ***(M)*** *Sigma wide range molecular weight markers consisting of aprotinin (6.5kDa), α-lactalbumin (14.2kDa), trypsin inhibitor (20kDa), trypsinogen (24kDa), carbonic anhydrase (29kDa), glyceraldehyde-3-phosphate dehydrogenase (36kDa), OVALBUMIN (45kDa), glutamic dehydrogenase (55kDa), bovine albumin (66kDa), fructose-6-phosphate kinase (84kDa), phosphorylase B (97kDa), β-galactosidase (116kDa) and myosin (205kDa);* ***(1-5)*** *small-scale expression cultures on clones 1-5, respectively. A band at approx. 35 kDa, which represents the expressed PLD, is visible in lanes 1-5.*

3.2.1.3 Optimisation of PLD expression conditions

In order to achieve high levels of protein expression, a number of parameters were optimised including protein solubility, IPTG concentration, sonication conditions and the length of time required for maximum protein expression post-induction, all of which are detailed in the following sections.

3.2.1.3.1 *Determination of protein solubility*

The high-level expression of recombinant proteins in *E. coli* may result in the formation of insoluble inclusion bodies, consisting of aggregates of the expressed protein. These insoluble inclusion bodies can be easily solubilised with denaturants such as guanidine hydrochloride or urea or with a variety of detergents. Protein solubility determination, as described in *Section 2.3.1.6.1*, enables high-level recombinant protein recovery, following optimisation of the buffer required for protein isolation.

Briefly, bacterial cells were pelleted by centrifugation four hours post-induction and resuspended in either a native buffer (distilled water) or a denaturing urea buffer (8M urea, 100mM NaH_2PO_4, 10mM Tris, pH 8) to isolate soluble and insoluble protein, respectively. Following centrifugation the supernatants were analysed by SDS-PAGE, as seen in *Figure 3.4.* It can be seen from the gel in *Figure 3.4.* that the majority of recombinant protein is expressed as insoluble material as solubilisation of the protein only occurs after lysing the cells with denaturing buffer, containing 8 M urea. This result was also confirmed using a web-based bioinformatics program (www.biotech.ou.edu) designed for protein solubility determination (Harrison, 2000). Based on the protein amino acid sequence, this program uses approximate charge average to determine protein solubility. From the protein sequence, it was determined that the PLD protein has a 67.3% chance of insolubility when over-expressed in *E. coli* cells.

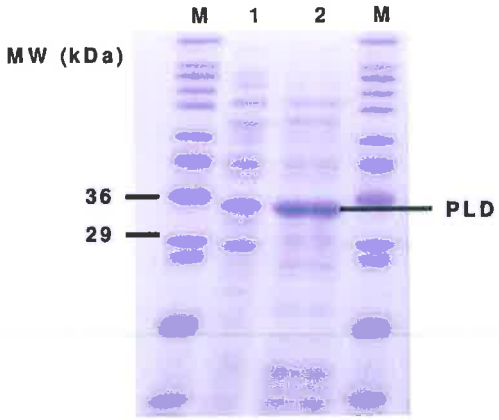


Figure 3.4. *Protein solubility determination. Lanes: **(M)** Sigma wide range molecular weight marker, as before; **(1)** isolation of soluble PLD under native conditions; **(2)** isolation of insoluble PLD under denaturing conditions.*

3.2.1.3.2 *Optimisation of IPTG concentration for induction of protein expression*

Expression of protein is induced upon addition of isopropyl-β-D-thiogalactoside (IPTG), which binds to the *lac* repressor protein resulting in its inactivation. This allows the host strains RNA polymerase to transcribe the sequence downstream of the promoter sequence with the resultant transcript being translated into recombinant protein. It is extremely important to optimise IPTG concentration for induction of protein expression so that the highest level of expression is attained without the IPTG concentration or level of protein expression becoming toxic to the cell. A concentration gradient of IPTG, ranging from 0.01 to 1mM, was set up and used to determine the optimal concentration required for high levels of protein expression (see *Section 2.3.1.6.2*). The varying levels of protein expression following induction with different IPTG concentrations can be seen in *Figure 3.5*, under denaturing conditions. The optimal concentration of IPTG for the expression of PLD was found to be 0.05mM.

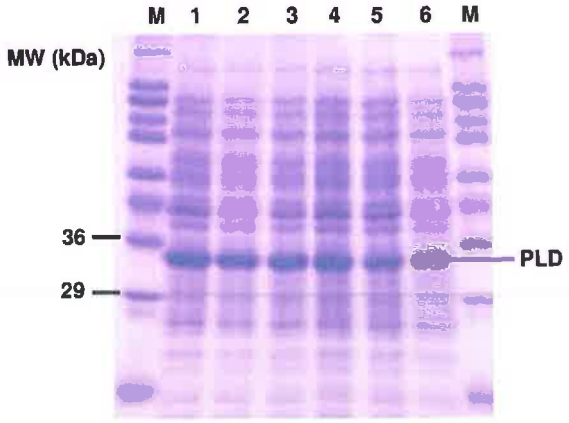


Figure 3.5. *Optimisation of IPTG concentration for the induction of PLD expression under denaturing conditions. Lanes: (**M**) Sigma wide range marker; (**1**) induction of PLD expression using 1mM IPTG; (**2**) induction of PLD expression using 0.5mM IPTG; (**3**) induction of PLD expression using 0.1mM IPTG; (**4**) induction of PLD expression using 0.05mM IPTG (**5**) induction of PLD expression using 0.01mM IPTG (**6**) induction of PLD expression in the absence of IPTG. The levels of expressed PLD are very low, indicating that E. coli XL10-Gold cells, harbouring the lacI^q gene, produce significant quantities of the lac repressor protein to prevent undesired levels of basal expression of PLD. The optimal concentration of IPTG for the expression of PLD was found to be 0.05mM.*

3.2.1.3.3 *Optimisation of sonication conditions for isolation of PLD*

Sonication is often necessary to isolate an expressed protein from within the cell cytoplasm. It involves pulsing cells with a high voltage current, which perforates the cell membrane, releasing the cell contents. For successful isolation of the desired protein, these conditions must also be optimised, as prolonged sonication may have an adverse effect on the recombinant protein. Briefly, a number of small-scale cultures were set up as per *Section 2.3.1.5*. Four hours post-induction, the bacterial cells were centrifuged and bacterial cell pellets resuspended in denaturing urea buffer. Each lysate was sonicated for various times, ranging from 0 to 60 seconds, centrifuged to remove all cell debris (*Section 2.3.1.6.3*) and the supernatants analysed by SDS-PAGE, as shown in *Figure 3.6*.

High levels of protein expression were observed but there was very little difference in PLD expression levels with or without sonication. Therefore, for large-scale expression, sonication was not necessary and cells could be lysed by gentle vortexing in urea-based buffer.

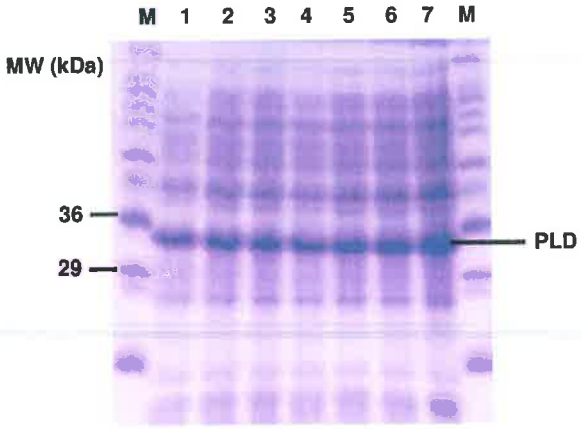


Figure 3.6. *Optimisation of sonication conditions for expression of PLD under denaturing conditions. Lanes:* ***(M)*** *Sigma wide range marker;* ***(1)*** *0 sec sonication;* ***(2)*** *10 sec sonication;* ***(3)*** *20 sec sonication;* ***(4)*** *30 sec sonication;* ***(5)*** *40 sec sonication;* ***(6)*** *50 sec sonication;* ***(7)*** *60 sec sonication.*

3.2.1.3.4 *Time-course analysis on PLD expression*

Following optimisation of all other parameters, a time-course experiment was performed, as described in *Section 2.3.1.6.4.* This experiment determined the length of time needed for maximum protein yield following induction with IPTG. Briefly, 1ml culture samples were taken at hourly intervals post-induction for up to five hours and following overnight induction (approx. 18 hours). The samples were then centrifuged and resuspended in denaturing urea buffer. The resulting cytoplasmic extracts were analysed by SDS-PAGE for levels of protein expression, as shown in *Figure 3.7.* The levels of protein expressed did not increase significantly after four hours so this was chosen as the optimal induction period for expression of PLD in subsequent experiments.

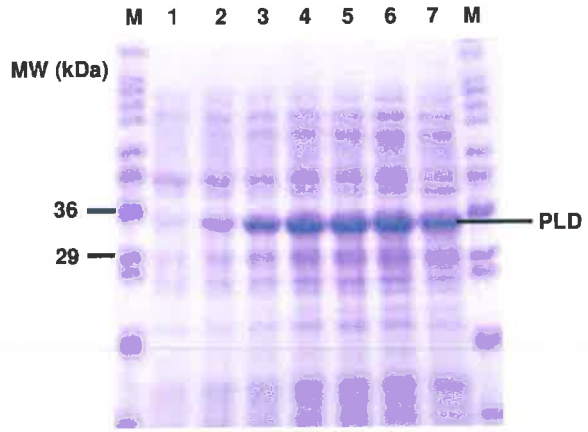


Figure 3.7. *Time-course analysis on the expression of PLD under denaturing conditions. Lanes:* ***(M)*** *Sigma wide range molecular weight marker;* ***(1)*** *non-induced control;* ***(2)*** *expressed PLD protein one hour post-induction;* ***(3)*** *PLD two hours post-induction;* ***(4)*** *PLD three hours post-induction;* ***(5)*** *PLD four hours post-induction;* ***(6)*** *PLD five hours post-induction;* ***(7)*** *PLD following overnight induction. The optimal induction period for expression of PLD under appeared to be four hours post-induction.*

3.2.1.4 Identification of $(His)_6$ tagged PLD by Western blotting

The PLD recombinant protein was analysed by Western blotting to determine whether the $(His)_6$ tag had been fully translated, was accessible and had not been adversely affected during cell lysis. Briefly, the recombinant protein was electrophoresed alongside a prestained molecular weight marker, as described in *Section 2.2.3.* Following separation by SDS-PAGE, the proteins were transferred to nitrocellulose for Western blot analysis, as described in *Section 2.2.4*, with the following amendments. Following transfer of the proteins onto the nitrocellulose, the membrane was blocked with PBS containing 5% (w/v) Marvel™ dried skimmed milk (Premier Foods Ltd., UK). It was then probed with a 1 in 5,000 dilution of a HRP-labelled anti-polyhistidine monoclonal antibody (Sigma-Aldrich Ireland Ltd.) in PBS containing 4% (w/v) Marvel™. The membrane was incubated for 1.5 hours with gentle shaking at room temperature and washed thoroughly as outlined in *Section 2.2.4.* Protein bands were visualised using 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) substrate and the reaction was allowed to proceed until bands were clearly visible. The recombinant PLD protein containing an N-terminal histidine tag was detected, as shown in *Figure 3.8*, confirming that the $(His)_6$ was fully intact and accessible.

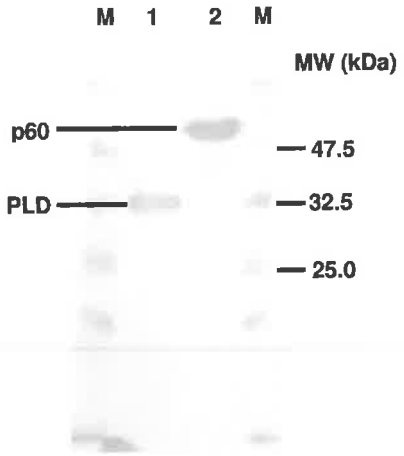


Figure 3.8. *Western blot analysis of PLD probed with anti-polyhistidine antibodies. Lanes: (M) Pierce BlueRanger™ prestained molecular weight markers; (1) PLD protein; (2) positive control, p60 recombinant protein (a pathogenicity marker for Listeria monocytogenes).*

3.2.1.5 Purification of recombinant PLD protein by immobilised metal affinity chromatography (IMAC)

Following optimisation of all parameters, the PLD recombinant protein was expressed on a large-scale as per *Section 2.3.1.7.1.* Once the protein was isolated from the host cell, purification was then necessary to remove any contaminating proteins that may interfere with subsequent assay development. The pTrcHisB vector encodes an N-terminal polyhistidine $(His)_6$ tag which allowed for the rapid purification of the PLD protein using immobilised metal ion affinity chromatography (IMAC).

Porath *et al.* (1975) first described metal chelate affinity chromatography as a tool to purify proteins. This type of chromatography involves a suitable support attached to a metal-chelating substance, which has the ability to form complexes with proteins facilitating separation. IMAC offers numerous advantages as a purification method, including low cost, ligand stability, simple regeneration and high protein loading (Arnold, 1991). Several different elution techniques including pH gradient, competitive ligands, organic solvents and chelating agents can also be used (Janson *et al.*, 1998). There are a number of commercially available chelating ligands but

nitrilotriacetic acid (NTA) bound to Sepharose® CL-6B and precharged with Ni^{2+}, supplied by Qiagen Ltd., was used in this case (*Figure 3.9*). One of the main advantages of this purification system is that it can be used under native or denaturing conditions since the interaction between Ni-NTA and the $(His)_6$ tagged recombinant protein does not depend on tertiary structure. The $(His)_6$ tag, with a molecular weight of only 840Da, is relatively small compared to other affinity tags but exhibits the strongest interaction with immobilised metal ion matrices. It is poorly immunogenic, and at pH 8.0, the tag is uncharged, and therefore does not generally affect secretion or folding of the fusion protein within the cell.

Considering the insolubility of the protein, a batch purification method (to maximise sample:column interaction) was optimised under denaturing conditions, as described in *Section 2.3.1.7.2*. In this case, the protein is denatured leaving the $(His)_6$ tag completely exposed and hence increasing it's ability to bind to the Ni-NTA matrix. This method can therefore lead to greater yields when compared to purification under native conditions. Fractions from each stage of the purification process were analysed using SDS-PAGE (*Figure 3.10 (A)*). From the gel picture it can be seen that the purity of the PLD protein increased following IMAC purification. The protein was then renatured by sufficient dilution in a native buffer, *i.e.* phosphate buffered saline (PBS), pH 7.4.

However, despite acceptable yields from the purification method under denaturing conditions, it is generally preferable to be able to purify recombinant proteins under native conditions. To maximise the yield of soluble product, a method described by Barbas *et al.* (2001) was modified as described in *Section 2.3.1.8.1* and *2.3.1.8.2*, and used for the expression and purification of PLD under native conditions. The expression method utilises a rich super broth (SB) media, containing high concentrations of tryptone and yeast extract to provide ample amount of nutrients and cofactors for cell growth and 3-(*N*-Morpholino)propanesulfonic acid (MOPS) acts as a buffer to prevent the pH decreasing and subsequent cell death. The Ni-NTA resin volume was reduced during purification, as an excess of resin can result in the inability to elute the protein, and the method was performed at 4°C. From a 500ml culture a yield of approximately 15mg of relatively pure protein was obtained. Considering the manufacturers estimate the NTA ligand is sufficient for the binding

of approximately 5–10 mg of $(His)_6$-tagged protein per milliliter of resin (Qiagen Ltd.), the yield was very acceptable. Purified protein stocks were diluted to approximately 0.5mg/ml in PBS to ensure the protein did not precipitate.

Although the yield of pure protein is high, it can be seen from *Figure 3.10 (B)* that some protein was lost during washes with 20mM imidazole and the eluate does contain some contaminating proteins. The concentration of imidazole in the wash buffer can be altered depending on whether purity or yield is more important. The higher the concentration of imidazole tolerated by the protein in the wash solution, the cleaner the eluate. In this case, an imidazole concentration of 20mM was used in the lysis and wash buffers, to reduce non-specific binding of untagged contaminant proteins to the resin, and the PLD protein was eluted with 250mM imidazole. The protein was subsequently purified under native conditions for the development of subsequent assays.

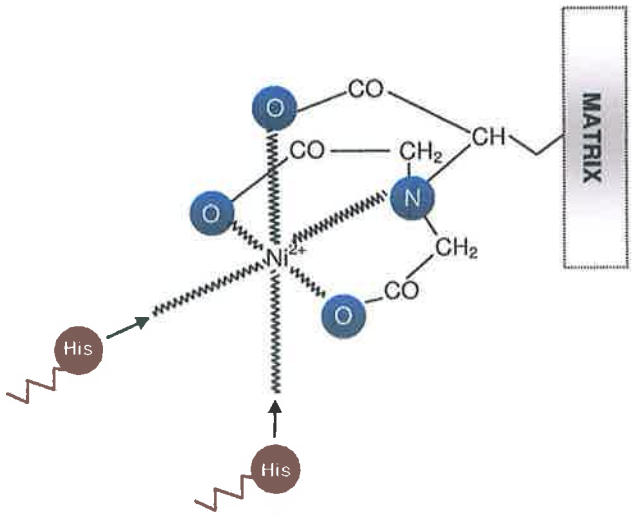


Figure 3.9. *Monomeric subunit of Nickel-charged NTA resin attached to a support. The NTA occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the* $(His)_6$ *tag.*

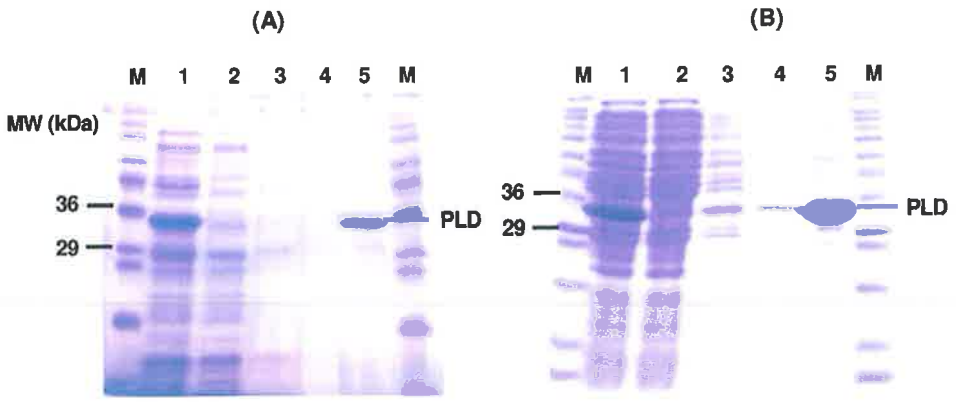


Figure 3.10. *SDS-PAGE analysis on the IMAC purification of PLD under* ***(A)*** *native and* ***(B)*** *denaturing conditions. Lanes:* ***(M)*** *Sigma wide range molecular weight marker;* ***(1)*** *crude cytoplasmic lysate;* ***(2)*** *flow-through from IMAC resin following application of lysates;* ***(3-4)*** *wash fractions;* ***(5)*** *eluted fraction containing purified PLD.*

3.2.1.6 Nucleotide and amino acid sequence analysis on the PLD protein

The plasmid pJGS90 containing the PLD insert was isolated from the DH5α *E.coli* cells, as described in *Section 2.3.1.1* and sent to MWG-Biotech (UK) Ltd for sequencing. Nucleotide comparison between the recombinant PLD and the previously sequenced PLD gene (accession number L16587) revealed that they demonstrated 100% homology, as seen in *Figure 3.11*. The amino acid sequence was then deduced using a web-based translate tool (Expasy) and sequences aligned using a web-based alignment tool (PBIL) (*Section 2.4.3.9.2*). Results revealed that the two amino acid sequences were identical (*Figure 3.12*).

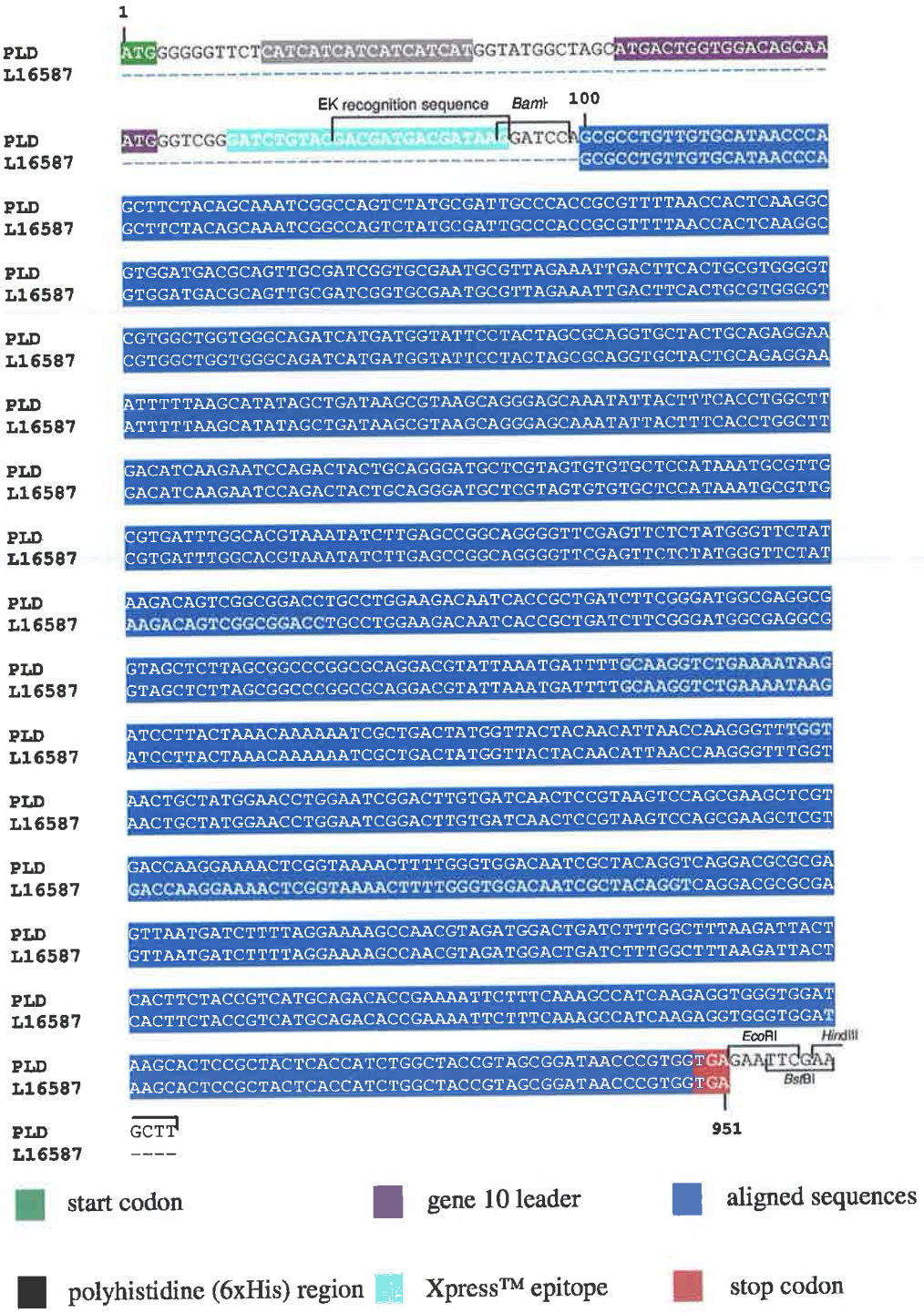


Figure 3.11. *Nucleotide sequence alignment of PLD. The mature PLD nucleotide sequence was aligned with the previously sequenced PLD gene from C. pseudotuberculosis, L16587 (Songer et al., 1990). The two sequences demonstrated 100% homology.*

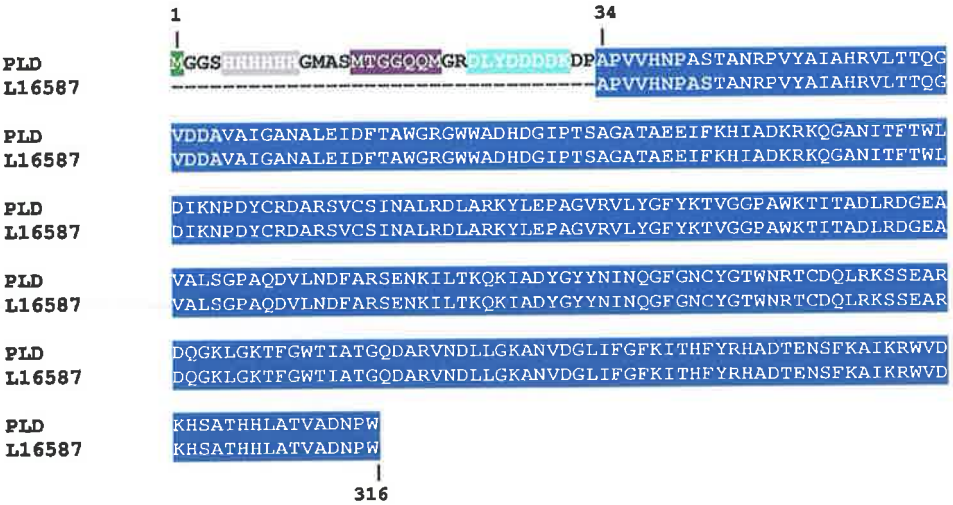


Figure 3.12. *Alignment of the deduced PLD amino acid sequence with the PLD amino acid sequence from C. pseudotuberculosis (accession number L16587). The nucleotide sequences (Figure 3.11.) were translated into their respective amino acid sequences and aligned. The two sequences were found to be identical.*

3.2.1.7 Identification of recombinant PLD by Western blotting

The PLD recombinant protein was analysed by Western blotting to determine the whether the PLD recombinant protein had the ability to detect antibodies to *C. pseudotuberculosis* in sheep infected with CLA. The PLD protein was probed with serum from CLA-infected sheep (CLA-positive) and serum from sheep with no prior history of CLA infection (CLA-negative) to establish whether or not CLA-specific antibodies recognised the protein.

The recombinant protein was electrophoresed alongside a prestained molecular weight marker, as described in *Section 2.2.3.* Following separation by SDS-PAGE, the proteins were transferred to nitrocellulose for Western blot analysis, as described in *Section 2.2.4*, with the following amendments. Following transfer of the proteins onto the nitrocellulose, the membrane was blocked with PBS containing 5% (w/v) Marvel™. It was then probed with a 1 in 200 dilution of sheep sera in PBS containing 4% (w/v) Marvel™, which had previously been incubated at 37°C for 1 hour. Following the addition of serum, the membrane was incubated for 1.5 hours with gentle shaking at room temperature and washed thoroughly as outlined in *Section*

2.2.4. A 1 in 2,000 dilution of goat anti-sheep horseradish peroxidase (HRP)-labelled antibody in PBS containing 4% (w/v) Marvel™ was added to the nitrocellulose for 1.5 hours with gentle shaking at room temperature. The nitrocellulose was washed thoroughly as before and protein bands were visualised using 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) substrate. The reaction was allowed to proceed until bands were clearly visible and was stopped by rinsing the membrane with water. The recombinant protein was able to bind antibodies to *C. pseudotuberculosis* and distinguish between CLA infected and uninfected sheep, as illustrated in *Figure 3.13*. A band, between 30 and 35kDa, can be clearly seen in *Figure 3.13 (B)*. This represents the PLD recombinant protein. No bands were visible when the proteins were incubated with CLA-negative serum (*Figure 3.13 (C)*).

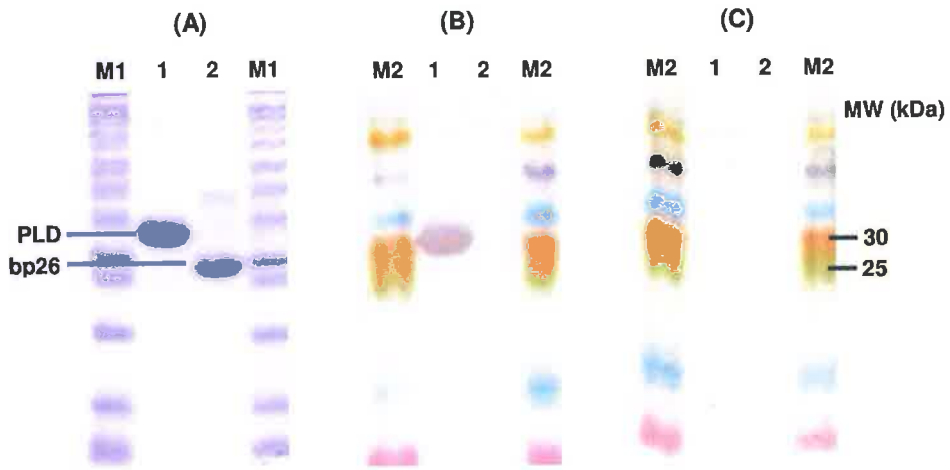


Figure 3.13. *Immunoreactivity of recombinant PLD towards CLA-specific antibodies. Lanes: **(M1)** Sigma wide range molecular weight marker (as before); **(M2)** Amersham Biosciences full range molecular weight markers consisting of red (10kDa), blue (15kDa), green (25kDa), orange (30kDa), blue (35kDa), purple (50kDa), yellow (75kDa), green (105kDa), red (160kDa) and blue (250kDa) markers. **(A)** SDS-PAGE analysis on IMAC-purified PLD and negative control bp26 (a Brucella-specific recombinant protein), with bands clearly visible at approx 35kDa and 26kDa, respectively. **(B)** Western blot probed with a CLA-positive sheep serum sample, with a band clearly visible at between 30 and 35kDa representing the PLD recombinant protein. **(C)** Western blot probed with a CLA-negative sheep serum sample, with no bands visible.*

3.2.2 Development of an ELISA for the detection of antibodies to *Corynebacterium pseudotuberculosis* in sheep

The ultimate aim of this research was to develop and validate a sensitive and specific assay for the detection of antibodies to *C. pseudotuberculosis* in sheep serum samples. This section focuses on the development of this assay to detect CLA in sheep using a process described by Jacobson (1998) for assay validation. This process involves two stages. Firstly, to establish the parameters and characteristics of the assay, and secondly, to assure constant validity of test results. A number of variables can affect the performance of such an assay, including the serum sample, the assay system and the actual test result (whether or not it can accurately predict the status of infection in an individual animal). The host can have a major influence on the composition and concentration of the serum sample, due to inherent (*e.g.* age, sex, breed, nutritional status and immunological responsiveness) or acquired (*e.g.* passively acquired antibody or active immunity) factors (Jacobson, 1998). Other factors, such as contamination or deterioration of the serum sample may also affect the analyte of interest. All of these issues must be considered in the development of the assay.

The following section concentrates on the development of an indirect ELISA and the process, including feasibility studies, optimisation and standardisation of protocols and reagents and finally, the performance characteristics of the assay. A number of control sera ($n=9$) were obtained from the Central Veterinary Research Laboratory (CVRL), Abbotstown, Dublin, Ireland to standardise the assay parameters. Controls were selected from sheep "on-site" that were experimentally infected with *C. pseudotuberculosis* and from sheep with no history of infection. Five positive serum samples, that ranged from high to low levels of antibodies against CLA infection (*GB04-013280*, *GB04-013281*, *GB04-013282*, *GB04-013283*, *GB04-013284*) and four samples that should contain no antibody, *i.e.* free of infection, (*GB04-013276*, *GB04-013277*, *GB04-0013278*, *GB04-013279*) were obtained. The five positive controls sera were taken from two male and three female sheep. Each serum sample was divided into 500µl aliquots and stored at –20°C. Working stocks were thawed and stored at 4°C until they had been depleted. These samples were employed initially in the optimisation of the assay reagents and protocols, and later as controls during runs of the assay for data normalisation.

3.2.2.1 Feasibility studies

Initially feasibility studies were carried out on the nine control sera to determine if the recombinant PLD antigen had the capacity to discriminate between a range of antibody concentrations to the disease and provide minimal background activity. The indirect ELISA, incorporating the purified recombinant PLD, as described in *Section 2.3.2.1*, was used to test the nine control sera. Briefly, an immunoplate was coated with PLD and blocked with PBS containing 4% (w/v) Marvel™. Following incubation, CLA-positive and CLA-negative serum, diluted in PBS containing 4% (w/v) Marvel™, were pre-incubated and added to the wells in triplicate. Specifically bound sera antibodies were detected using a rabbit anti-sheep HRP-labelled antibody (Dako) and the absorbance recorded following the addition of a chromogenic substrate, *o*-PD (*o*-phenylenediamine). The results were normalised by dividing the absorbance values (A) by the absorbance value obtained for zero concentration of serum (A_0) and plotted on a bar chart, as shown in *Figure 3.14*. It is evident from these results that the indirect ELISA could distinguish between sera from sheep infected or not infected with CLA.

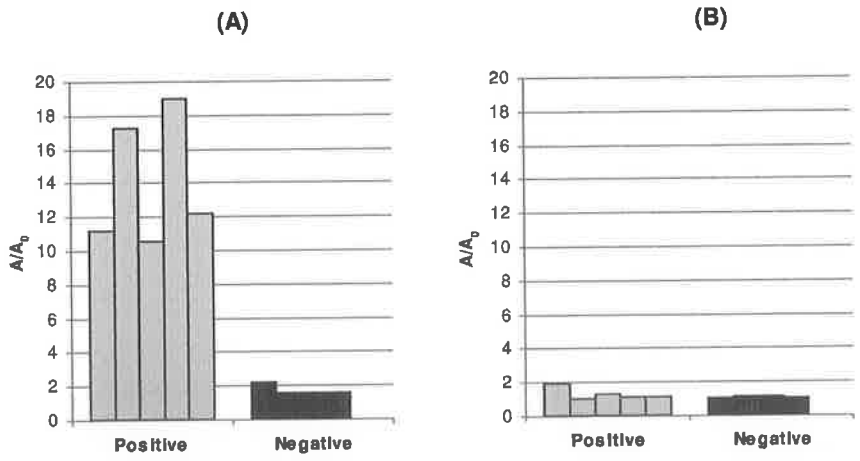


Figure 3.14. *Initial feasibility studies on the indirect ELISA for the detection of caseous lymphadenitis (CLA). Five standard positive and four negative control serum samples were tested against* ***(A)*** *PLD antigen and* ***(B)*** *no PLD antigen (control). The purpose of the control test was to determine if there were any non-specific interactions between the CLA-positive and negative sera and the plate or blocking reagent. Negligible non-specific binding was observed, as seen in graph (B).*

3.2.2.2 Assay development and standardisation

A number of factors can affect the analytical accuracy of an assay, including reagent choice, diluents, incubation temperatures and duration. It was therefore necessary to optimise blocking and diluent reagents, and the concentration of antigen, serum and enzyme-labelled secondary antibody due to the presence of polyclonal antibodies and the complexities of the serum matrix.

3.2.2.2.1 *Optimisation of blocking reagents and antibody diluents*

Marvel™, chicken egg albumin (ovalbumin-OVA) and bovine serum albumin (BSA) were investigated as the blocking reagent and antibody diluents. In order to establish the optimum blocking reagent and antibody diluent, immunoplates were coated with PBS containing 4% (w/v) Marvel™, PBS containing 4% (w/v) OVA or PBS containing 4% (w/v) BSA. Each of the nine control serum samples were prepared using each of the following diluents; PBS containing 4% (w/v) Marvel™, PBS containing 4% (w/v) OVA and PBS containing 4% (w/v) BSA. Each of the serum samples were pre-incubated at 37°C for one hour and then added to the three immunoplates, in triplicate. A 1 in 2,000 dilution of HRP-labelled anti-sheep antibody was then prepared using the three different diluents, PBS containing 4% (w/v) Marvel™, PBS containing 4% (w/v) OVA and PBS containing 4% (w/v) BSA. The anti-sheep HRP-labelled antibody dilutions were pre-incubated first at 37°C for one hour and then added to the three immunoplates. Bound serum antibodies were detected following the addition of *o*-PD and the normalised absorbance values (A/A_0) were plotted on bar graphs as shown in *Figures 3.15, 3.16* and *3.17.* Marvel™ and BSA were found to be the most suitable blocking reagents and serum antibody diluents as both protein solutions significantly reduced non-specific binding of the serum to the immunoplates. However, Marvel™ was chosen in subsequent assay development, as it was more economically viable.

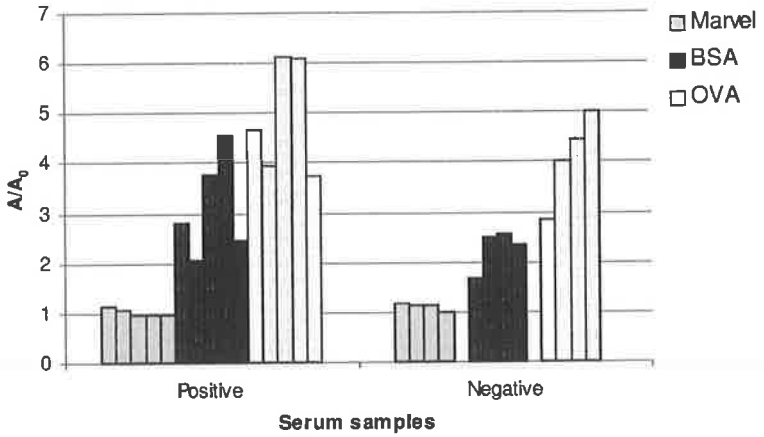


Figure 3.15. *Optimisation of blocking reagent and antibody diluent. Results obtained for plate 1, which was coated with 4% (w/v) Marvel™. Each of the control serum samples was added in triplicate to the immunoplate, diluted 1/50 with 3 different diluents; PBS containing 4% (w/v) Marvel™, PBS containing 4% (w/v) OVA, PBS containing 4% (w/v) BSA. A high degree of non-specific interactions was observed between the Marvel™ blocking solution and serum samples diluted in OVA and BSA. However, negligible non-specific interactions were observed between the Marvel™ blocking solution and serum samples diluted in Marvel™.*

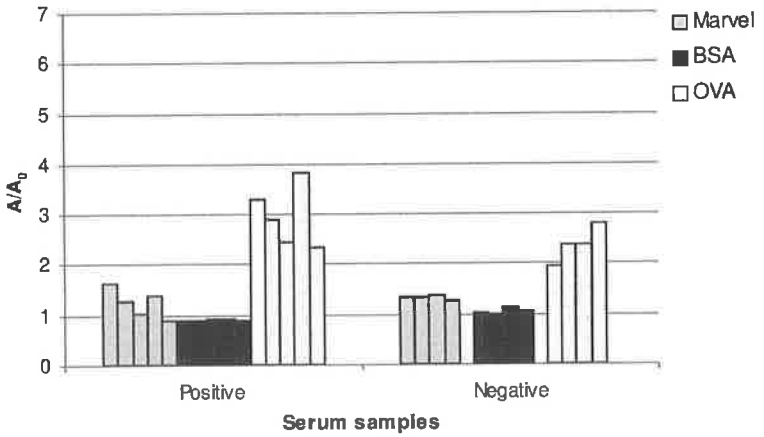


Figure 3.16. *Optimisation of blocking reagent and antibody diluent. Results obtained for plate 2, which was coated with 4% (w/v) BSA. Each of the control serum samples was added in triplicate to the immunoplate, diluted 1/50 with 3 different diluents; PBS containing 4% (w/v) Marvel™, PBS containing 4% (w/v) OVA, PBS containing 4% (w/v) BSA. A high degree of non-specific interactions was observed between the BSA blocking solution and serum samples diluted in OVA. However, negligible non-specific interactions were observed between the BSA blocking solution and serum samples diluted in Marvel™ and BSA.*

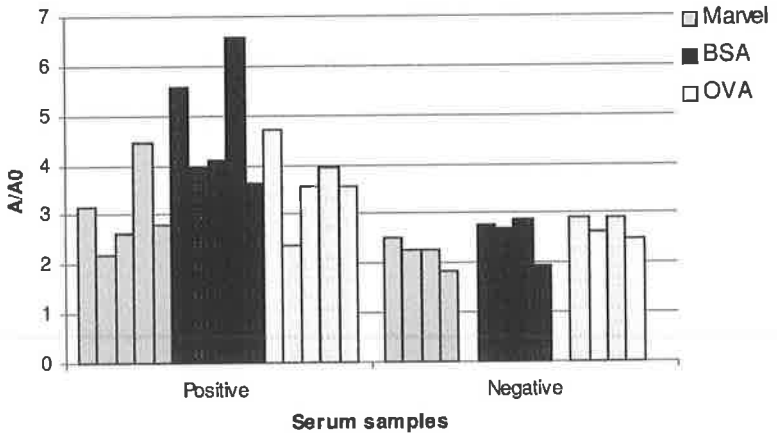


Figure 3.17. *Optimisation of blocking reagent and antibody diluent. Results obtained for plate 3, which was coated with 4% (w/v) OVA. Each of the standard serum samples was added in triplicate to the immunoplate, diluted 1/50 with 3 different diluents; PBS containing 4% (w/v) Marvel™, PBS containing 4% (w/v) OVA, PBS containing 4% (w/v) BSA. A high degree of non-specific interactions was observed between the OVA blocking solution and serum samples diluted in Marvel™, OVA and BSA.*

3.2.2.2.2 *Optimisation of antigen coating dilution for use in the indirect ELISA*

Following optimisation of the blocking reagent and antibody diluent, the next step in the assay development focused on optimising the antigen coating concentration. The indirect ELISA was carried out, as described in *Section 2.3.2.1*, and the results obtained are illustrated in *Figure 3.18*. Varying PLD coating dilutions ranging from 1/4 to 1/512 were investigated for use in the indirect ELISA. A 1 in 32 dilution of the PLD antigen enabled optimal discrimination between the CLA-positive and negative serum samples.

3.2.2.2.3 *Optimisation of sheep serum dilutions for use in the indirect ELISA*

Following optimisation of the antigen coating dilution, the subsequent step in assay development involved the determination of the optimal sheep serum dilution for use in the indirect ELISA. Sheep serum dilutions, ranging from 1/25 to 1/3,200, were investigated to determine which enabled optimal discrimination between the positive and negative sera. *Figure 3.19* illustrates the results obtained for the indirect ELISA, where a 1 in 50 serum dilution was chosen as the optimal dilution for use in the indirect ELISA.

3.2.2.2.4 *Optimisation of anti-sheep secondary antibody dilution for use in the indirect ELISA*

The final step in optimisation of the assay reagents was the determination of the optimal dilution of secondary antibody, *i.e.* anti-sheep HRP-conjugated antibody (Dako), for use in the indirect ELISA. The nine control sera were tested with varying anti-sheep dilutions and a 1 in 4,000 dilution offered the best discriminatory capability, while being the most economically viable (*Figure 3.20*).

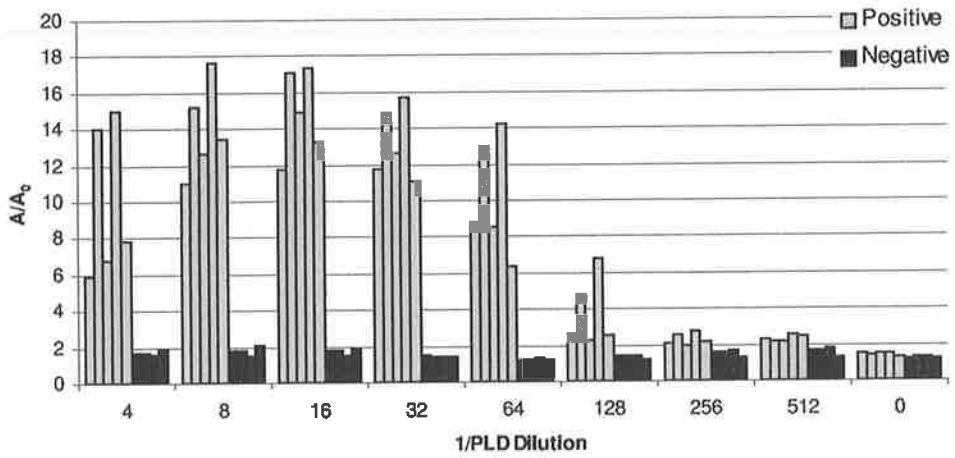


Figure 3.18. *Optimisation of the PLD antigen coating dilution for use in the indirect ELISA. PLD coating dilutions, ranging from 1/4 to 1/512, were investigated to determine which dilution enabled optimal discrimination between the CLA-positive and negative serum samples. A 1/32 dilution of PLD was selected as the optimal dilution.*

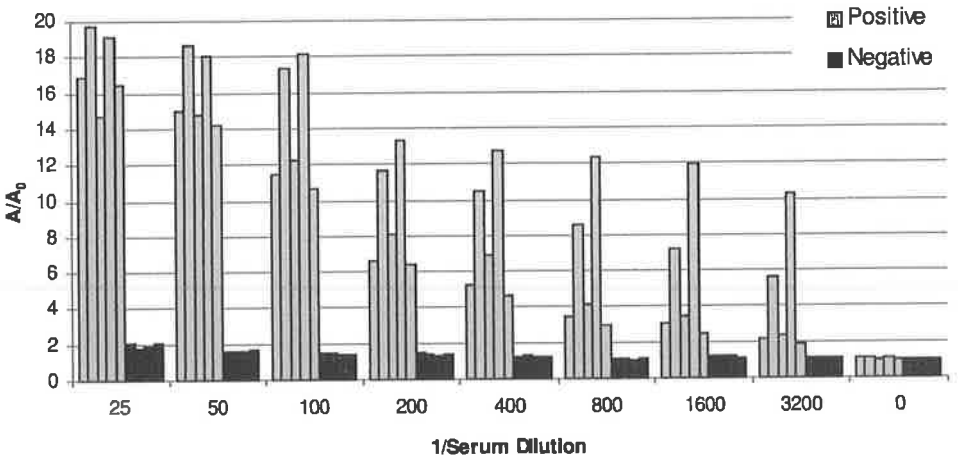


Figure 3.19. *Optimisation of the serum dilution for use in the indirect ELISA. Serum dilutions, ranging from 1/25 to 1/3200, were tested in order to determine which concentration enabled optimal discrimination between the positive and negative serum. A 1/50 dilution of the serum samples was selected as optimal.*

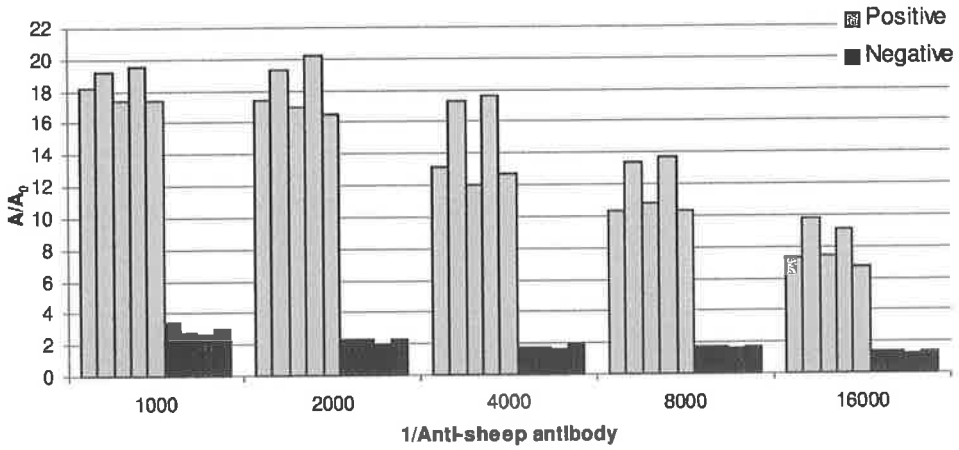


Figure 3.20. *Optimisation of the anti-sheep antibody dilution for use in the indirect ELISA. Anti-sheep antibody dilutions, ranging from 1/1000 to 1/16000, were tested in order to determine which concentration enabled optimal discrimination between the standard positive and negative serum. A 1/4,000 dilution of anti-sheep antibody was selected as optimal.*

3.2.2.2.5 Repeatability studies on the indirect ELISA

Following optimisation on the various assay parameters, intra-assay (variation within an assay) and inter-assay (variation between assays) studies were performed to ensure the assay repeatedly achieved the same results for a serum control. Three replicates of each control sera were assayed over ten individual days to provide estimates on repeatability and the coefficients of variation (CV) were determined by expressing the standard deviation of the replicates over the mean of the replicates.

For preliminary estimates of repeatability within and between runs of the assay, values less than 20% for raw absorbance values indicate adequate repeatability (Jacobson, 1998). For intra-assay studies, CVs ranged from 1.47 to 9.94% and 0.3 to 11.84%, for the positive and negative serum controls, respectively. For inter-assay studies, CVs ranged from 3.8 to 9.37% and 8.95 to 11.75% for the positive and negative serum controls, respectively. The CV values for the negative sera were found to be higher but this was due to some of the mean values approaching zero, in which case the CVs are not meaningful. Values for the positive sera did not exceed 10% indicating excellent reproducibility. The graphical representation of the inter-day studies on the PLD indirect ELISA is shown in *Figure 3.21.* The positive serum sample, *GB04-031283* and negative serum, *GB04-031279*, were chosen as controls for subsequent runs of the assay, as they proved to be the most reproducible, with inter-assay CVs of 3.80 and 8.95%, respectively.

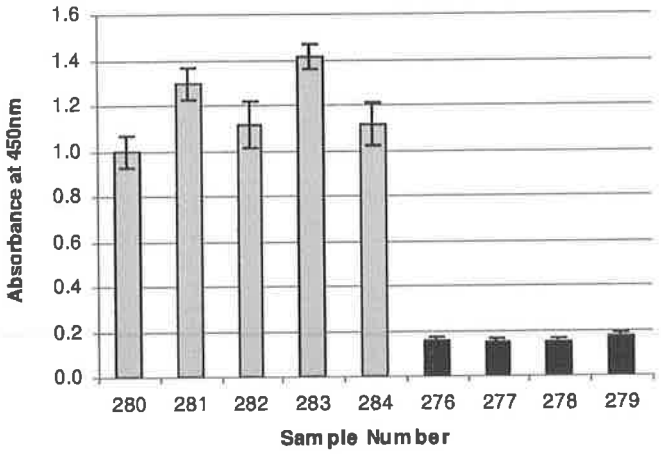


Figure 3.21. *Inter-assay (n=10) graph obtained for the PLD indirect ELISAs. The optimised assay format was capable of discriminating between the CLA-positive and negative serum samples. The bar graph represents the average absorbance values obtained over ten individual days for the nine control sera. The error bars correspond to the standard deviation of the mean calculated between the ten day replicates.*

3.2.2.3 Characterisation of indirect ELISA performance

Following optimisation of the various assay parameters required to successfully carry out the indirect ELISA, the performance of the assay was determined using a panel of sera samples (*n=60*) and the two control serum samples (positive *GB04-013283* and negative *GB04-013279*). The sixty serum samples tested were taken from three different flocks, two of which were infected with CLA (*n= 33*) (G. Killilea and Lyons Estate) and one flock that had no history of infection (*n=27*) (Abbotstown). To account for assay variability, results were normalised by expressing all absorbance values as a percentage the positive serum control, which was included on each plate. A cut-off point was established by calculating the mean plus four standard deviations (SD) obtained for the negative control sample during repeatability studies. This value was found to be 11.4% when expressed as a percentage of the positive serum control and used to discriminate between CLA-positive and negative samples, where any samples with a value above 11.4% were regarded as positive and any values below regarded as negative. *Figure 3.22* shows the graphical representation of the results obtained for analysis of the sixty sera using the PLD indirect ELISA. Applying the

cut-off of 11.4%, twenty-one of the samples were found to be positive and thirty-nine were found to be negative. Although the infection status of each individual animal was unknown, three of the sheep had developed lesions, all of which sera gave a high positive result when applied to the indirect ELISA. One of the sera from the uninfected flock gave a positive result. Preliminary studies did indicate that the assay could distinguish between some of the infected and uninfected sheep. However, since the infection status of each individual animal was unknown, the diagnostic sensitivity (the proportion of known infected animals that give positive results) and the diagnostic specificity (the proportion of known uninfected animals that give negative results) could not be calculated.

In order to calculate the diagnostic sensitivity (DSn) and diagnostic specificity (DSp), ninety-two serum samples, previously tested at the Central Veterinary Institute, Lelystad, the Netherlands were evaluated (ter Laak *et al.*, 1992; Dercksen *et al.*, 2000). All samples were tested in triplicate and results were normalised by expressing their respective absorbance values as a percentage of the positive serum control, which was included in each immunoplate. The intra-assay coefficients of variation (CVs) were found to be below 20% for all the serum samples that gave a positive result in the indirect ELISA. Of these values, 86.4% were found to be below 10%. 90% of the serum samples that gave negative results were found to have CVs below 20%. The CVs for the negative sera were found to be higher but this was due to some of the mean values approaching zero, in which case the CVs are not meaningful. However, data analysed from the results obtained from Lelystad revealed that only 57% of the serum samples that tested positive in the ELISA had CV values below 10%, 25% below 20%, 9% below 30% and 9% greater than 50%, indicating inadequate reproducibility. No data from the serum samples that gave a negative result in the ELISA used in Lelystad was received so CVs for ELISA-negative samples could not be calculated.

To ensure the results obtained from the PLD indirect ELISA were reproducible, each of the ninety-two samples was tested again in triplicate on a different day. The absorbance values were normalised by expressing them as a percentage of the positive control and compared to the first set of data obtained. The mean of both sets of normalized results was found and the CVs determined by expressing the standard

deviation of the replicates over the mean of the replicates. 95.5% of all the samples tested in triplicate on two separate occasions that gave positive results were found to have CV values below 20%. The CVs for the negative sera were found to be higher, with only 83% of samples tested found to have values below 20%. Although, again, this was due to some of the mean values approaching zero, in which case the CVs are irrelevant. Overall data for each sample tested in triplicate on two different occasions revealed excellent reproducibility, with 89% of all sera tested having CVs below 20%.

To determine the diagnostic sensitivity (DSn) and diagnostic specificity (DSp) of the indirect ELISA, a cut-off value was selected by visual inspection of the frequency distributions (Jacobson, 1998). A value of 25%, at the intersection of the two distribution results (CLA-positive and CLA-negative as determined by the ELISA performed at Lelystad) was chosen. *Figure 3.22* shows the graphical representation of the results obtained for the PLD indirect ELISA, where a cut-off point of 25% was used as it gave optimal discrimination between CLA-positive and negative sera. By comparing both sets of results, a DSn of 82% and a DSp of 83% were calculated as outlined in *Figure 3.24.*

However, the reference sera analysed were taken from animals where the history and infection status relative to the disease was unknown and the DSn and DSp was calculated on previous ELISA-positive and -negative results received from Lelystad, as opposed to clinical infection. Therefore, these values are not a true representation of the sensitivity and specificity of the PLD indirect ELISA, as estimated DSn and DSp values are entirely dependent upon the characteristics of the reference population.

Applying this cut-off of 25% to the previous series of samples tested (*n=60*) from the G. Killilea, Lyons Estate and Abbotstown flocks (*Figure 3.22*), seventeen of the samples were found to be positive and forty-three were found to be negative. Although the infection status of each individual animal was unknown, this cut-off may be more accurate as none of the sera from the uninfected flock gave a positive result. The sera taken from the three sheep that had developed lesions still gave a high positive result when applied to the indirect ELISA.

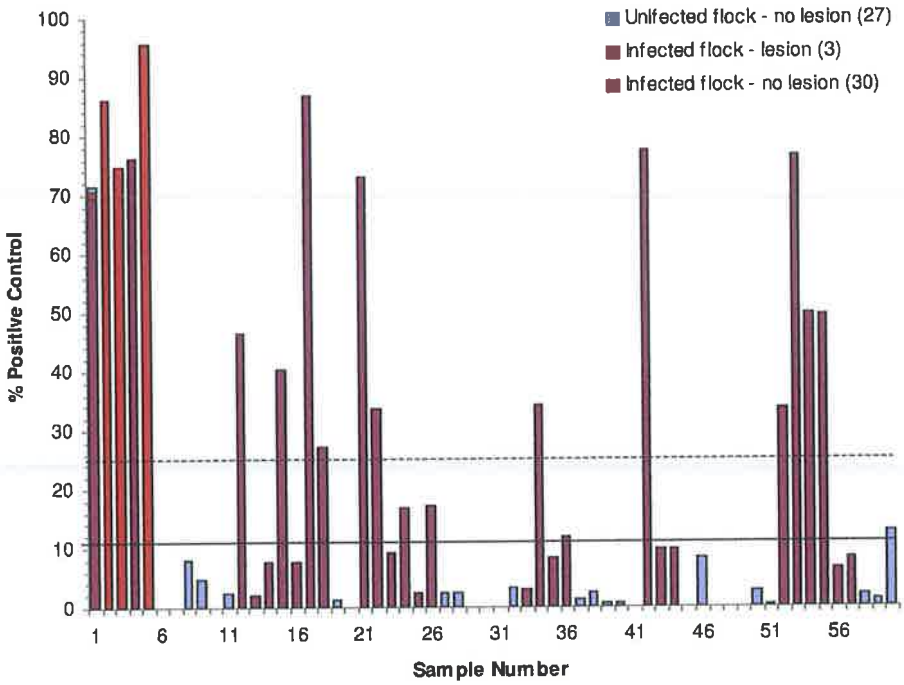


Figure 3.22. *Characterisation of PLD indirect ELISA performance. The indirect ELISA was assessed for use as a diagnostic assay for caseous lymphadenitis (CLA). The indirect ELISA was performed using a selection of 60 serum samples and a cut-off point of 11.4% was chosen to allow discrimination between CLA-positive and negative sera. Applying this cut-off, 21 samples were found to be positive and 39 found to be negative. However, applying a cut-off point of 25% proved to be more accurate, as none of the sheep from the uninfected flock gave a positive result, while the three sheep that had developed lesions still gave a high positive result.*

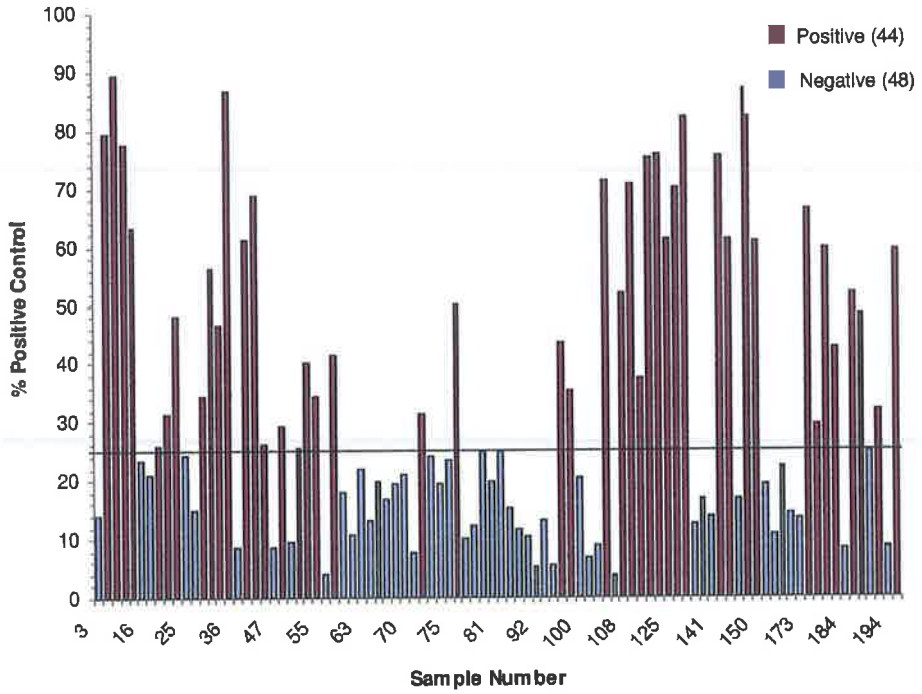


Figure 3.23. *Characterisation of the PLD ELISA performance. The PLD indirect ELISA was assessed for use as a diagnostic assay for caseous lymphadenitis (CLA) in sheep serum samples. The indirect ELISA was performed using a panel of 44 previously determined Lelystad ELISA-positive and 48 ELISA-negative reference samples. The absorbance value of each sample was recorded at 405nm and the mean plotted as a percentage of the mean absorbance obtained for the positive control sample. Applying the cut-off point of 25%, 44 of the serum samples tested were found to be positive and 48 negative.*

		Reference animals	
		Lelystad-Positive	**Lelystad-Negative**
Test Result	**Positive**	36 (A)	8 (B)
	Negative	8 (C)	40 (D)
		Diagnostic sensitivity $\frac{A}{A+C} = \frac{36}{44} = 82\%$	***Diagnostic specificity*** $\frac{D}{D+B} = \frac{40}{48} = 83\%$

Figure 3.24. *Diagnostic sensitivity (DSn) and diagnostic specificity (DSp) calculations for the PLD indirect ELISA. Results yielded a diagnostic sensitivity of 82% and specificity of 83%.*

3.2.3 Development of a Biacore-based assay for the detection of antibodies to *Corynebacterium pseudotuberculosis* in sheep

The SPR-based biosensor, Biacore™, has been proved to be a valuable alternative to ELISA procedures. As in ELISA, surface plasmon resonance (SPR) detection is rapid, cheap and standardisation is relatively straightforward. However, the Biacore system does have additional advantages. It allows the researcher to monitor biomolecular interactions in "real-time", is label-free, fully automated and its rapid analysis makes it ideal for testing a large number of samples. For these reasons, it was decided to transfer the assay principle to the surface plasmon resonance biosensor, Biacore 3000™, to determine whether this optical biosensor system would be suitable to detect antibodies in sheep sera that were specific for *C. pseudotuberculosis*.

This section focuses on the development of such an assay using a carboxymethylated-5 (CM5) dextran chip (Biacore AB) immobilised with the PLD recombinant protein. For the successful development of such an assay, the same parameters were optimised as before in the development of the indirect ELISA. However, for this system, a number of other parameters had to be considered, including preconcentration studies for subsequent immobilisation of antigen and stability studies on the immobilised surface for the assessment of binding capacity and regeneration of the chip surface.

3.2.3.1 Preconcentration studies

Preconcentration studies are essential for efficient immobilisation of the protein onto the surface of the CM5 sensor chip. The CM dextran surface has a negative charge at pH values ≥ 3.5. Therefore, in order to maximise the electrostatic interaction between the negatively charged CM dextran sensor surface and the positively charged protein the pH of the immobilisation buffer should be higher than 3.5 but should be one or two units lower than the isoelectric point (pI) of the protein.

Briefly, the PLD protein was prepared in a low ionic strength buffer (10mM sodium acetate) at a range of pH values, as described in *Section 2.3.3.1.* It was then passed over an unactivated chip surface and the amount of electrostatic interaction monitored. A pH of 4.4 gave the highest degree of electrostatic interaction, *i.e.* the

maximum binding, as seen in *Figure 3.25*, and was therefore chosen for subsequent immobilisations of the PLD protein.

3.2.3.2 Immobilisation of PLD onto the the CM5 sensor chip surface

PLD, at a concentration of 10μg/ml in 10mM sodium acetate buffer, pH 4.4, was immobilised on to the chip surface using standard amine coupling chemistry, as described in *Section 2.3.3.2*. The CM dextran surface of the chip was activated with a mixture of EDC and NHS and the protein was passed over the surface allowing the activated NHS esters on the surface to react with the amino groups on the protein. This was followed by an injection of ethanolamine to cap any remaining NHS unreacted sites and any loosely bound protein was removed with 5mM NaOH. A final immobilised level of approximately 7,000RU was achieved (*Figure 3.26*).

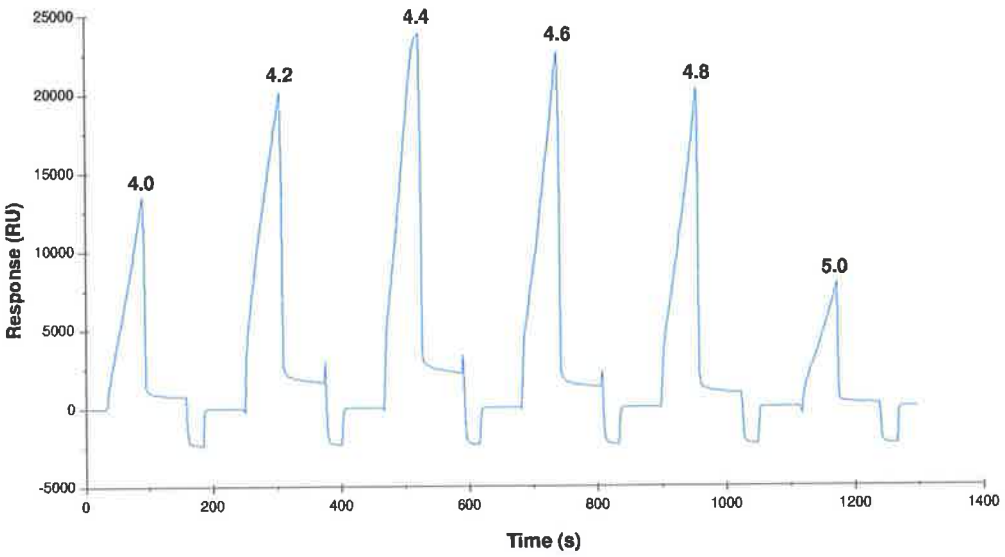


Figure 3.25. *Sensogram illustrating the preconcentration analysis on the recombinant PLD. Protein was passed over a CM5 dextran chip surface in 10mM sodium acetate buffer, ranging from pH values 4.0 to 5.0, at a flow rate of 10ul/min for 1 min. After each injection of protein a 30 second pulse of 5mM NaOH was used to remove electrostatically associated protein. Maximum preconcentration was observed at pH 4.4 (approx. 22,500 RU) and was, therefore, chosen as the immobilisation buffer.*

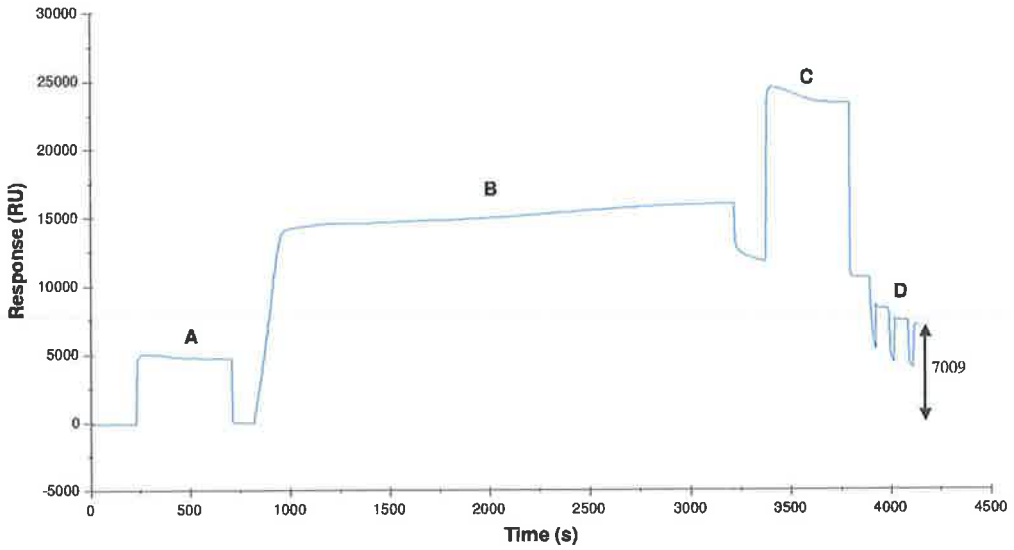


Figure 3.26. *Sensogram showing the amine coupling of the IMAC-purified PLD onto the CM5 sensor chip surface.* ***(A)*** *NHS/EDC activation,* ***(B)*** *covalent binding of PLD (10µg/ml in 10mM sodium acetate, pH 4.4),* ***(C)*** *capping of unreacted groups and* ***(D)*** *three 30 sec regeneration pulses of 5mM NaOH. A final level of 7,009 RU of covalently attached PLD was achieved.*

3.2.3.3 Assessment of non-specific binding of sheep serum to CM5 sensor chip surface

To investigate if there were any non-specific interactions between the sera and the CM dextran chip surface, the positive and negative control sera were diluted 30-fold in HBS buffer and passed over both the immobilised PLD and CM dextran (reference) flow cell surfaces as described in *Section 2.3.3.3.* Throughout the sera analyses, the final report response was corrected for the response of the reference flow cell. As seen in *Figure 3.27 (A)* and *3.28 (A)*, both the positive and negative sera bound non-specifically to the CM dextran surface. For the positive control, a response of 1,394RU was observed when sample was passed over the PLD surface; however, a response of 1,266RU was observed when it was injected over the reference surface (*Figure 3.27 (A)*).

To overcome this non-specific binding, carboxymethyl dextran sodium salt (Fluka Chemie) was added to the HBS diluent buffer to compete with the surface dextran for

binding to the serum antibodies. CM dextran sodium salt is commonly used in Biacore assays when dealing with complex matrices (Jongerius-Gortemaker *et al.*, 2002; Yang *et al.*, 2005). The serum controls were diluted in HBS containing 12mg/ml CM dextran sodium salt and passed over the sensor surface at a flowrate of 5µl/min for 2 minutes and the binding response monitored. The results, shown in *Figure 3.27 (B)* and *3.28 (B),* illustrates that addition of CM dextran to the serum diluent greatly reduced the non-specific binding and interference from the serum matrix.

3.2.3.4 Feasibility studies

Initial studies were carried out on the serum control samples to determine the feasibility of the Biacore assay, *i.e.* whether the PLD immobilised on the chip surface could discriminate between sera from CLA infected and uninfected sheep. The nine sera controls, as used previously in the development of the indirect ELISA, were each injected over the immobilised PLD chip surface and the response monitored. The results, illustrated in *Figure 3.29*, demonstrate the immobilised protein could distinguish between CLA-positive and negative sera.

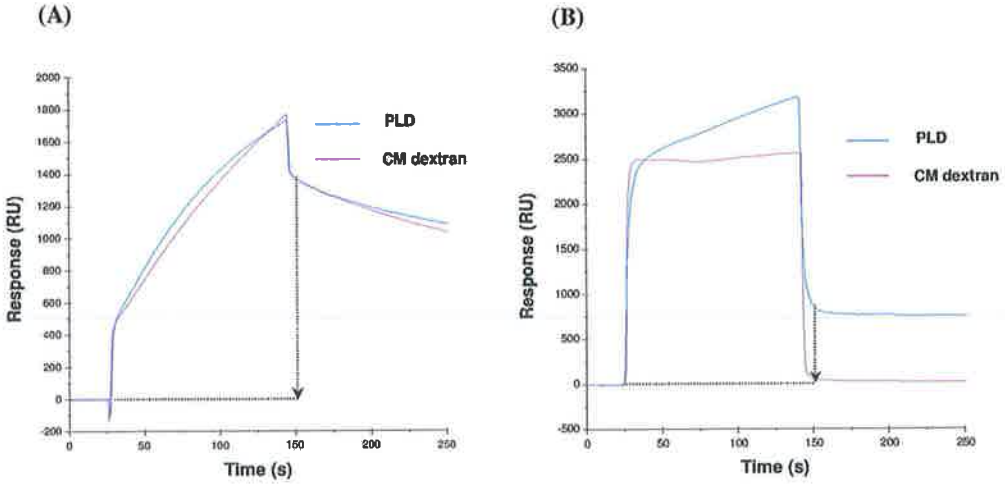


Figure 3.27. *Sensograms demonstrating the difference in response of the positive serum control* ***(A)*** *without or* ***(B)*** *with CM dextran sodium salt. The serum, at a dilution of 1/30, was passed over the PLD immobilised surface and the reference dextran surface at a flowrate of 5µl/min for 2 min.* ***(A)*** *Approximately 1,394RU was observed when the positive control was passed over the immobilised PLD surface. However, a response of 1,266 units was observed when the serum was passed over the unactivated dextran surface indicating a high degree of non-specific binding.* ***(B)*** *Following addition of CM dextran to the sample diluent, a response of 769RU and 30RU was observed when the positive control was passed over the immobilised and reference flow cell, respectively.*

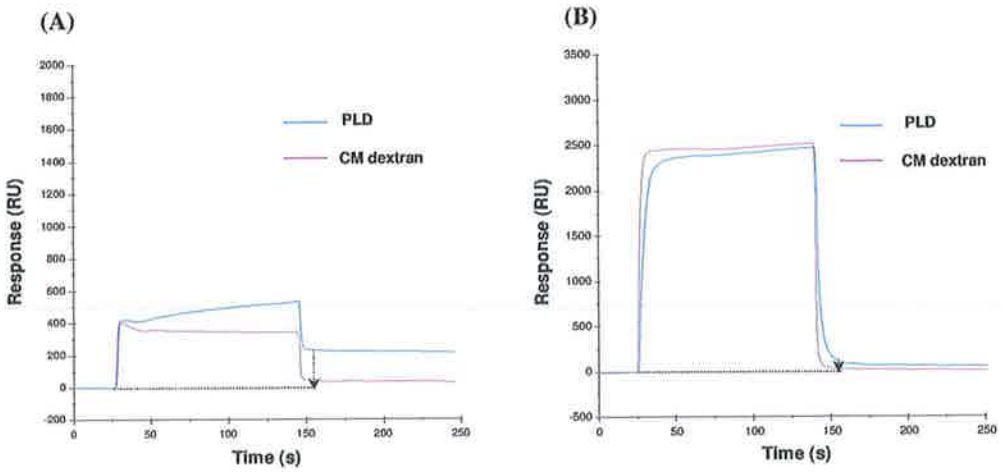


Figure 3.28. *Sensograms demonstrating the difference in response of the negative serum control* ***(A)*** *without or* ***(B)*** *with CM dextran sodium salt. The serum, at a dilution of 1/30, was passed over the PLD immobilised surface and the reference dextran surface at a flowrate of 5µl/min for 2 min.* ***(A)*** *Approximately 225RU was observed when the negative control was passed over the immobilised PLD surface. However, a response of 39 units was observed when the negative control was passed over the unactivated dextran surface indicating a high degree of non-specific binding of the serum antibodies to the dextran surface.* ***(B)*** *Following addition of CM dextran to the sample diluent, a response of 70RU and 24RU was observed when the negative control was passed over the immobilised and reference flow cell, respectively.*

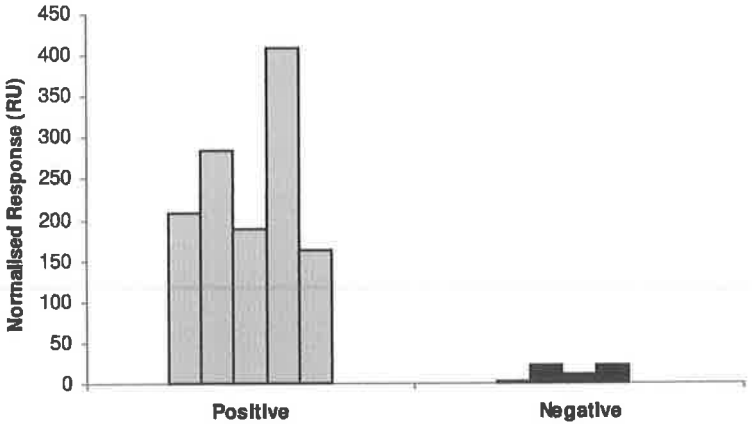


Figure 3.29. *Initial feasibility studies on the Biacore for the detection of caseous lymphadenitis. The five positive and four negative control serum samples were tested against the PLD immobilised surface. The normalised response units (RU) were obtained for each control and plotted on a bar chart. The PLD antigen, immobilised on the sensor chip surface was found to be capable of discriminating between CLA-positive and negative sera.*

3.2.3.5 Regeneration studies

Once it was established that the Biacore system had potential as a diagnostic tool for the detection of CLA, the next step was to optimise the regeneration of the chip surface so it could be used for testing numerous serum samples. Mild regeneration of the chip surface is essential for sustainable and optimal use with a large sample series. A number of regeneration solutions were evaluated but sodium hydroxide was found to be suitable for complete regeneration of the surface. When sera samples were diluted 40-fold, the normalised response for the positive and negative controls was found to be 640 and 25RU, respectively (*Figure 3.29*). All responses were normalised by subtracting the response obtained from the flow cell with no immobilised antigen (reference flow cell). The serum was passed over the sensor chip surface at a flowrate of 5µl/min for 2 minutes and the response recorded 30 seconds after the end of injection. This was followed by a 5µl injection of 20mM sodium hydroxide, which allowed complete regeneration of the chip surface, as seen in *Figure 3.30.*

The efficiency of the regeneration process was then evaluated by performing multiple (*i.e.* 20) binding-regeneration cycles on the PLD immobilised surface, as shown in *Figure 3.31.* The positive serum control, diluted 40-fold was injected over the sensor surface at a flowrate of 5µl/min for 2 minutes and specific bound antibodies removed with a 30 second pulse of 20mM NaOH at a flowrate of 10µl/min. Over the twenty binding-regeneration cycles, an increase of 14.5% in response signal was observed. This indicated that the regeneration solution was not effective in completely removing all bound serum antibodies. It was therefore decided to use a higher dilution of sera in subsequent analyses, as more concentrated sera samples would require harsher solutions to fully regenerate the chip surface and would ultimately affect the lifetime of the PLD-immobilised surface.

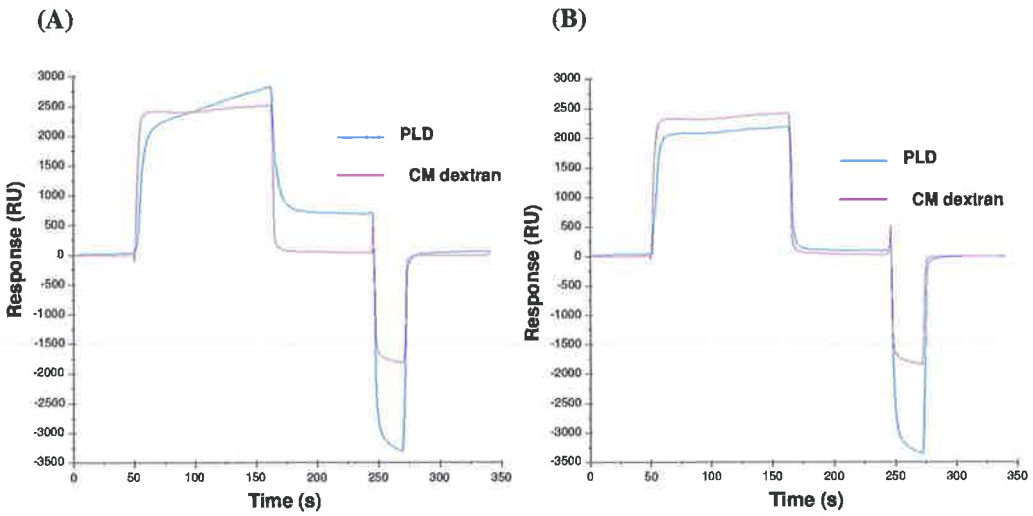


Figure 3.30. *Sensograms showing* ***(A)*** *the positive and* ***(B)*** *the negative sera being passed over the chip surface. The sera was passed over at a flowrate of 5µl/min for 2min, the specific serum antibodies bound to PLD and the surface was regenerated with 5µl of 20mM NaOH, which allowed complete regeneration of the surface.*

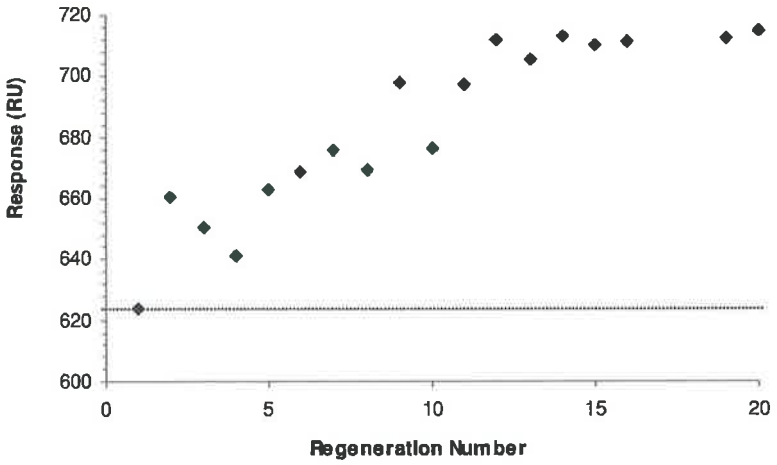


Figure 3.31. *Regeneration profile. Twenty consecutive binding-regeneration cycles were carried out with the positive serum control, GB04-013283. The serum was injected over both the PLD-immobilised and reference CM dextran surface at a flowrate of 5µl/min for 2 minutes, followed by a pulse of 20mM NaOH at a flowrate of 10µl/min for 30 seconds, to remove bound CLA-specific antibodies after each binding cycle. An increase in binding response was observed indicating the regeneration solution did not enable complete regeneration of the chip surface.*

3.2.3.6 Optimisation of sheep serum dilution for use in a Biacore assay

Although higher responses can be obtained with less dilution of sera, it complicates the regeneration of the chip surface, as mentioned previously. For this reason, a higher dilution of sera, which allowed complete regeneration of the surface without the need for harsh regeneration solutions, was required. Serial two-fold dilutions of the sheep positive and negative controls were prepared to determine which dilution enabled optimal discrimination between positive and negative sera. Dilutions, ranging from 1/10 to 1/160 were prepared in HBS containing 12mg/ml CM dextran sodium salt and injected over the chip surface. Response values were recorded and subsequently plotted on a bar chart, illustrated in *Figure 3.32*. From the results it can be seen that even diluting the controls 160-fold, the PLD Biacore assay still enabled discrimination between positive and negative sera. These results were then graphed to assess the linearity of the assay, where each two-fold dilution demonstrated roughly a two-fold decrease in response units as seen in *Figure 3.33*. As discussed previously, a 1/40 dilution of the positive control would require harsher solutions to fully regenerate the chip surface. Therefore, it was decided to use a higher dilution of sera, *i.e.* a 1/80 dilution, in subsequent analyses. At this dilution the response from the positive control is still quite high (approx. 400RU), which can be easily distinguished from the response from the negative serum control (approx. 20RU).

To ensure the linearity of response, as seen with the control sera, would be observed when testing the reference sera, the first eighteen of the reference samples (*n=92*) were examined. The positive and negative controls and reference serum samples (*n=18*) were analysed in triplicate at a 40 and 80-fold dilution, as seen in *Figure 3.34 (A)*. The values were then normalised by expressing their respective response units (RU) as a percentage the positive serum control, which was included in each run, as seen in *Figure 3.34 (B)*. The CVs were then determined by expressing the standard deviation of the replicates over the mean of the replicates. The CVs were found to be all below 20%, which the exception of two samples, number 18 and 40, which gave CV values of 36.85 and 32.69%, respectively. However, these response values for the negative sera were approaching zero, in which case the CVs are not significant. It was thus decided to use a dilution of 1/80 for subsequent screening of the reference samples.

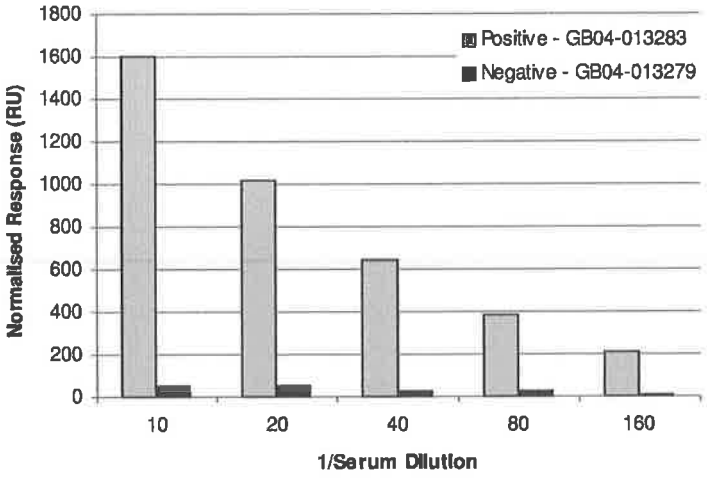


Figure 3.32. *Optimisation of the serum dilution for use in the PLD Biacore assay. Serum dilutions, ranging from 1/10 to 1/160, were tested in order to determine which concentration enabled optimal discrimination between the positive and negative serum controls.*

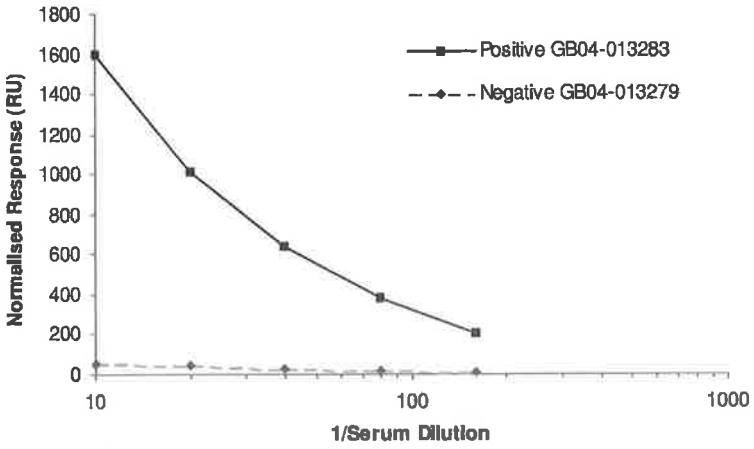
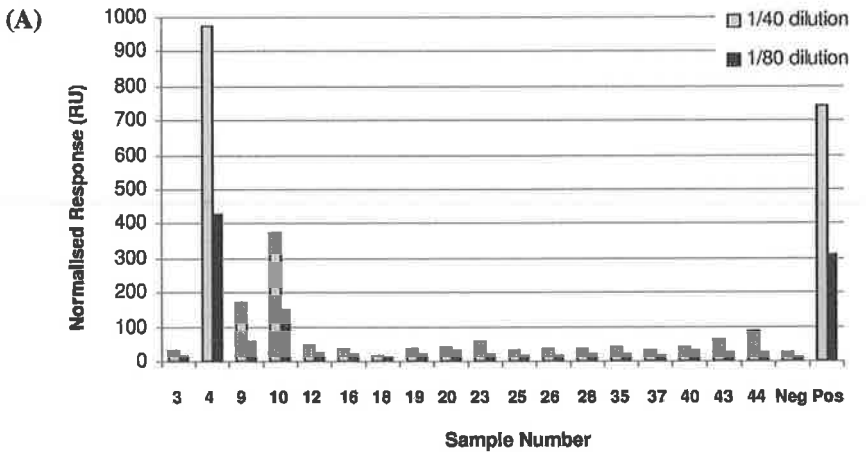


Figure 3.33. *Serial two-fold dilutions of the sheep positive and negative controls were prepared to examine the linearity of the assay. The series of dilutions, ranging from 1/10 to 1/160, revealed linear responses for anti-C. pseudotuberculosis antibodies binding to the PLD-immobilised surface.*



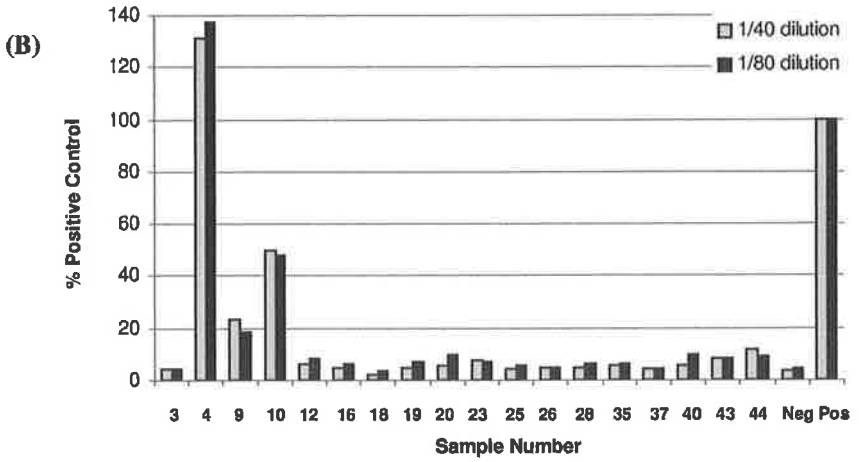


Figure 3.34. *Assessment of the linearity of response of the positive and negative controls and 18 of the reference sera.* ***(A)*** *Each serums sample was analysed in triplicate at a 40 and 80-fold dilution.* ***(B)*** *The values were then normalised by expressing their respective absorbance values as a percentage the positive serum control, which was included in each run. The CVs were then determined by expressing the standard deviation of the replicates over the mean of the replicates. The CVs were found to be all below 20%, which the exception of two samples, number 18 and 40, which gave CV values of 36.85 and 32.69%, respectively.*

3.2.3.7 Characterisation of assay performance

Serum samples (*n=92*), diluted 80-fold in HBS containing 12mg/ml CM dextran sodium salt, were transferred into individual glass vials, which were positioned in the auto sampler of the Biacore 3000™. Samples were then injected over the sensor surface at a flowrate of 5µl/min for 2 minutes and the response recorded 30 seconds after the end of injection. This was followed by a 5µl injection of 20mM NaOH, which allowed the complete regeneration of the chip surface. All ninety-two samples were tested in triplicate and results were normalised by expressing their respective response units (RU) values as a percentage of the positive control (*Figure 3.36*).

The non-specific binding responses of the sera were relatively constant in terms of response units (RU) with an average of approximately 20RU. The CVs were determined by expressing the standard deviation of the replicates over the mean of the replicates. The intra-assay (within the run) coefficients of variation (CVs) ranged from 0.33 to 9.76%, with a single outlier at 13.48%. 83% of all the ninety-two samples tested had a CV of less than 5%. All ninety-two sera samples were tested in a continuous run, with negative and positive controls included after every twenty serum samples. In total 306 analyses per flow cell were conducted successfully in a continuous series. The immobilised PLD surface went through a 264 binding-regeneration cycles (*i.e.* equivalent to analysing 80 serum samples before a significant (>20% original binding capacity) decrease in binding capacity was observed and the baseline remained stable, as illustrated in *Figure 3.35*.

The next step was to determine the cut-off point, which requires much attention as its level determines the false-positive/false-negative ratio. A cut-off point 8.1% was determined as the average response of the negative sera control throughout the analyses plus three times the standard deviation. As seen in *Figure 3.36*, using this cut-off point, forty-nine of the samples were determined positive and forty-three negative. To determine the diagnostic sensitivity (DSn) and diagnostic specificity (DSp) of the Biacore assay, results were compared to that obtained in Lelystad. A DSn of 86% and a DSp of 76% were calculated as outlined in *Fig 3.37*. Comparing the results to that found in the indirect PLD ELISA, described in *Section 3.2.2*, it was calculated that 95.5% of serum samples that gave a positive result in the ELISA also gave a positive result in the Biacore assay. Overall 91.3% of the results obtained in

the Biacore assay gave the same result as that found in the ELISA. Of the ninety two sera analysed, only eight samples gave conflicting results in the Biacore assay, six of these were within ±1% of the cut-off point, so therefore could be considered inconclusive.

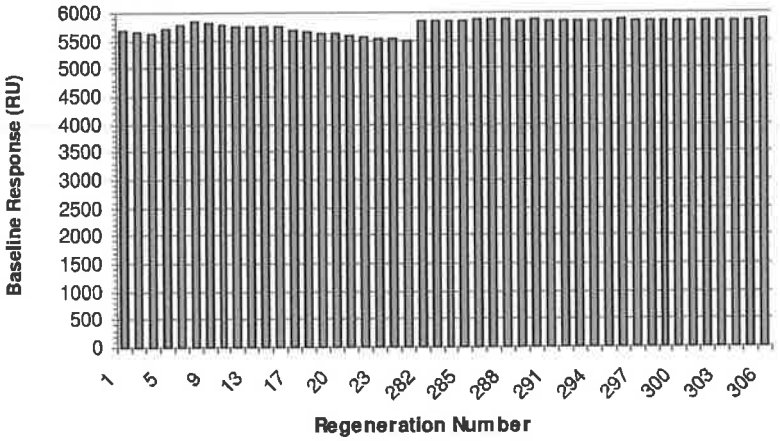


Figure 3.35. *Regeneration profile for the first (1-25) and last (282-306) twenty-five serum samples analysed. 306 consecutive binding-regeneration cycles were carried out in a continuous series. Each serum sample was injected over both the PLD-immobilised and reference surface at a flowrate of 5µl/min for 2 minutes, followed by a pulse of 20mM NaOH at a flowrate of 10µl/min for 30 seconds, to remove bound CLA-specific antibodies after each binding cycle. The baseline remained stable throughout the run, with only a 3% increase in observed.*

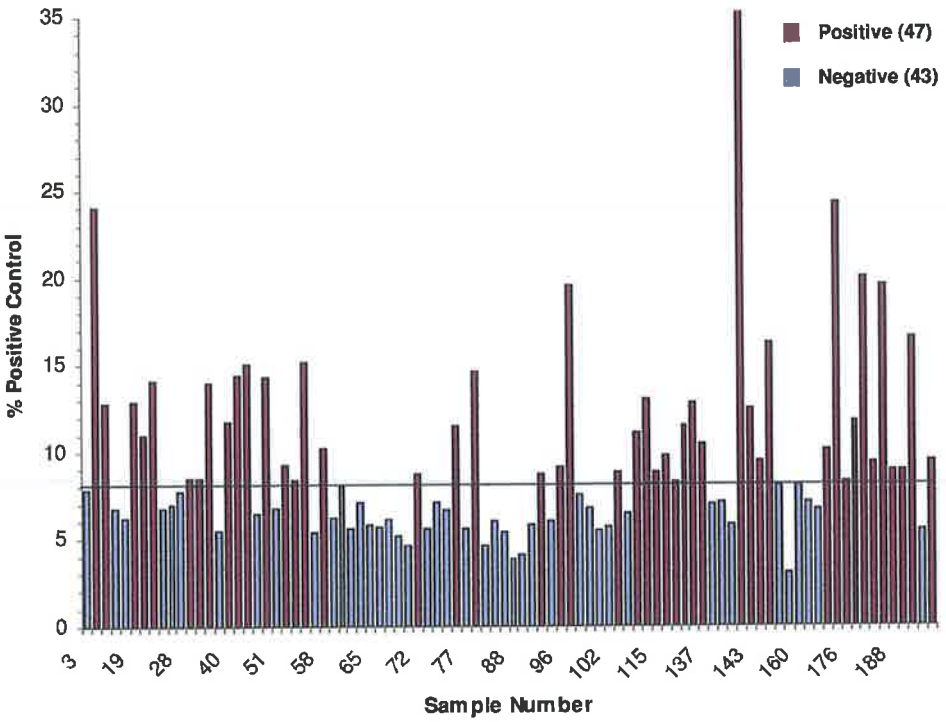


Figure 3.36. *Characterisation of the Biacore assay performance. The assay was assessed for use as a diagnostic assay for caseous lymphadenitis (CLA) in sheep serum samples. The assay was performed using a panel of 44 previously determined Lelystad ELISA-positive and 48 ELISA-negative reference samples. The absorbance value of each sample was recorded at 405nm and the mean plotted as a percentage of the mean absorbance obtained for the positive control sample. Using a cut-off point of 8.1%, 49 of the serum samples tested were found to be positive and 43 negative. Two samples, number 4 and 10, which were not included on the bar chart, gave a response as high as 169.3% and 53.83% of the positive control, which correlated with the response from the same samples tested previously in the indirect ELISA.*

		Reference animals	
		Lelystad-Positive	**Lelystad-Negative**
Test Result	**Positive**	37 A	12 B
	Negative	6 C	37 D
		Diagnostic sensitivity $\frac{A}{A+C} = \frac{37}{43} = 86\%$	***Diagnostic specificity*** $\frac{D}{D+B} = \frac{37}{49} = 76\%$

Figure 3.37. *Diagnostic sensitivity (DSn) and diagnostic specificity (DSp) calculations for the PLD Biacore assay. Results yielded a diagnostic sensitivity of 86% and specificity of 76%.*

3.3 Discussion

The main focus of this chapter was the expression of a recombinant form of the phospholipase D (PLD) exotoxin from *C. pseudotuberculosis* and its subsequent use in the development of an ELISA and Biacore-based assay for the detection of CLA infection in sheep.

The PLD gene from *C. pseudotuberculosis* had previously been cloned into the commercially available vector pTrcHisB (Songer *et al.*, 1990; Menzies *et al.*, 2004). This pTrcHisB plasmid bearing the PLD gene (referred to as pJGS90) was isolated from *E. coli* DH5α cells and restriction analysis confirmed the presence of the PLD gene at approximately 850bp. The plasmid was subsequently transformed into competent XL10-Gold *E. coli* cells for high-level protein expression and a number of transformants were selected for initial small-scale expression. This confirmed the presence of the PLD protein at a molecular weight of approximately 35kDa, a size which corresponds with previously published research (Menzies *et al.*, 2004). Once the presence of the protein was verified, protein solubility analysis was required for high-level protein recovery. Results indicated that over-expression of PLD resulted in the formation of inclusion bodies, which could be solubilised with a denaturant containing 8M urea. This result was also confirmed using a web-based program designed for protein solubility determination (Harrison, 2000). From the protein amino acid sequence, the program concluded that PLD has a 67% chance of insolubility when over-expressed in *E. coli.* The urea denaturant was therefore used for the subsequent isolation of PLD, following expression in XL10-Gold *E. coli.*

Several parameters were then optimised for high-level expression of the recombinant protein, including IPTG concentrations for induction of protein expression, sonication conditions and incubation time post induction. Optimisation of IPTG concentration is vital to ensure so that the highest level of expression is attained without the levels of IPTG or protein expression becoming toxic to the host cell (Liu *et al.*, 1999). For this reason, a concentration gradient of IPTG was set up to determine the optimal concentration, which was found to be 0.05mM. For successful isolation of the desired protein, sonication conditions were also optimised. Prolonged sonication times can have adverse effects and may lead to breakdown of fusion proteins (Ausubel *et al.*, 1995). For that reason, varying sonication times were investigated, however, very

little difference in PLD expression levels was observed with or without sonication. Hence, for large-scale expression sonication was not necessary and cells were lysed by gentle vortexing. A time-course experiment determined the optimal length of time needed for maximum protein expression was four hours post-induction with IPTG. Four hours was chosen as optimal as it minimised proteolytic degradation and $(His)_6$ tag cleavage, although after five hours the same levels of protein expression were observed,. The PLD recombinant protein was then examined by Western blotting to ensure the N-terminal $(His)_6$ tag was fully translated and had not been adversely affected during cell lysis. The protein reacted specifically with the anti-His tag antibody, confirming the histidine tag was accessible and fully intact.

The optimised parameters, as discussed above, were then applied for the large-scale production of the PLD recombinant protein. The $(His)_6$-tagged PLD protein was subsequently purified using immobilised metal affinity chromatography (IMAC) to remove any contaminating proteins remaining within the cytoplasmic lysate. This is an essential step if the recombinant protein is to be used in assay development as any contaminating proteins may cause interference. The protein was denatured with 8M urea, applied to the Ni-NTA resin and following pH shock elution the purity of the protein greatly increased in comparison to the denatured cell lysate. The denaturant was the removed under optimal conditions allowing the protein to refold. However succeeding the refolding process the yield of protein was relatively low. Consequently, the recombinant protein was also expressed and purified under native conditions. To maximise the yield of soluble product, an adapted method described by Barbas *et al.* (2001) was used. The method employs a super broth (SB) media for high-level expression of recombinant proteins and native purification was facilitated using imidazole. A yield of approximately 15mg of pure protein was obtained from a 500ml culture, which is very satisfactory as the manufacturers (Qiagen Ltd.) estimate a yield of approximately 5–10 mg of $(His)_6$-tagged protein per milliliter of resin.

The gene encoding the PLD recombinant protein was then sequenced and analysis indicated 100% homology with a published sequence, submitted by Songer *et al.* (1990) (accession number L16587). The amino acid sequence was then deduced using a web-based translate tool (Expasy) and the results revealed that the two amino acid sequences were identical.

Once the protein was successfully purified, the potential of the PLD recombinant protein for use as a diagnostic marker of caseous lymphadenitis (CLA) was then investigated by Western blotting. The PLD protein was probed with a CLA-positive and negative sheep serum sample to establish whether CLA-specific antibodies recognised the protein. Results showed that the recombinant protein was able to detect antibodies to *C. pseudotuberculosis* and distinguish between CLA-positive and negative serum. Following this, the protein was then applied to the development of an indirect ELISA for the diagnosis of caseous lymphadenitis (CLA) in sheep sera.

The indirect ELISA was based on the detection of anti-*C. pseudotuberculosis* serum antibodies specific for the recombinant protein phospholipase D. The ELISA validation was carried out using a method, previously described by Jacobson (1998), for the validation of serological assays for the diagnosis of infectious diseases This process involves determining the feasibility of the method; the development of the assay through standardisation of reagents and protocols; and finally, determining the performance characteristics of the assay.

The first stage in validating the indirect ELISA was to study the feasibility of the assay. Studies were carried out in order to determine if the selected reagents and protocols had the capacity to distinguish between CLA-infected and uninfected samples. Feasibility studies were carried out on nine serum controls received from the Central Veterinary Research Laboratory (CVRL). Controls were selected from sheep "on-site" that were experimentally infected with the CLA disease and from sheep free of infection. The indirect ELISA was found to be capable of discriminating between sera from infected and uninfected sheep.

Following establishment of the feasibility of the PLD indirect ELISA, the assay was developed and standardised. Optimal reagent concentrations and protocol parameters were selected and the repeatability of the assay was estimated. Various assay parameters were optimised, including blocking reagent and serum antibody diluent. Optimal dilutions for antigen, serum samples and secondary antibody were also determined.

The choice of blocking reagent and antibody diluent can greatly influence the sensitivity of an assay. The possibility of non-specific interactions between the serum antibodies and the blocking reagent is greatly increased due to the polyclonal nature of serum. For this reason, three protein blocking reagents, Marvel™, BSA and OVA, were investigated to determine which would minimise the non-specific interactions. Each of the protein solutions was added to the immunoplate to block the surface and to both the sera and secondary antibody diluent to reduce any non-specific binding to the blocking reagent. A significant degree of non-specific interaction between the sera antibodies and the OVA blocking solution was observed. However, negligible non-specific binding was observed when Marvel™ and BSA were used as both a blocking reagent and antibody diluent. Marvel™ was chosen for use in subsequent assay development, as it was more cost effective.

The optimal dilution of the PLD antigen, sera and secondary antibody were then determined by checkerboard titrations. The optimal dilutions were selected as the one, which enabled the greatest discrimination between the positive and negative serum samples. A PLD coating dilution of 1/32 was found to be optimal. The optimal dilution of sera and secondary antibody were found to be 1/50 and 1/4,000 dilution, respectively.

Following reagent optimisation, intra-assay (variation within an assay) and inter-assay (variation between assays) studies were performed to ensure the assay repeatedly achieved the same results for a serum control. Three replicates of each control were assayed over ten individual days and the coefficients of variation (CVs) were determined by expressing the standard deviation of the replicates as a percentage of the mean of the replicates. CVs within and between runs of the assay were all found to be less than 12% for raw absorbance values indicating adequate reproducibility (Jacobson, 1998). The positive serum sample, *GB04-031283*, and negative serum, *GB04-031279*, were chosen as controls for subsequent runs of the assay, as they proved to be the most reproducible, with inter-assay values of 3.80 and 8.95%, respectively.

The final stage of validation is characterising the assay in accordance to performance in categorising predetermined serum samples as positive and negative. This involves

determining the diagnostic sensitivity (DSn) and diagnostic specificity (DSp) of the assay. DSn is estimated as the proportion of known infected animals that give a positive result to infected animals that yield negative results (false negative (FN)). Whereas, DSp is the proportion of uninfected animals that give negative results to uninfected that display positive results (false positives (FP)). A panel of reference serum samples (n=92), previously tested by an ELISA developed in Lelystad (ter Laak *et al.*, 1992; Dercksen *et al.*, 2000) were tested using the PLD indirect ELISA in order to determine the DSn and DSp of the assay. Each sample was tested in triplicate on two separate occasions and results were normalised by expressing their respective absorbance values as a percentage the positive serum control, which was included in each immunoplate. 86.4% of the serum samples that gave a positive result in the indirect ELISA were found to have intra-assay CVs below 10% and the remaining 13.6% below 20%. 90% of the serum samples that gave negative results were found to have CVs below 20%. These values suggest the indirect ELISA was more reproducible than the Lelystad ELISA, which had much higher CV values. The inter-assay CVs also demonstrated the excellent reproducibility of the indirect ELISA with 89% of all the samples tested (n=92) on two different occasions having values below 20%. To determine the diagnostic sensitivity (DSn) and diagnostic specificity (DSp) of the indirect ELISA, a cut-off value of 25% was used as it gave optimal discrimination between CLA-positive and negative sera. By comparing both sets of results, from the PLD indirect ELISA and the Lelystad ELISA, a DSn of 82% and a DSp of 83% were calculated. However, the reference sera analysed were taken from animals where the history and infection status relative to the disease was unknown and the DSn and DSp was calculated on previous ELISA-positive and -negative results received from Lelystad, as opposed to clinical infection. Therefore, these values are not a true representation of the sensitivity and specificity of the PLD indirect ELISA, as estimated DSn and DSp values are entirely dependent upon the characteristics of the reference population. It is also recommended that a minimum of approximately 300 infected and 1,000 to 5,000 uninfected samples should be tested to provide confidence in estimates of DSn and DSp (Jacobson, 1998). However, in this case only a small number of samples were sent to Lelystad for testing so initial evaluation of the assay was only possible with this small panel of sera.

Once it had been established that PLD had proven useful as a serological diagnostic marker for CLA infection in the ELISA, the potential of an SPR biosensor-based system (Biacore 3000^{TM}) as a diagnostic tool was evaluated. The Biacore has proved to be a valuable alternative to ELISA procedures and facilitates automated, label-free detection of biomolecules in "real-time". A CM5 dextran chip immobilised with the PLD recombinant protein was used for the development of the assay and all parameters were optimised. A high degree of non-specific binding of the serum antibodies to the CM dextran surface of the chip was observed. However, this was overcome with the addition of CM dextran sodium salt to the serum diluent. Feasibility studies carried out on the nine control sera indicated the significant potential of the Biacore assay for the detection of *C. pseudotuberculosis* specific antibodies. The binding capacity and regeneration of the chip surface was also assessed. At a higher dilution of sera (1/80), a less concentrated solution of NaOH (*i.e.* 20mM) could be used for complete regeneration of the chip surface while still obtaining a high response (approx. 400RU) for the positive control.

Once all assay influencing factors were optimised, the Biacore assay was employed to analyse the reference panel of sera (n=92). Serum samples, diluted 80-fold, were injected over the sensor surface at a flowrate of 5µl/min for 2 minutes, the response recorded and the chip surface regenerated following a 5µl injection of 20mM NaOH. All ninety-two samples were tested in triplicate in a continuous run and results were normalised by expressing their respective response units (RU) values as a percentage of the positive control, included after every twenty samples analysed. The non-specific binding responses of the sera were found to be relatively constant with an average response of approximately 20RU. The intra-assay CVs of all sera analysed were found to be below 10%, with the exception of one sample with a value 13.48%. 83% of all serum samples tested had a CV of less than 5%. In total 306 analyses per flow cell were conducted successfully in a continuous series. The immobilised surface was also found to be stable, with only a 21% decrease observed for the binding response of the positive control after 264 binding-regeneration cycles (*i.e.* equivalent to analysing 80 serum samples).

To establish the sensitivity and specificity of the assay a cut-off point of 8.1% was determined as the average response of the negative sera control throughout the

analyses plus three times the standard deviation. Applying this cut-off value, forty-nine of the samples were determined positive and forty-three negative. When compared to the Lelystad ELISA results, a DSn of 86% and a DSp of 76% were calculated.

When the results obtained for the Biacore assay were compared to the indirect PLD ELISA, it was calculated that 95.5% of serum samples that gave a positive result in the ELISA also gave a positive result in the Biacore assay. Overall 91.3% of the results obtained in the Biacore assay gave the same result as that found in the ELISA. Of the ninety two sera analysed, only eight samples gave conflicting results in the Biacore assay, six of these were within ±1% of the cut-off point, so therefore could be considered inconclusive. Comparing the results from all three assays (*i.e.* Lelystad ELISA, indirect PLD ELISA and Biacore assay), it was found that 80% of the samples determined as positive in the Lelystad ELISA, were also found to be positive in both the PLD ELISA and Biacore assay. Seven of these nine samples gave conflicting results in both the ELISA and Biacore assay (*i.e.* found to be negative in both assays). All of these samples, except one, gave results that could be considered inconclusive or invalid. Results obtained for two of these samples (ID no. 3 and 151) were found to have CV values above 20% when tested by the Lelystad ELISA, suggesting the positive result may be questioned. Four of the samples were found to have values within close proximity of the cut-off point established for either the ELISA or Biacore assay, so therefore could be considered inconclusive. Two samples gave conflicting results in the ELISA and Biacore so therefore could not be determined either definitely positive or negative. Finally one sample was determined definitely negative in both the ELISA and Biacore assay, with values well below the cut-off points.

However, it must be noted that all the reference sera analysed were taken from animals where the history and infection status relative to the disease was unknown. Further confirmation of the clinical status of the samples analysed was not possible as no more sera were available and access to the flocks was no longer possible via the National Veterinary Research Laboratory. Therefore, all data analysis was calculated on previous results obtained from the Lelystad ELISA, as opposed to clinical

infection. Hence, these values are not a true representation of the sensitivity and specificity of the PLD Biacore assay, as estimated DSn and DSp values are entirely dependent upon the reliability of the Lelystad assay, which can be questioned.

The original double-antibody sandwich ELISA developed in Lelystad, developed by ter Laak *et al.* (1992), claimed a specificity of 99.9% and sensitivity of 100%. However, there were a number of inconclusive samples taken from CLA-positive flocks. Dercksen *et al.* (2000) later evaluated the assay and reported a sensitivity of 51±6% and a specificity of 97±2%, which differs greatly of what was previously reported by ter Laak *et al.* (1992). Dercksen and colleagues also modified the assay to improve sensitivity and specificity, which were found to be 79±5% and 99±1% in sheep, respectively. The number of false-positive results were thought to be due to the possible cross-reactivity of the toxin-specific serum, used as the coating antigen, with other bacteria including other *Corynebacterium* species, *Listeria monocytogenes* or *Mycobacterium avium* subsp. *paratuberculosis*. However, the double antibody sandwich ELISA, modified by Dercksen *et al.* (2000), was further investigated by Malone and colleagues in 2006. In this case, the sensitivity and specificity of the ELISA was found to be 88% and 55%, respectively. These figures differ greatly from the previously determined diagnostic sensitivity and specificity of 79±5% and 99±1%, respectively, reported by Dercksen and colleagues (2000). Although Dercksen *et al.* (2000) had suggested that false-positive results could be due to possible cross-reactivity with other bacteria, Malone *et al.* (2006) found no evidence of these bacterial infections in any of the flocks examined.

Although other published research on the development of a variety of ELISA formats for diagnosis in CLA in sheep, to the best of my knowledge this is the first time Biacore has been used for this purpose.

Chapter 4

Production and characterisation of genetically derived Fab fragments directed against aflatoxin B_1

4.1 Introduction

4.1.1 The aflatoxins

Aflatoxin molecules were first discovered in the early 1960s when one hundred thousand turkey poults died in England after consuming contaminated Brazilian peanut meal (Hartley *et al.*, 1963). These mycotoxins were found to be toxic metabolites produced by the fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. Fungal growth and hence, the production of aflatoxins, occurs at high temperatures (*i.e.* greater than 30°C) and humidity (greater than 70%) and is, therefore, most common in tropical and subtropical countries. These naturally occurring mycotoxins are most commonly found in tree nuts, peanuts and oil seeds including corn and cottonseed. However, contamination has also been reported in other nuts (Brazil nuts, almonds, walnuts, pecans), wheat, sorghum, dried fruits, legumes, peppers, potatoes, rice, copra, filberts, milk and milk products (Lee *et al.*, 2004). Contamination can occur in the field, during harvest, transportation and storage under conditions where conditions for growth of the mould is optimal. Research has also shown that host plants weakened by drought, high temperatures, and damage from insects or birds are more susceptible to fungal invasion (USDA, 2006).

Aflatoxins are crystalline structures and fluoresce under ultra violet (UV) radiation. Four main aflatoxins, B_1, B_2, G_1 and G_2, have been identified based on their fluorescence under UV light and their separation patterns on thin layer chromatography (TLC) plates (Keller *et al.*, 2005). AFB_1 and AFB_2 have been found to fluoresce blue under UV light, whereas AFG_1 and AFG_2 fluoresce yellow-green. Of all the aflatoxins, aflatoxin B_1 (AFB_1) has proven to be the most potent and is generally found in the highest concentration in food and animal feeds. Two additional other metabolites, aflatoxins M_1 and M_2, have also been found in milk. The chemical structures of these aflatoxins are outlined in *Figure 4.1.*

Aflatoxins are heat stable, even at temperatures in excess of 100°C and are hard to destroy. However, they are broken down in the presence of oxidising reagents, such as sodium hypochlorite, hydrogen peroxide and potassium permanganate. Aflatoxin molecules are also hydrophobic in nature and soluble in solvents such as chloroform, methanol and dimethyl sulfoxide (DMSO).

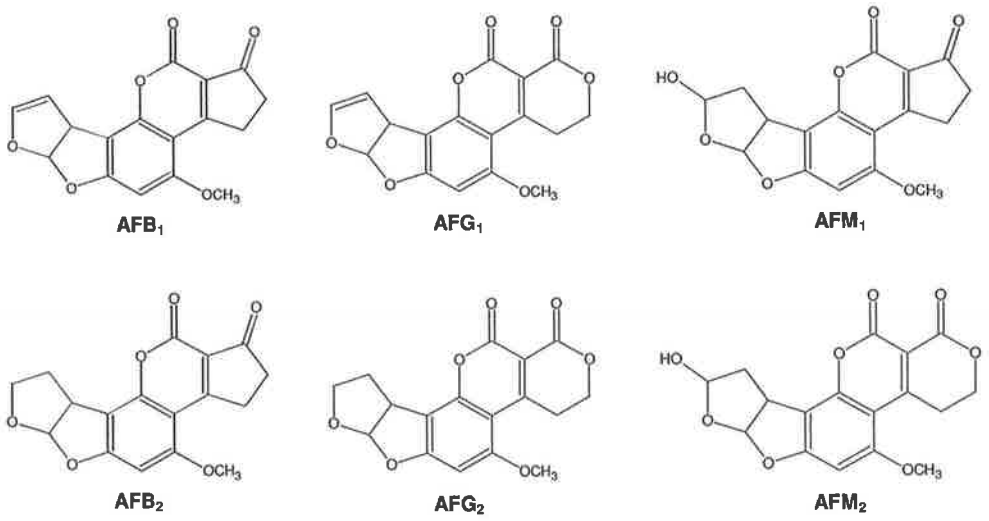


Figure 4.1. *Chemical structures of the most frequently occurring aflatoxins. Aspergillus flavus produces AFB_1 and AFB_2, whereas Aspergillus parasiticus produces AFB_1, AFB_2, AFG_1 and AFG_2. Two other metabolites, AFM_1 and AFM_2 have also been found in the milk and urine of lactating animals. AFM_1 and AFM_2 are produced from AFB_1 and AFB_2, respectively, by hydroxylation in lactating animals.*

4.1.2 Aflatoxin metabolism

Aflatoxin B_1 (AFB_1) is one of the most potent naturally occurring carcinogens. Its carcinogenicity derives from its ability to induce specific mutations in specific mammalian genes. However, AFB_1 is not mutagenic in its pure form and requires bioactivation to render it a mutagenic and carcinogenic metabolite. AFB_1 exerts its toxic effects when metabolised in mammalian tissues by microsomal cytochrome P450 enzymes to AFB_1-8,9-epoxide (Pelkonen *et al.*, 1997). This highly reactive metabolite binds to the N-7 position of guanine residues of DNA to form AFB_1-N7-guanine adducts in target cells. This initial adduct is unstable and either depurinates to give an AFB_1-guanine residue or a more stable formamidopyrimidine derivative (AFB_1-FAPY) (*Figure 4.2*) (Smela *et al.*, 2001; Jeffrey *et al*, 2005). These adducts are then responsible for subsequent heritable genetic changes. A change in base sequence, most commonly G→T transversions, or even a frame shift can often occur when the aflatoxin bound DNA is repaired or duplicated (Goodsell *et al.*, 2001). Detoxification

of the AFB_1-8,9-epoxide and AFB_1 in mammalian tissues is carried out by way of conjugation by glutathione (Hussein *et al.*, 2001). However, unless the highly reactive epoxide is immediately disarmed by glutathione it can attack DNA.

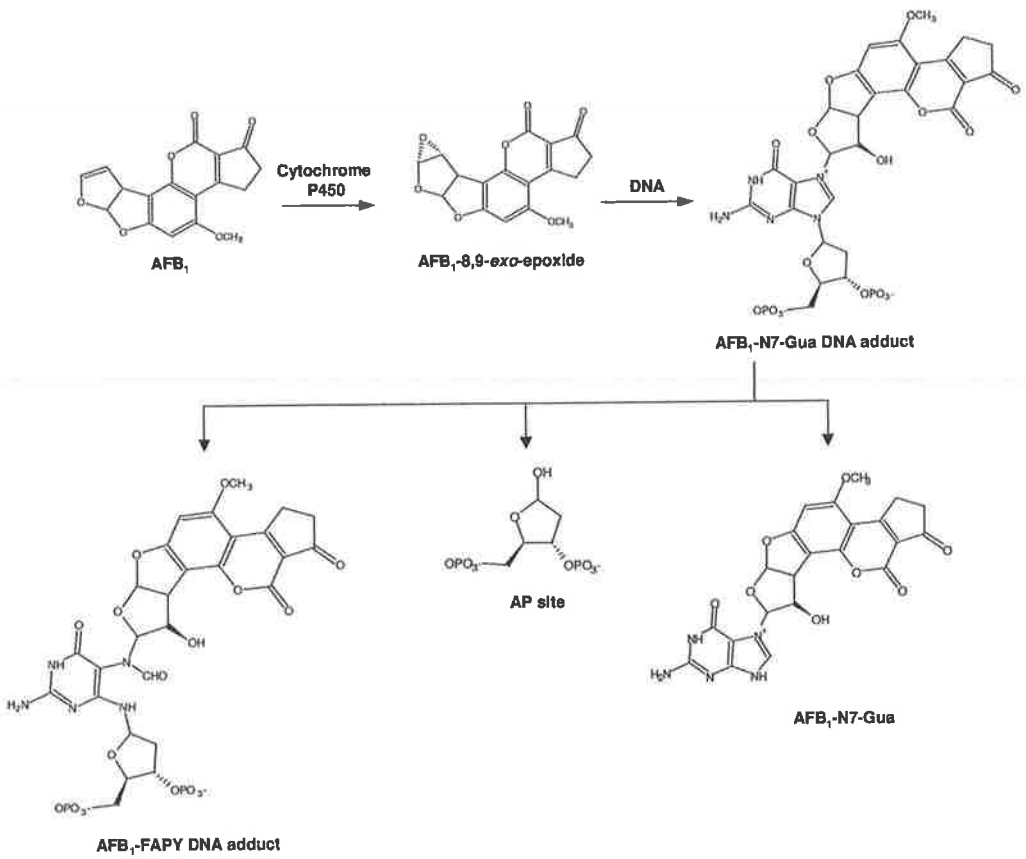


Figure 4.2. *Pathway of AFB_1 metabolic activation. Cytochrome P450 enzymes, found predominantly in the liver, are responsible for the metabolic activation of AFB_1 to 8,9-epoxide. This activated AFB_1 metabolite reacts with the N-7 position of guanine residues of DNA to form AFB_1-N7-Gua DNA adducts in the target cells. The adduct promotes depurinated giving rise to an apurinic (AP) site. Alternatively, the imidazole ring of the AFB_1-N7-Gua adduct opens to form the more chemically and biologically stable AFB_1 formamidopyrimidine adduct, AFB_1-FAPY (adapted from Smela et al., 2001).*

4.1.3 Effects of aflatoxin contamination

Aflatoxins have been linked to human hepatocellular carcinoma and are listed as group I carcinogens by the International Agency for Research on Cancer (IARC, 1993). Aflatoxins primarily attack the liver with secondary effects shown in decreased lymphocyte activity and immune system suppression. Studies have shown the association between aflatoxin contaminated food consumption and the incidence of liver cancer in Africa (Kenya, Mozambique, Uganda and Swaziland) and in Asia (China and Thailand) (USDA, 2006). In Europe and North America health risks associated with mycotoxin contamination in humans is rarely seen. This is thought to be due to better control of food and feed storage and higher levels of general health.

Aflatoxin poisoning (aflatoxicosis) can result from ingestion of aflatoxins in contaminated food or feed. Aflatoxicosis can occur from acute exposure of very high doses of contaminated grain over a short period of time or from chronic ingestion of low levels of aflatoxin over longer periods of time. Symptoms include vomiting, abdominal pain, pulmonary edema, convulsions, coma and cerebral edema (USDA, 2006). The effects of aflatoxicosis can vary from species to species with pigs, guinea pigs, calves, chicks and ducklings being more susceptible to AFB_1. Sheep, goats, rats and mice have shown to be relatively resistant (Gourama and Bullerman, 1995). Chickens with aflatoxin poisoning have found to lay fewer eggs, which can also contain aflatoxin metabolites. While aflatoxicosis in cows can result in the hydroxylated metabolite of AFB_1, aflatoxin M_1 (AFM_1), to be excreted in the milk of dairy cattle following consumption of contaminated feed (Yaroglu *et al.*, 2005). This leaves both calves and humans vulnerable to aflatoxin M_1 exposure through the mother's milk and its associated milk products.

Although acute aflatoxicosis in humans is extremely unlikely, a few cases have been reported, mainly in Africa and Asia. In 1967, twenty-six people in farming communities in Taiwan became ill after consuming contaminated rice. Further studies on the rice taken from affected households revealed it contained 200ppb (ng/g) of aflatoxin (USDA, 2006). One of the most serious reports of aflatoxicosis in humans occurred in India in 1974 when almost four hundred people became ill, after consumption of contaminated corn. One hundred of these people later died. Aflatoxin levels of up to 15,000ppb were found in the corn, suggesting the affected adults may

have consumed two to six milligrams of aflatoxin per day (USDA, 2006). Single doses of aflatoxins are not thought to be as effective in humans as long-term doses. It has been postulated that extended subacute doses, resulting from dietary exposure, may be required for inducing lethal acute toxic effects. An example of this was a case involving a young woman who attempted suicide by ingesting 5.5mg of aflatoxin over two days followed by 35mg over 2 weeks, 6 months later. The woman recovered completely with no signs of liver damage when examined fourteen years later (Hussein *et al.*, 2001).

In addition to the serious health threats posed, aflatoxin contamination can have huge economical impacts on all sectors of the production and consumption of grain products. It has been estimated that approximately 25% of the worlds food supply may be contaminated by mycotoxins annually (Hussein *et al.*, 2001). Contamination can cause major losses for food producers, processors and associated industries if large quantities of food or feeds require disposal. Other losses can occur due to restricted storage options, the cost of testing grain lots and the reduction in livestock production. Various companies and researchers have attempted to detoxify contaminated grains but treatments are often too expensive and can result in side effects on the end products. Detoxification strategies can also affect the nutritional qualities of the resulting food products (Das and Mishra, 2000). Essentially to reduce incidences of contamination, good field management and cool and dry (less than 14% moisture content) conditions during storage and transport are imperative. Animal feeds need to be continually monitored to prevent and control the spread of mycotoxins. To ensure that food is free from aflatoxin contamination, several international agencies have established limits for aflatoxins in food and feed, which vary from country to country. The EU have set maximum limits for aflatoxins in various food products (EEC, 2006), all of which are outlined in *Table 4.1*. Therefore, in order to monitor feed and foodstuffs for contamination and ensure they are safe for human consumption, a rapid, sensitive assay capable of detecting these contaminants in a range of matrices is essential.

Table 4.1. *Maximum levels for aflatoxins (total aflatoxins is sum of B_1, B_2, G_1 and G_2) in a variety of food types established as guidelines by the EU (No.1881/2006) (EEC, 2006).*

	Products	**B_1 (µg/kg)**	**Total aflatoxins* (µg/kg)**	**M_1 (µg/kg)**
2.1.1	Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	8	15	-
2.1.2	Nuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5	10	-
2.1.3	Groundnuts and nuts and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2	4	-
2.1.4	Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5	10	-
2.1.5	Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2	4	-
2.1.6	All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in *2.1.7*, *2.1.10* and *2.1.12*	2	4	-
2.1.7	Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	5	10	-
2.1.8	Raw milk, heat-treated milk and milk for the manufacture of milk-based products	-	-	0.05
2.1.9	Following species of spices: *Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika); *Piper* spp. (fruits thereof, including white and black pepper); *Myristica fragrans* (nutmeg); *Zingiber officinale* (ginger); *Curcuma longa* (turmeric)	5	10	-
2.1.10	Processed cereal-based foods and baby foods for infants and young children	0.1	-	-
2.1.11	Infant formulae and follow-on formulae, including infant milk and follow-on milk	-	-	0.025
2.1.12	Dietary foods for special medical purposes intended specifically for infants	0.1	-	0.025

4.1.4 Current methods of detection for aflatoxins

Chromatographic analysis employing methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas-liquid chromatography (GLC) are widely accepted as the official methods for aflatoxin determination. These conventional analytical techniques in conjunction with multi-step cleanup procedures have been applied for the sensitive and accurate detection of aflatoxins in various matrices (Hunt *et al.*, 1978; Rosen *et al.*, 1984; Tavčar-Kalcher *et al.*, 2005). However the methods are time consuming, laborious, expensive, require specially trained personnel and are generally unsuitable for high throughput sample analysis. For these reasons a number of immunoassay-based tests have been developed as an alternative to these techniques. Immunoassays are simple, sensitive, cost-effective and generally specific; making them a suitable method for use in developing countries, where aflatoxin contamination is more common.

A number of ELISAs have been developed employing antibodies with high sensitivities and specificities to aflatoxin. Kolosova *et al.* (2006) applied a specific monoclonal antibody to the development of a direct competitive ELISA for the detection of AFB_1 in spiked rice samples. The assay range of detection was found to be 0.1 to 10μg/L for AFB_1, with an IC_{50} value of 0.62μg/L. However, further research is required to validate the assay in naturally occurring contaminated samples. Devi *et al.* (1999) described the production and characterisation of monoclonal antibodies to AFB_1. One of the antibodies proved to be extremely sensitive and when applied to an ELISA format it was reported to be able to detect as little as 1pg/ml of AFB_1. Gathumbi *et al.* (2001) also generated highly sensitive polyclonal antibodies against AFB_1. When applied to the development of an ELISA, the antibody was shown to have a detection limit of 15.8pg/ml for AFB_1 in PBS buffer. The same antibody was later applied to a direct ELISA format for the detection of AFB_1 in spiked chicken liver tissues, which had been previously purified by immunoaffinity chromatography (Gathumbi *et al.* (2003). The assay was found to have a detection limit of below 20pg/ml for AFB_1. In 2004, Lee and colleagues described the development and comparison of four direct competitive immunoassays ELISAs for the detection of AFB_1 spiked in corn, peanuts, pistachios and soybeans. Rapid assays were developed utilising two horseradish peroxidase (HRP)-labelled aflatoxin conjugates (AFB_1-HRP and AFB_2-HRP). The conjugates were added simultaneously with sample extracts to

polyclonal antibody-coated wells. Employing this assay format, analysis could be completed within a shorter time-frame (within 15 minutes) than standard ELISA protocols. However, lower sensitivities were observed with the rapid assays in comparison to the standard assays (longer assays), with detection limits of 7.1μg/kg and 4.1μg/kg, respectively. The assays would still be suitable to detect AFB_1 below the maximum residue limit of 10μg/kg currently enforced in Australia and South East Asia. Other antibodies with high sensitivities to aflatoxin have been developed by Candlish *et al.* (1985) and Ward *et al.* (1990), which could detect 200pg/ml and 10pg/ml of AFB_1, respectively.

Lateral flow immunoassays (LFIA) or immunochromatographic assays have also proven to be useful tools for the rapid detection of AFB_1. Delmulle *et al.* (2005) developed an immunoassay-based lateral flow dipstick for rapid detection of AFB_1 in pig feed. The assay, with a detection limit of 5μg/kg in pig feed, facilitated a test result to be attained within 10 minutes Xiulan *et al.* (2005) also described the immunochromatographic technique for the detection of AFB_1 in buffer solution. The authors described the preparation of an antibody-colloidal gold conjugate and its subsequent use in the lateral flow immunoassay (LFIA). The time required for analysis was less than ten minutes representing a 6-10 fold decrease in detection time when compared to an ELISA format. The LFIA also exhibited a two-fold increase in sensitivity for AFB_1 in buffer comparative with the ELISA, with a limit of detection of 2.5ng/ml for AFB_1 reported. The immunochromatography assay was later described by Xiulan *et al.* (2006) for the detection of AFB_1 in spiked samples of rice, corn and wheat. The assay, employing an AFB_1-specific polyclonal antibody, was reported to have a detection limit of approx. 2μg/kg for AFB_1 in food extract.

With the advent of array and sensor technology, a number of biosensors have been developed in recent years for the rapid detection of aflatoxins in a variety of food matrices. Maragos *et al.* (1999) described the use of a fibre-optic immunosensor for the detection of aflatoxins in maize. This non-competitive assay utilised the native fluorescence properties of the mycotoxins. The sensor allowed for the rapid detection if AFB_1 concentrations as low as 2ng/ml. Although the assay proved to be a sensitive means of detecting aflatoxin, it did lack specificity. Carlson *et al.* (2000) demonstrated the use of an automated immunoaffinity fluorometric handheld

biosensor for the detection of aflatoxin. The sensor-based assay, with a detection range of 0.1 to 50ppb detection range, gave a result within less than 2 minutes with a sample requirement of one millilitre. In 2006, Ammida and colleagues compared the use of an immunosensor and HPLC for the detection of AFB_1 in barley. The indirect competitive electrochemical ELISA employed a specific monoclonal antibody and disposable screen-printed carbon electrodes. The immunosensor assay had a linear range of 0.1 to 10ng/ml for AFB_1 with a limit of detection of 90pg/ml. All results obtained were confirmed by HPLC. Pemberton *et al.* (2006) described the use of a screen-printed carbon electrochemical immunosensor array for the detection of mycotoxins. The assay employed a competitive format between free AFB_1 and biotinylated AFB_1 for binding to specific antibodies immobilised on the sensor platform. A detection limit of 0.15ng/ml was reported. Sapsford and colleagues (2006) developed an array biosensor for the detection of AFB_1 in corn and nut products. Neutravidin slides were chosen as the array platform and were patterned with biotinylated AFB_1. Free AFB_1 and an AFB_1-specific Cy5-labeleld monoclonal mouse antibody were applied to the slides where both free and biotinylated AFB_1 competed for antibody binding sites. The assay was found to have a limit of detection of 1.5-5ng/g for corn products, depending on the food matrix, which included popcorn, cornflakes and cornmeal. A limit of detection of 0.6-1.4ng/g was reported for nut products (peanuts, pecans and peanut butter).

In recent years the commercially available surface plasmon-based biosensors, developed by Biacore^{TM}, have also proven to be valuable for the automated screening of aflatoxin-contaminated samples. The system enables rapid analysis with minimal sample handling and consumables, and its reusable sensor surface makes it ideal for screening a large number of samples. In 2000, Daly *et al.* demonstrated the use of the biosensor for the detection of AFB_1. Samples spiked with AFB_1 were incubated with antibody and the solution was subsequently passed over an AFB_1 conjugate immobilised surface. The binding of remaining free polyclonal antibody, in terms of response units, could then be monitored in 'real-time'. One of the polyclonal antibodies tested demonstrated high avidity for AFB_1-BSA conjugate surface, a common problem encountered with the use of anti-hapten antibodies generated with hapten-protein conjugates. This lead to associated problems with regeneration of the aflatoxin conjugate immobilised surface. However, an assay was successfully

developed with the second polyclonal antibody, which displayed a limit of detection of 3ng/ml for AFB_1. Van der Gaag *et al.* (2003) developed a Biacore-based assay for the simultaneous detection of four mycotoxins. A test result was achievable within ten minutes, following a sample extraction and clean-up procedure of approximately fifteen minutes. The assay limit of detection was found to be 0.2ng/g for AFB_1.

The immunoassays described have incorporated either polyclonal or monoclonal antibodies. However, recent developments in recombinant antibody technology have yielded novel antibody fragments with desirable affinities and specificities. Recombinant antibodies have several advantages over their conventional counterparts, including the speed of antibody production, the possibility of altering affinity and specificity, and the ability to generate novel functionalities. Unlike polyclonal antibodies recombinant antibodies also provide an infinite supply. Single chain variable fragments (scFv) specific for AFB_1 have been isolated from a phage display library (Daly *et al.*, 2002; Dunne *et al.*, 2005). These specific antibody fragments have been applied to the development of ELISA and Biacore-based assays. The competitive ELISA employing an AFB_1-specific scFv, described by Daly *et al.* (2002), offered limited sensitivity with a limit of detection of 98ng/ml for AFB_1. However, increased sensitivity was displayed when applied to a Biacore assay format with limits of detection at 3 and 0.75ng/ml for AFB_1 in spiked PBS and grain, respectively. Dunne *et al.* (2005) later described the use of monomeric and dimeric scFvs for the detection of AFB_1 in a Biacore-based assay. The highly sensitive recombinant antibody fragments were found to have detection ranges of 390 to 12,000pg/ml and 190 to 24,000pg/ml for the monomeric and dimeric scFv, respectively. The following chapter describes the conversion of this antibody fragment to a chimeric Fab fragment by the addition of human constant domains, which may improve the overall stability of the fragment. The stability of biological components facilitates long-term storage, which is vital for future commercial immunoassay applications. In addition, the sensitivity can also be improved with this conversion (Townsend *et al.*, 2006) and with changes in legislation and more stringent quality control; there is a definite need for antibodies with even greater sensitivity

4.1.5 Chapter outline

This chapter focuses on the conversion of a previously isolated murine anti-AFB_1 single chain variable fragment (scFv) to a chimeric Fab construct by the addition of human constant regions, using PCR assembly methods combined with phage display techniques. Two clones were isolated from the chimeric mouse/human Fab antibody library and applied to the development of several immunoassay formats and compared with regard to sensitivity to the parent scFv. The potential benefits of converting and scFv to a Fab format in terms of expression, stability and specificity were also assessed.

Finally, the antibodies were transformed into the pAK400Bio vector for subsequent expression of *in vivo* biotinylated recombinant fragments. This method would facilitate immobilisation of fragments onto avidin surfaces in an orientated manner and could be potentially useful for future antibody array applications.

4.2 Results

4.2.1 Construction of an anti-AFB_1 chimeric mouse/human Fab library

An anti-AFB_1 murine scFv (Dunne *et al.*, 2005) was converted to a chimeric Fab format by the addition of human constant regions using a number of overlap extension polymerase chain reactions (PCRs), as described by Barbas *et al.* (2001). A schematic representation of the PCR steps is shown in *Figure 4.3*. The resulting Fab fragments were ligated into the phagemid vector pComb3X to allow phage-display selection of AFB_1-specific Fab fragments.

4.2.1.1 Amplification of the murine variable and human constant regions

The murine variable heavy (V_H1) and light (V_λ) chain antibody genes of the AFB_1-specific scFv produced and characterised by Dr. Lynsey Dunne (Dunne *et al.*, 2004; Dunne *et al.*, 2005) were amplified using the primers listed in *Section 2.4.2.1.1*. Although the kappa (κ) light chain is dominant in mice, with a κ:λ ratio of approximately 20:1 in Balb/c mice, sequence analysis of the scFv sequence revealed it contained a variable lambda light (V_λ) chain. Therefore, chimeric Fab constructs were generated with the V_λ region in association with constant lambda (C_λ) regions (rather than C_κ regions, as described by Barbas *et al.* (2001). It was suggested that a more compatible pairing of the light chain constant and variable regions might improve stability and activity of resulting Fab fragments (Townsend *et al.*, 2006). For the generation of the C_λ-associated Fab, the reverse V_λ primer was designed to incorporate a human C_λ sequence tail (*Section 2.4.2.1.1*), allowing subsequent assembly of variable light chains (V_λ) with human constant regions (C_λ) by overlap extension PCR. The reverse V_H primer introduces a human C_H1 sequence tail (*Section 2.4.2.1.1*), facilitating generation of the chimeric heavy chain (Fd) fragment by overlap extension PCR. PCR components and conditions for amplification of the variable regions of the anti-AFB_1 scFv are described in *Section 2.4.2.1.2* and *2.4.2.1.3*. The constant heavy (C_H1) and light (C_λ) chain antibody genes were amplified from the vectors pComb3XTT and pComb3Xλ, respectively, using the primers and conditions outlined in *Section 2.4.2.2*. These pComb3 series vectors contain standard human Fab fragment inserts (Andris-Widhopf *et al.*, 2000). Variable and constant region PCR products were electrophoresed on a 2% (w/v) agarose gel, as described in *Section 2.2.5*. The resulting gel images are shown in *Figure 4.4* and

Figure 4.5 with bands visible at approximately 350-450bp for both the variable and constant regions. Following successful amplification of both variable and constant region PCR products were pooled, ethanol precipitated and purified from an agarose gel (*Section 2.4.2.1.4* and *2.4.2.1.5*).

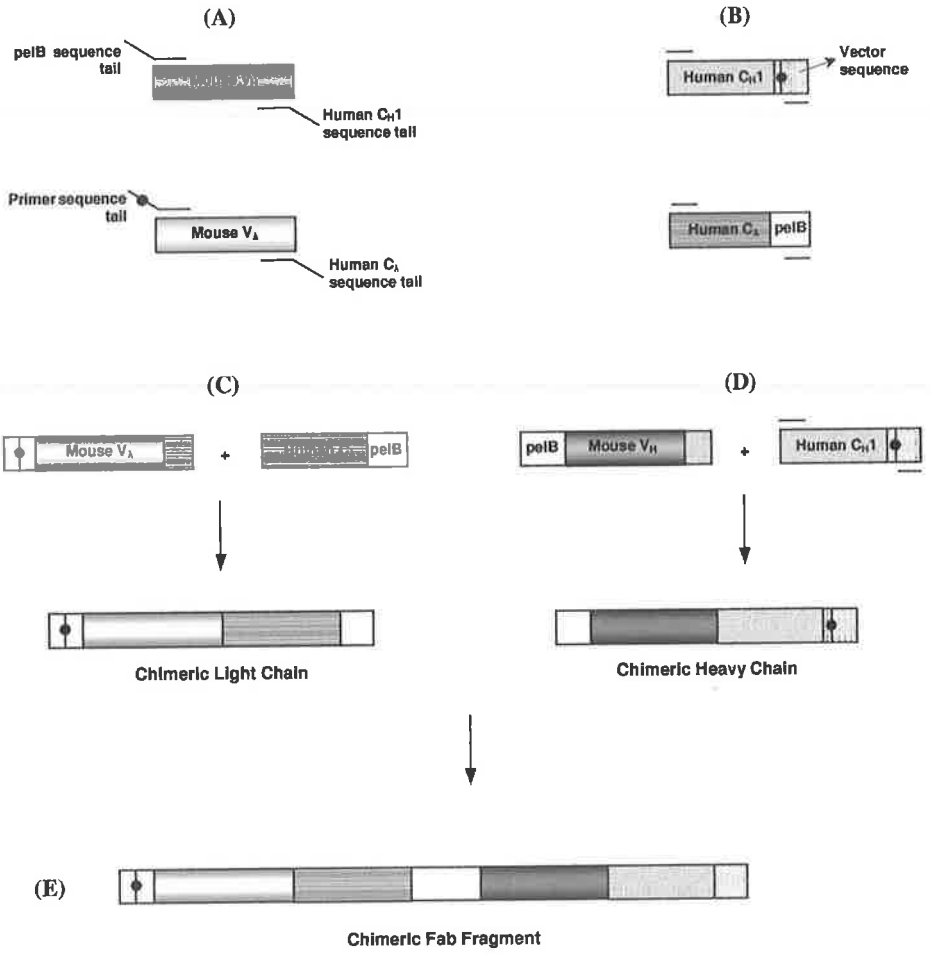


Figure 4.3. *Construction of chimeric Fab fragments by overlap extension PCR. (A) Variable regions (V_λ and V_H) were amplified from the murine scFv using specific primers, which introduce human C_H1 and C_λ tails, allowing subsequent overlap extension PCR with constant regions. (B) Human constant regions (C_λ and C_H1) were amplified from the pComb3Xλ and pComb3XTT vectors, respectively. (C) Assembly of murine V_λ regions with C_λ regions to create chimeric lambda light chains by overlap extension PCR. (D) Assembly of murine V_H regions with human C_H1 regions to create chimeric heavy chains (Fd) by overlap extension PCR. (E) Assembly of the chimeric Fab fragment by overlap extension PCR, where the pelB leader sequence serves as the overlap region for the two products.*

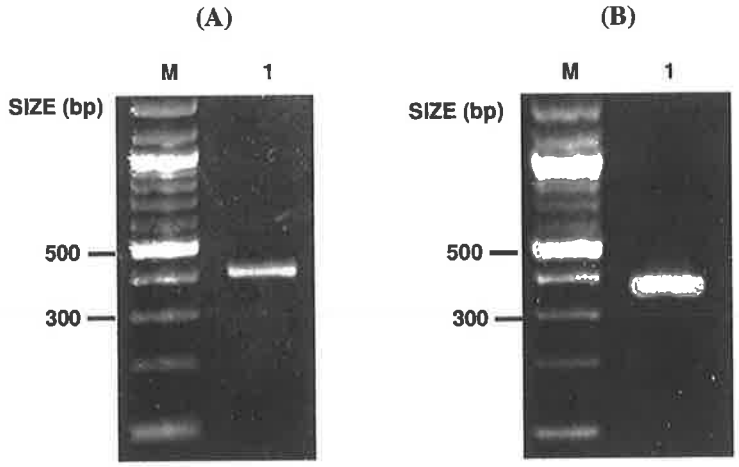


Figure 4.4. *Amplification of* **(A)** *variable heavy (V_H) and* **(B)** *variable light (V_L) chain genes of anti-AFB_1 scFv. A specific band can be seen at approx. 400bp and 350bp for both the V_H and V_L fragments, respectively.* **(A)** *Lanes:* **(M)** *1Kb plus DNA ladder;* **(1)** *amplified variable heavy chain DNA* **(B)** *Lanes:* **(M)** *1Kb plus DNA ladder;* **(1)** *amplified variable light chain DNA.*

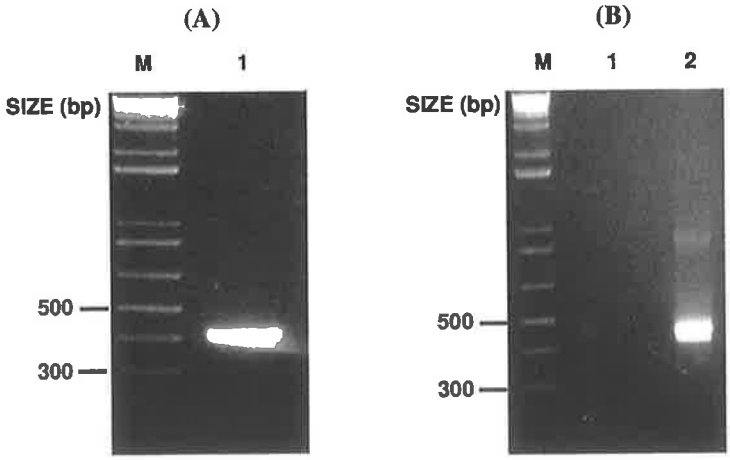


Figure 4.4. *Amplification of* **(A)** *constant heavy (C_H1) and* **(B)** *constant light-pelB (C_λ-pelB) chain genes from previously cloned human Fab fragments.* **(A)** *Lanes:* **(M)** *1Kb plus DNA ladder;* **(1)** *amplified human constant heavy chain (C_H1) DNA at approx. 400bp.* **(B)** *Lanes:* **(M)** *1Kb plus DNA ladder;* **(1)** *amplified human constant light chain – pelB (C_λ-pelB) product at approx. 450bp.*

4.2.1.2 Assembly of murine variable regions with human constant regions

Overlap extension PCR was used to assemble mouse variable region PCR products with the human constant region PCR products to generate chimeric light- and heavy-chain fragments. The murine V_λ PCR products, which have a human C_λ sequence tail, were combined with the human C_λ-pelB products to generate chimeric lambda light chain-pelB fragments using the primers and conditions outlined in *Section 2.4.2.3.1* and *2.4.2.3.2*. The murine V_H PCR products, which have a human C_H1 sequence tail, were combined with the human C_H1 PCR products to create chimeric Fd fragments using the primers and conditions outlined in *Section 2.4.2.3.3* and *2.4.2.3.4*. The amplified PCR products were electrophoresed on a 1.5% (v/v) agarose gel and the resulting gel images are shown in *Figure 4.6* with bands visible at approximately 750-800bp for both the chimeric light- and heavy-chain fragments. Following successful amplification of both chimeric fragments, PCR products were pooled, ethanol precipitated and purified from an agarose gel (*Section 2.4.2.3.5*).

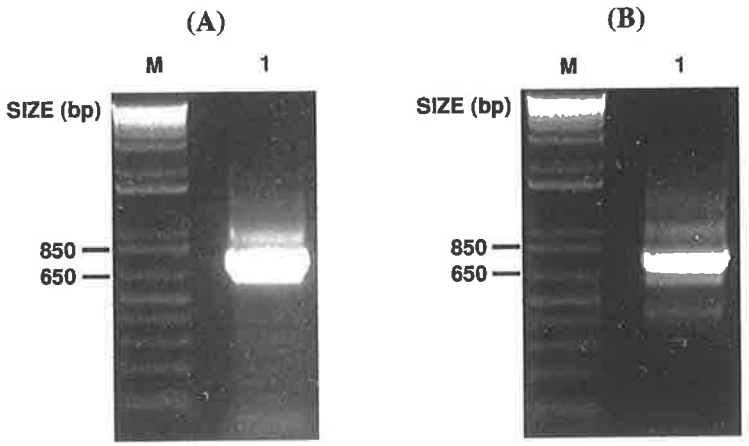


Figure 4.6. *Overlap extension PCR for amplification of* ***(A)*** *lambda light chain and* ***(B)*** *heavy chain (Fd) fragment. A specific band can be seen at approx. 750-800bp for both the light and heavy chain fragments.* ***(A)*** *Lanes:* ***(M)*** *1Kb plus DNA ladder;* ***(1)*** *amplified chimeric lambda light chain-pelB fragment* ***(B)*** *Lanes:* ***(M)*** *1Kb plus DNA ladder;* ***(1)*** *amplified chimeric heavy chain (Fd) fragment.*

4.2.1.3 Assembly of chimeric Fab fragment

In the final overlap PCR reaction the chimeric heavy (Fd) fragment and light chain-pelB fragment were combined to form the chimeric Fab fragment, where the pelB leader sequence served as the overlap region (*Section 2.4.2.4).* The overlap PCR reaction was analysed on a 1.5% (v/v) agarose gel and the resulting gel image is shown in *Figure 4.7,* with bands visible at approximately 1,500bp representing the chimeric Fab fragments. Following successful amplification, the PCR reaction was scaled up, PCR products pooled, ethanol precipitated and purified from an agarose gel (*Section 2.4.2.4.3*).

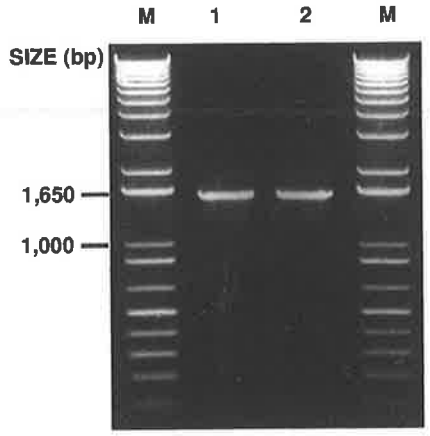


Figure 4.7. *Final overlap extension PCR for the generation of the chimeric Fab fragment. Lanes:* ***(M)*** *1Kb plus DNA ladder;* ***(1-2)*** *chimeric Fab fragment, where specific bands can be seen at approx. 1,600bp.*

4.2.1.3 Digestion of pComb3X vector and Fab overlap PCR product

The resulting chimeric Fab fragments and the pComb3X vector were digested with *Sfi*I as described in *Section 2.4.2.5.* The digested overlap product and vector DNA were then purified from a 1% (w/v) and 0.6% (w/v) agarose gel by electroelution and the resulting purified DNA quantified as per *Section 2.4.2.5.*

4.2.1.4 Library ligation and transformation

The purified digested Fab fragment was ligated into the pComb3X vector and high efficiency electrocompetent *E. coli* ER2738 cells were prepared for subsequent library

transformation (*Section 2.4.2.6* and *2.4.2.7*). The vector containing the insert was transformed into the *E. coli* ER2738 cells by electroporation (*Section 2.4.2.8*), resulting in a library size of 6.3 x 10^7 cells, with a vector self-ligation background of <0.1%.

4.2.2 Isolation of Fab fragments against AFB_1 from chimeric Fab library

In order to enrich the constructed Fab library for antibodies specific to AFB_1, a series of selections on Nunc Maxisorp™ immunotubes (round 1) and on Nunc Maxisorp™ microtitre plates (subsequent rounds), were performed. This selection process, known as bio-panning, involves incubating the phage library in an immunotube (or well of an immunoplate) coated with a specific antigen. Unbound phage particles are washed away, bound phage are recovered from the surface, reinfected into *E. coli* cells and re-grown for further enrichment.

Phage displaying Fab fragments were rescued from the library as per *Section 2.4.3.1* and concentrated by polyethylene glycol/ sodium chloride (PEG/NaCl) precipitation, as described in *Section 2.4.3.2*. Re-suspended phage was added to an immunotube that had been previously coated with 10μg/ml of AFB_1-BSA and blocked with PBS containing 3% (w/v) BSA (*Section 2.4.3.3.1*). Non-specific phage was removed by washing and bound phage eluted with 1μg/ml AFB_1 in PBS containing 5% (v/v) methanol. Eluted phage was re-infected into *E. coli* ER2738 cells (*Section 2.4.3.4*) for subsequent propagation and selection by bio-panning. Succeeding rounds of bio-panning were performed in a single microtitre plate well (Nunc Maxisorp™), as described in *Section 2.4.3.3.1*, and specifically bound phage eluted with 10mg/ml trypsin prepared in PBS.

Three rounds of panning were performed and phage pools obtained after each round of panning were analysed by polyclonal phage ELISA to monitor the enrichment of the phage population. As seen in *Figure 4.8*, phage pools showed specific binding to AFB_1 and negligible binding to BSA after two rounds of panning. A number of clones (n=94) were then picked from output plates from the third round of bio-panning and analysed by monoclonal phage ELISA for specific binding to AFB_1 (*Section 2.4.3.6* and *2.4.3.7*). Two clones (D11 and G6), which displayed specific binding to AFB_1, were isolated (*Figure 4.9*).

For the soluble expression of the two Fab clones (G6 and D11) in pComb3X, plasmid preparations were transformed into a nonsuppressor strain of *E. coli* (*i.e.* Top10 F′ cells (Invitrogen)) by electroporation, as per *Section 2.4.3.8*. In this nonsuppressor strain, the amber stop codon between the Fab constructs and the gIII gene is not suppressed, allowing production of soluble Fab antibodies fused to a C-terminal histidine (His_6) and influenza hemagglutinin (HA) tag (YPYDVPDYAS) tag. Single transformants were grown, induced with IPTG for expression of Fab antibody fragments and the cell lysates analysed by ELISA. Cell lysates were incubated with decreasing concentrations of AFB_1 (100,000 to 32ng/ml) and added to Maxisorb™ microtitre plates (Nunc) coated with 10μg/ml AFB_1-BSA conjugate. Bound antibody was detected with the addition of a HRP-labelled anti-hemagglutin (HA) antibody (Roche) and absorption values were normalised by dividing the absorbance at each AFB_1 concentration (A) by the absorbance in the absence of AFB_1 (A_0). Both the D11 and G6 clones were inhibited from binding immobilised aflatoxin by free toxin, indicating they were specific to AFB_1 (*Figure 4.10*).

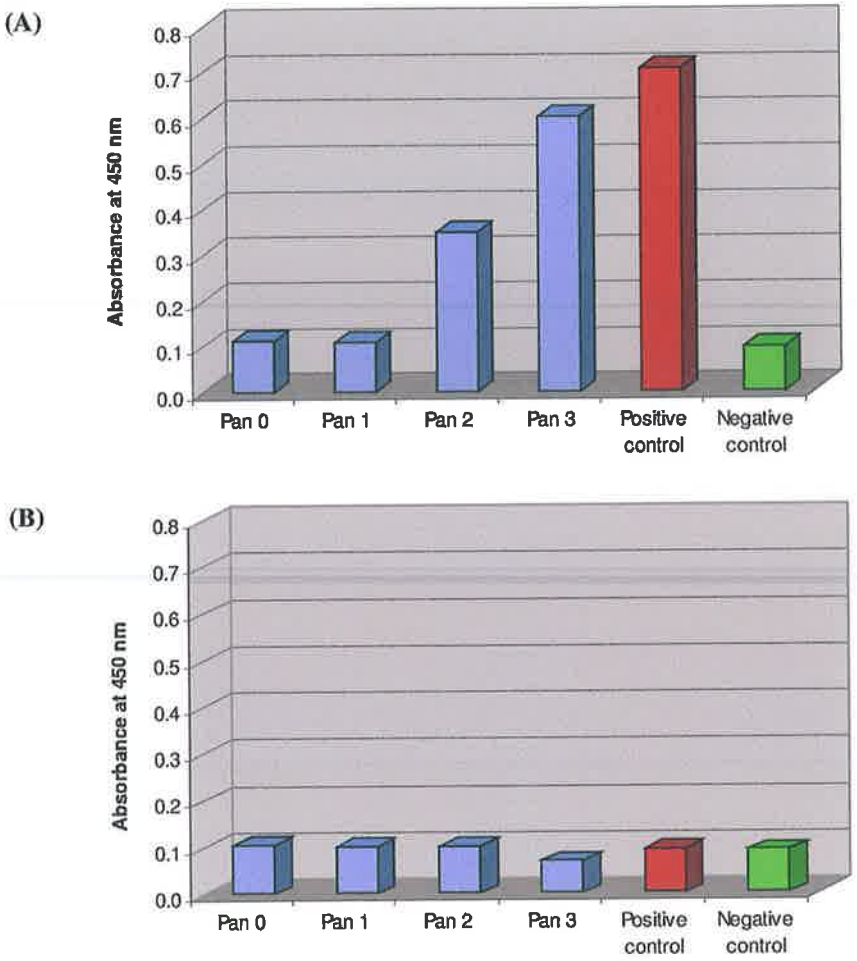


Figure 4.8. *Polyclonal phage ELISA following three rounds of panning. Phage pools obtained after each round of panning were analysed in parallel with phage from the unpanned library. Maxisorp™ plates (Nunc) were coated with* **(A)** *10µg/ml AFB_1-BSA and* **(B)** *10µg/ml BSA (control). Bound phage were detected using a HRP-labelled anti-HA antibody (Roche). Phage pools did not bind to negative control antigen (BSA), indicating that phage were specific for AFB_1 rather than the conjugate carrier protein.*

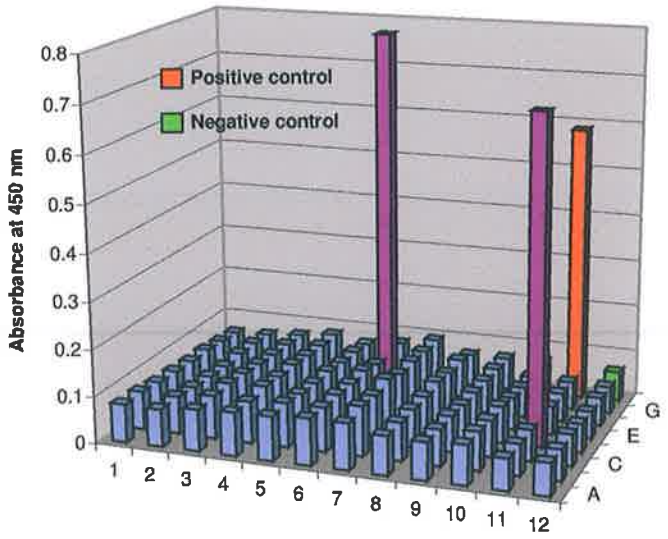


Figure 4.9. *Monoclonal phage ELISA following three rounds of bio-panning the chimeric Fab library. Plates were coated with 10μg/ml AFB_1-BSA and blocked. Phage were detected using a HRP-labelled anti-HA antibody. Two clones (D11 and G6) were found to be specific for AFB_1.*

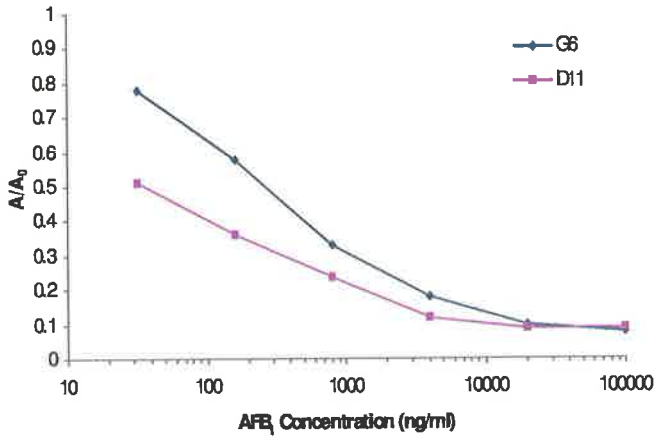


Figure 4.10. *ELISA analysis of D11 and G6 periplasmic lysates following soluble expression of Fab fragments selected form Fab library. Plates were coated with 10μg/ml AFB_1-BSA conjugate and blocked with PBS containing 5 % (w/v) Marvel™. Cell lysate (50μl) in PBS containing 5% (w/v) Marvel™ was added to each well containing 50 μl of free AFB_1. Absorbance values were normalised by dividing the mean absorbance obtained at each AFB_1 concentration (A) by the mean absorbance value determined in the presence of zero AFB_1 (A_0) and plotted against the logarithm of AFB_1. Both clones were inhibited from binding the immobilised conjugated with the addition of free AFB_1*

4.2.3 Sequence analysis on AFB_1-specific recombinant antibodies

Stab cultures of *E. coli* Top10F′ (containing the pComb3X bearing the anti-AFB_1 G6 and D11 Fab genes) and *E. coli* JM83 (containing the plasmid pAK400 bearing the anti-AFB_1 scFv gene) were prepared as described in *Section 2.4.3.9.1*. The cultures were sent to Qiagen (Germany) Ltd for plasmid preparation and sequencing. Sequence analysis of the antibody fragment clones was performed with the oligonucleotide primers listed in *Table 2.2* (*Section 2.4.3.9.2*).

The nucleotide sequences were translated to amino acid sequences using a web-based translate tool (Expasy) and the amino acid sequences aligned using a web-based protein alignment tool (PBIL) (*Table 2.3*, *Section 2.4.3.9.2*). The alignment of the variable region sequences of the Fab clones selected from the Fab library is shown *Figure 4.11* in comparison to the parent scFv variable regions. The light and heavy chain complementarity determining regions (CDRs) were defined according to Kabat *et al.* (1991) and are highlighted in yellow and red, respectively. All three variable light chain sequences displayed 100% homology. The heavy chain CDRs of the scFv and D11 were found to be identical, as expected. However, the G6 Fab has single amino acid difference in the CDRH3 due to a single base substitution (guanine for an adenine) and resulting in glycine (G) being translated instead of aspartic acid (D). This base substitution was due to an error in PCR amplification rather than an error in sequencing as the heavy chain regions of the both the D11 and G6 Fab were sequenced a number of times (n=6) and all sequence data provided the same result.

The CDRH3 is of critical importance in the interaction of an antibody with its respective antigen. This region, along with the CDRL3, is considered to make the most significant contributions to affinity and specificity. Although mutating residues in this region can result in an antibody with increased affinity, it can also abolish antigen binding. Therefore, to investigate whether the amino acid substitution would result in varied antigen binding ability, the functional characteristics of the G6 Fab fragment were examined (in parallel with the D11 Fab) by ELISA and Biacore and compared to the parent scFv

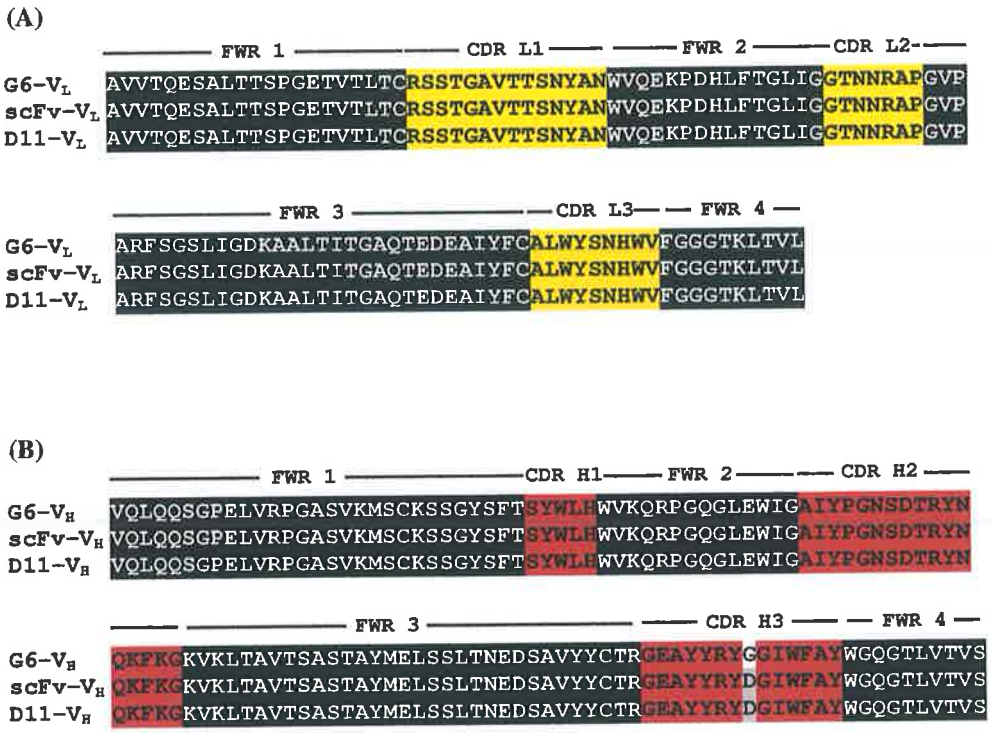


Figure 4.11. *Anti-AFB$_1$ scFv, G6 Fab and D11 Fab variable gene sequences were translated into amino acid sequences using a web-based translate tool (Expasy) and the amino acid sequences aligned using a web-based protein alignment tool (PBIL) (Section 2.4.3.9). The light and heavy chain complementarity determining regions (CDRs) were defined according to Kabat et al. (1991) and are highlighted in yellow and red, respectively. The variable regions of the three antibodies were found to be identical with the exception of one amino acid difference in the CDRH3 region of G6 Fab (substitution of a glycine (G) for an aspartic acid (D)).*

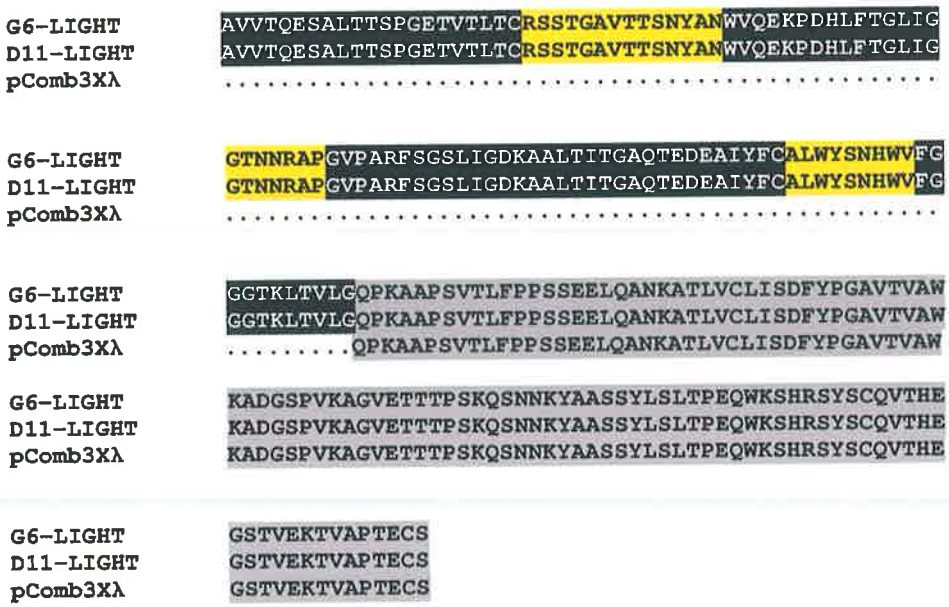


Figure 4.12. *Anti-AFB$_1$ G6 and D11 Fab light chain gene sequences were translated into amino acid sequences using a web-based translate tool (Expasy). The amino acid sequences were aligned with the C_λ region of the cloned human Fab (sourced from the pComb3Xλ) using a web-based protein alignment tool (PBIL) (Section 2.4.3.9). The light chain complementarity determining regions (CDRs) were defined according to Kabat et al. (1991) and are highlighted in yellow. The variable (highlighted in black) and constant (highlighted in grey) regions of the two antibodies displayed 100% homology with the lambda light chain of the cloned human Fab (in pComb3Xλ).*

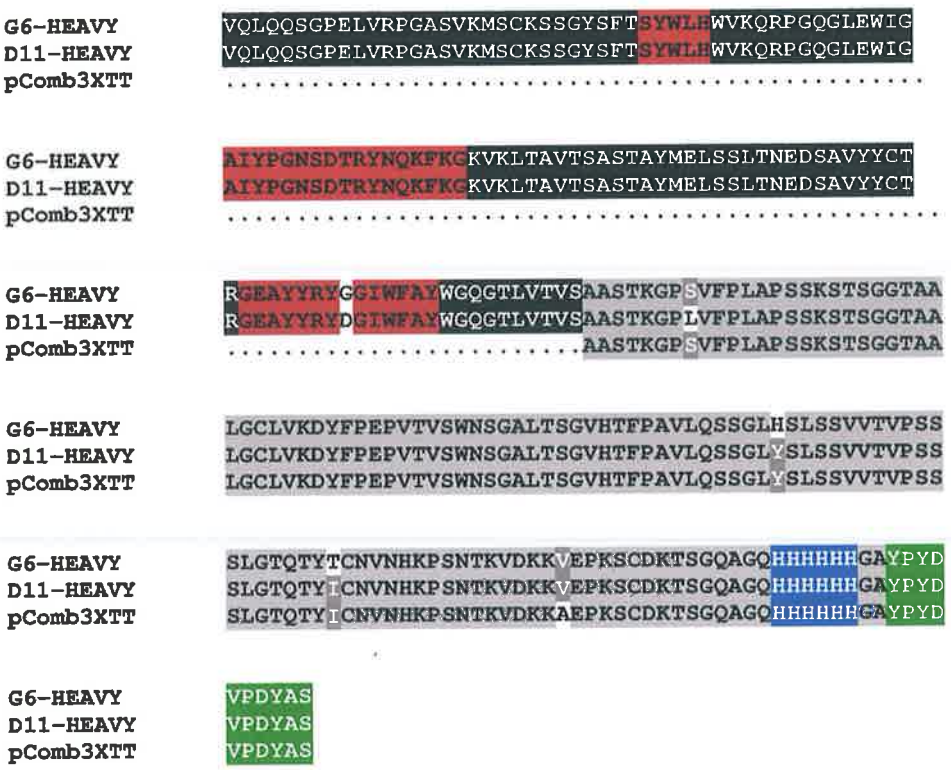


Figure 4.13. *Anti-AFB$_1$ G6 and D11 Fab heavy chain gene sequences were translated into amino acid sequences using a web-based translate tool (Expasy). The amino acid sequences were aligned with the C_H1 region of the cloned human Fab (sourced from the pComb3XTT) using a web-based protein alignment tool (PBIL) (Section 2.4.3.9). The heavy chain complementarity determining regions (CDRs) were defined according to Kabat et al. (1991) and are highlighted in red. The variable regions of the two antibodies were found to be identical with the exception of the substitution of a glycine (G) for an aspartic acid (D) in the CDRH3 region of the G6 Fab). The constant regions (highlighted in grey) regions were found to be identical to the heavy chain of the cloned human Fab (from pComb3XTT), with the exception of four residues. The C-terminal hexa-histidine tag (His$_6$) is highlighted in blue and the influenza hemagglutinin (HA) epitope tag (YPYDVPDYAS) is highlighted in green.*

4.2.4 Western blot analysis on the anti-AFB_1 recombinant antibodies

The scFv and Fab (clone G6 and D11) recombinant antibodies were analysed by Western blotting to determine whether the C-terminal hexa-histidine (His_6) had been fully translated, was accessible and had not been adversely affected during cell lysis. The Fab fragments were also probed with antibodies specific for the human lambda light chain and the C-terminal influenza hemagglutinin (HA) tag (YPYDVPDYAS).

Briefly, the recombinant antibodies were electrophoresed alongside a prestained molecular weight marker, as described in *Section 2.2.3.* Following separation by SDS-PAGE, the proteins were transferred to nitrocellulose for Western blot analysis, as described in *Section 2.2.4*, with the following amendments. Following transfer of the proteins onto the nitrocellulose, the membranes were blocked with PBS containing 5% (w/v) Marvel™. The blots were then probed with a 1 in 5,000 dilution of HRP-labelled anti-polyhistidine (Sigma) or HRP-labelled anti-HA (Roche) or HRP-labelled anti-human lambda (Sigma) antibodies. The membranes were incubated for 1.5 hours with gentle shaking at room temperature and washed thoroughly as outlined in *Section 2.2.4.* Protein bands were visualised using 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) substrate and the reaction was allowed to proceed until bands were clearly visible. The recombinant antibodies were all detected with the histidine-specific antibody (*Figure 4.14*), confirming that the histidine tag was fully intact and accessible. The heavy and light chains of the Fab fragment were also visible when probed with anti-HA and anti-human lambda antibodies, respectively (*Figure 4.15*).

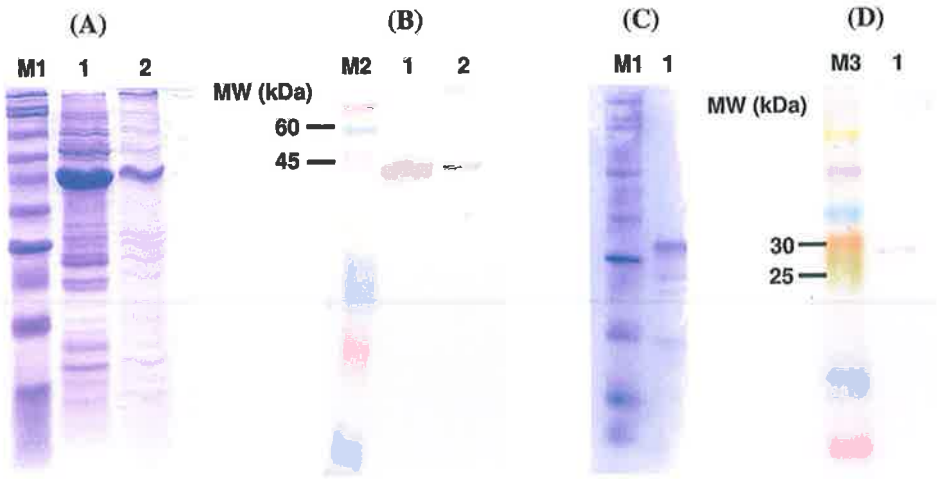


Figure 4.14. *(A) SDS-PAGE analysis on G6 and D11 Fab fragments with the two chains not reduced prior to gel electrophoresis. Lanes:* ***(M1)*** *Sigma wide range molecular weight marker;* ***(1)*** *G6 Fab fragment;* ***(2)*** *D11 Fab fragment, with bands at approx. 45kDa representing the non-reduced Fab fragments* ***(B)*** *Western blot probed with anti-histidine antibodies (Sigma). Lanes:* ***(M2)*** *Sigma ColorBurst™ molecular weight markers;* ***(1)*** *G6 Fab fragment;* ***(2)*** *D11 Fab fragment.* ***(C)*** *SDS-PAGE analysis on scFv. Lanes:* ***(M1)*** *Sigma wide range molecular weight marker;* ***(1)*** *scFv with single band visible at approximately 30kDa following expression from pAK400.* ***(D)*** *Western blot probed with anti-histidine antibodies (Sigma). Lanes:* ***(M3)*** *Amersham Biosciences full range molecular weight markers;* ***(1)*** *scFv.*

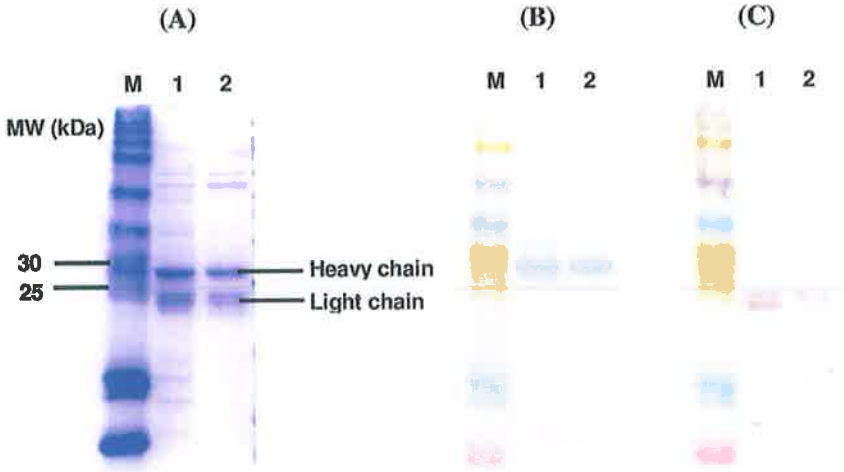


Figure 4.15. *(A) SDS-PAGE analysis on the reduced G6 and D11 Fab fragments, revealing light and heavy chains at approx. 25kDa and 30kDa, respectively. Lanes:* ***(M1)*** *Amersham Biosciences full range molecular weight markers;* ***(1)*** *G6 heavy chain;* ***(2)*** *D11 heavy chain;* ***(M2)*** *Sigma wide range molecular weight marker.* ***(B)*** *Western blot probed with anti-HA antibodies (Roche). Lanes:* ***(M1)*** *Amersham Biosciences full range molecular weight markers;* ***(1)*** *G6 heavy chain;* ***(2)*** *D11 heavy chain.* ***(C)*** *Western blot probed with anti-human lambda antibodies (Sigma). Lanes:* ***(M1)*** *Amersham Biosciences full range molecular weight markers;* ***(1)*** *G6 light chain;* ***(2)*** *D11 light chain.*

4.2.5 Expression and purification of anti-AFB_1 recombinant antibodies

The anti-AFB_1 scFv and Fab fragments (G6 and D11) recombinant antibodies were expressed on a large scale in shaker flasks. To maximise the yield of soluble product, a method described by Barbas *et al.* (2001) was modified as per *Section 2.4.3.10.1.* For high levels of expression, all antibodies were grown in super broth (SB) media, which contains high concentrations of tryptone and yeast extract to provide ample amount of nutrients and cofactors for cell growth.

The antibodies were isolated from the host cell by sonication and purified to remove any contaminating proteins. Both the pComb3X phagemid vector (for expression of Fab fragments) and pAK400 (for expression of scFv) encode an carboxy-terminal $(His)_6$ tag, which allowed for the rapid purification of the recombinant antibodies using immobilised metal ion affinity chromatography (IMAC) (*Section 2.4.3.10.4*). All factors affecting purification, including the quantity of resin and concentrations of imidazole in both the wash and elution buffers, were optimised. Wash buffers containing imidazole concentrations of 20mM and 40mM were found to be optimal for the scFv and Fab fragments, respectively. Imidazole, incorporated in the wash buffer, reduces the non-specific binding of untagged contaminant proteins to the resin, where the higher the concentration tolerated by the protein, the cleaner the eluate. All three antibodies were eluted with 250mM imidazole. Fractions from each stage of the purification process were analysed using SDS-PAGE (*Figure 4.16* and *4.17*) and results indicated that the purity of the antibodies greatly increased following elution with imidazole in comparison to the crude cell lysate. It can also be seen from *Figure 4.16* that the Fab light and heavy chains were expressed in approximately equimolar concentrations.

When all three antibodies were expressed and purified in parallel, high titres of functional antibodies were observed. In a direct ELISA against 6.25μg/ml of AFB_1-BSA conjugate, the IMAC-purified and concentrated antibodies displayed titres of approximately 1/300,000 and 1/50,000 for the Fab and scFv antibodies, respectively (*Figure 4.18* and *4.19*). The antibodies were quantified spectrophotometrically (Nanodrop ND-1000) and the yield of His-purified Fab fragments was found to be as high as 3.5mg/L of shaker flask culture. This compares favourably to yields of Fab recombinant antibodies cited in the literature (Kramer *et al.*, 2002). However, yields

of scFv were significantly lower (<1mg/L culture) so therefore the anti-AFB_1 scFv was subsequently expressed in terrific broth (TB) as described in *Section 2.4.3.10.2.* The use of terrific broth (TB) has been reported to improve the yield of plasmid-bearing *E. coli* by extending the period of log-phase cell growth and has shown to increase expression levels of scFv antibodies (Brennan *et al.*, 2005). The rich tryptone and yeast extract media also includes glycerol as a source of carbon and potassium phosphate salts as a buffering agent to prevent cell death at decreased pHs.

For large-scale expression, the G6 Fab fragment was also produced in a 10L fermenter, as described in *Section 2.4.3.10.3*. The yield of purified Fab antibody was approx. 1mg/L of fermenter culture (6mg total), lower than obtained from shaker flasks cultures. However, the expression conditions were not optimised and therefore yields could be increased, allowing higher quantities of antibody to be produced.

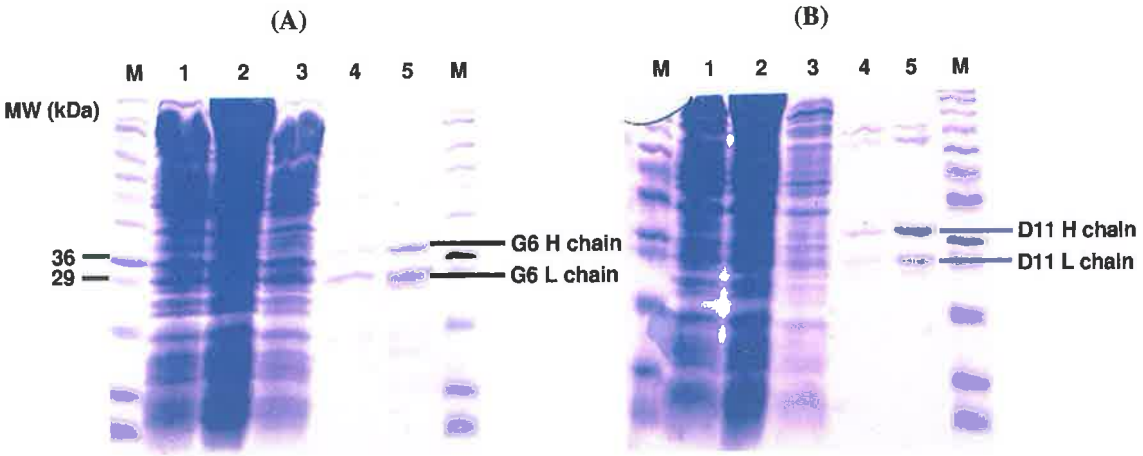


Figure 4.16. *SDS-PAGE analysis on the IMAC purification of* ***(A)*** *G6 and* ***(B)*** *D11 Fab fragments.* ***(A)*** *Lanes:* ***(M)*** *Sigma wide range molecular weight marker;* ***(1)*** *crude cytoplasmic lysate;* ***(2)*** *flow-through from IMAC resin following application of lysates;* ***(3-4)*** *wash fractions;* ***(5)*** *eluted fraction containing purified G6 Fab.* ***(B)*** *Lanes:* ***(M)*** *Sigma wide range molecular weight marker;* ***(1)*** *crude cytoplasmic lysate;* ***(2)*** *flow-through from IMAC resin following application of lysates;* ***(3-4)*** *wash fractions;* ***(5)*** *eluted fraction containing purified D11 Fab.*

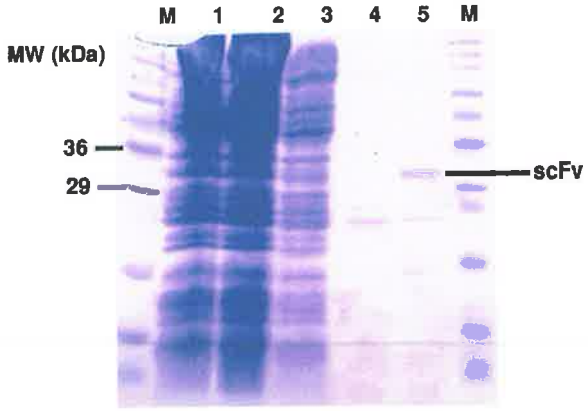


Figure 4.17. *SDS-PAGE analysis on the IMAC purification of scFv. Lanes:* ***(M)*** *Sigma wide range molecular weight marker;* ***(1)*** *crude cytoplasmic lysate;* ***(2)*** *flow-through from IMAC resin following application of lysates;* ***(3-4)*** *wash fractions;* ***(5)*** *eluted fraction containing purified scFv antibody.*

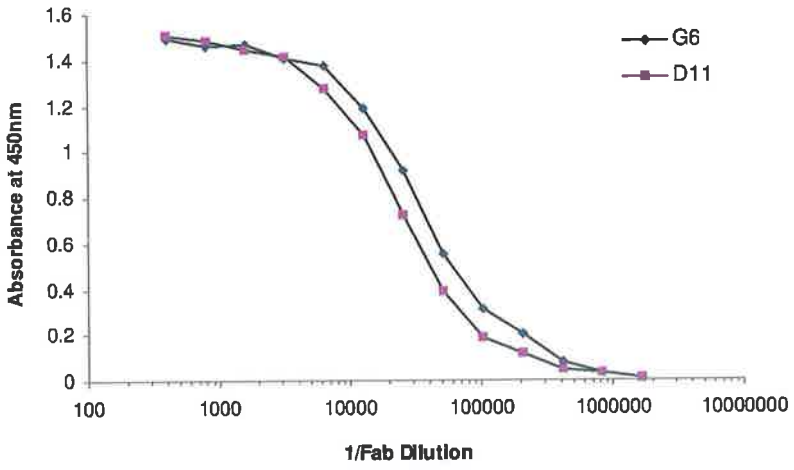


Figure 4.18. *Titres of the IMAC-purified and concentrated G6 and D11 Fab fragments against 6.25μg/ml AFB_1-BSA. The final antibody titre recorded was approximately 1 in 300,000.*

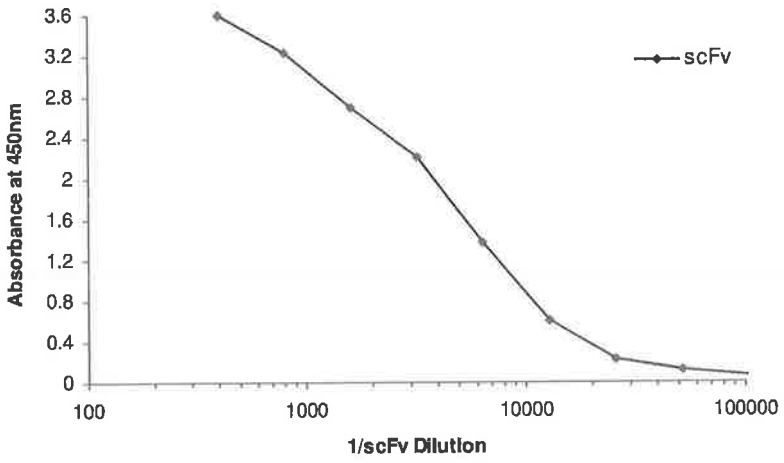


Figure 4.19. *Titre of the IMAC-purified and concentrated scFv antibody fragment against 6.25μg/ml AFB_1-BSA. The final antibody titre recorded was approximately 1 in 50,000.*

4.2.6 Development of an inhibition ELISA for the detection of AFB_1

Following generation and purification of the anti-AFB_1 Fab fragments, they were applied to the development of an ELISA for the detection of AFB_1. Their assay performance was investigated and compared to the parent scFv in terms of sensitivity.

4.2.6.1 *Checkerboard ELISA for the determination of optimal coating conjugate concentration and antibody dilution*

A checkerboard ELISA was performed, as described in *Section 2.4.3.11.1,* with the affinity-purified recombinant antibodies and plates coated with AFB_1-BSA to determine the concentrations of both the coating concentration of the conjugate and the optimal antibody dilution that would give the greatest assay sensitivity. Higher concentrations of conjugate can result in unfair binding bias towards the immobilised conjugate and reduce sensitivity to free antigen in solution. Similarly, higher concentrations of antibody will require higher concentrations of free toxin in solution to inhibit its binding to the immobilised conjugate, resulting in decreased sensitivity. These results were plotted, as shown in *Figure 4.20-4.22.* For all three antibodies an optimum conjugate coating concentration of 6.25μg/ml of AFB_1-BSA was determined as that which gave the highest absorbance, using the most economical conjugate

concentration. The optimal antibody dilutions, *i.e.* which gave the greatest change in absorbance per change in antibody dilution, were found to be 1/5,000, 1/40,000 and 1/30,000 for the scFv, G6 and D11 Fab fragments, respectively.

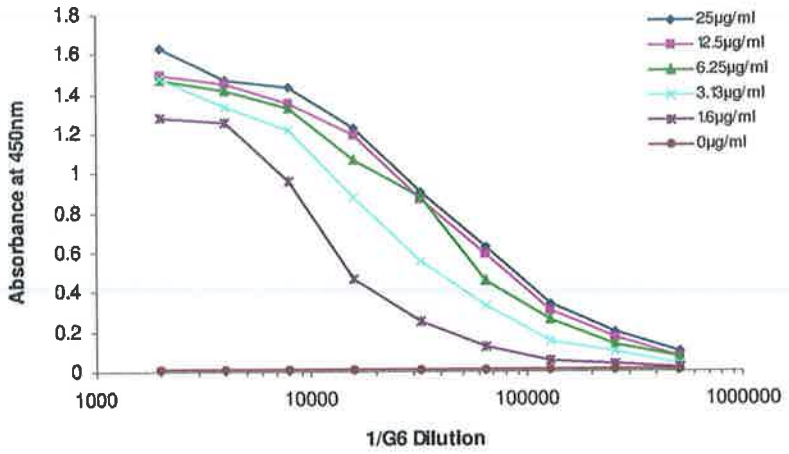


Figure 4.20. *Checkerboard ELISA for the determination of optimal conjugate coating concentration and G6 Fab antibody dilution for use in an inhibition ELISA. Varying AFB_1-BSA coating concentrations ranging from 0 to 25µg/ml and antibody dilutions from 1/2,000 - 1/512,000 were assayed. A 6.25µg/ml conjugate concentration and an antibody dilution of 1/40,000 was chosen for use in an ELISA.*

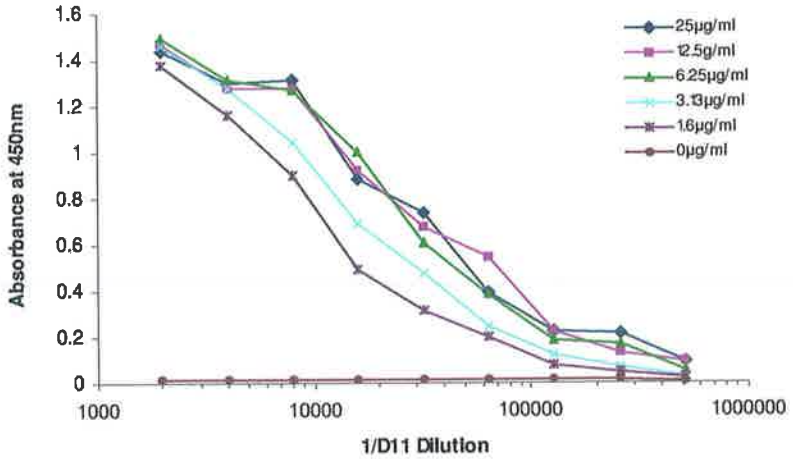


Figure 4.21. *Checkerboard ELISA for the determination of optimal conjugate coating concentration and D11 Fab antibody dilution for use in an inhibition ELISA. Varying AFB_1-BSA coating concentrations ranging from 0 to 25µg/ml and antibody dilutions from 1/2,000 - 1/512,000 were assayed. A 6.25µg/ml conjugate concentration and an antibody dilution of 1/30,000 was chosen for use in an ELISA.*

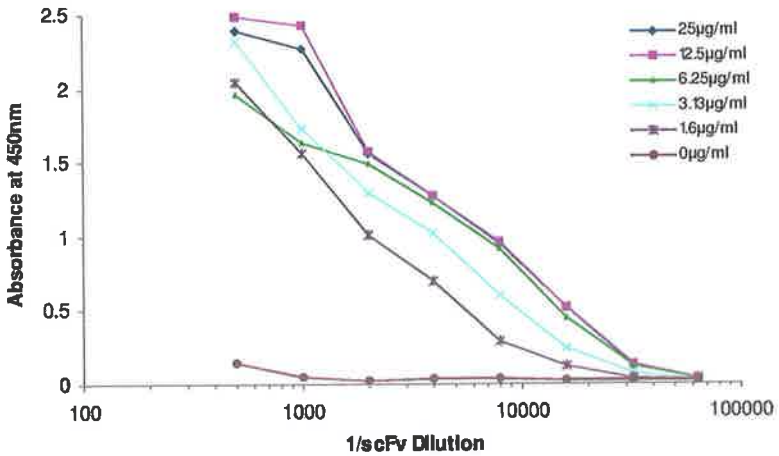


Figure 4.22. *Checkerboard ELISA for the determination of optimal conjugate coating concentration and scFv antibody dilution for use in an inhibition ELISA. Varying AFB_1-BSA coating concentrations ranging from 0 to 25µg/ml and antibody dilutions from 1/500 - 1/64,000 were assayed. A 12.5µg/ml conjugate concentration and an antibody dilution of 1/5,000 was chosen for use in an inhibition ELISA.*

4.2.6.2 Inhibition ELISA for the detection of AFB_1

Using the optimised dilutions determined, an inhibition assay was performed employing the Fab recombinant antibodies to determine their range of detection for AFB_1. Standards of AFB_1, ranging from 0.38 to 781ng/ml, were prepared in PBS containing 5% (v/v) methanol (methanol was required in the diluent for the preparation of AFB_1 due to its strong hydrophobic nature). Standards were mixed with an equal volume of antibody, at the optimal dilution and added to a coated and blocked immunoplate, as described in *Section 2.4.3.11.2.*

4.2.6.2.1 *Intra- and inter-assay variability studies*

Intra-assay (variation within an assay) and inter-assay (variation between assays) variability studies were also performed to demonstrate the reproducibility of the ELISA. Three sets of each standard were assayed on the same day and the coefficients of variation (CV's) were determined by expressing standard deviation as a percentage of the mean values. Intra-assay CV's ranged from 0.72 to 10.76% for the G6 Fab fragment, while inter-assay CV's ranged from 0.63 to 8.25%. Intra- and inter-assay CV's for the ELISA employing the D11 Fab ranged from 3.40 to 20% and 2.62 to 5.58%, respectively. Both assays displayed acceptable reproducibility (*i.e.* below the recommended precision of 20%) (Findlay *et al.*, 2000).

To determine assay accuracy (*i.e.* whether measured concentrations agreed with theoretical values), percentage recovery values were also calculated by expressing the measured concentration as a percentage of the actual concentration. For the linear range of the assays employing Fab fragments, the percentage recoveries were ±10%, indicating adequate accuracy (within 20% recommended by Findlay *et al.*, 2000). Percentage recoveries at non-linear parts of the curve were found to be higher. Both percentage recovery and CV values for G6 and D11 Fab ELISAs are shown in *Tables 4.2 and 4.3*, respectively.

Figure 4.23 shows the inter-day calibration curve using the G6 Fab fragment, where normalised absorbance values (A/A_0) were plotted against the logarithm of AFB_1 concentration using BIAevaluation 4.0.1 software. The range of detection was found to be between 0.76 and 391ng/ml. The limit of detection (LOD), also termed the least detectable dose (LDD), is the smallest concentration of the analyte that produces a

signal which can be significantly distinguished from zero for a given sample matrix (Hennion and Barcelo, 1998). This value was determined by selecting the mean normalised absorbance minus three standard deviations for the negative standard (no AFB_1) and was found to be approximately 1.3ng/ml. *Figure 4.24* shows the inter-day calibration curve using the D11 Fab fragment, where normalised absorbance values (A/A_0) were plotted against the logarithm of AFB_1 concentration using BIAevaluation 4.0.1 software. The range of detection was found to be between 6 and 391ng/ml. The limit of detection (LOD), determined as before was found to be approximately 4.1ng/ml.

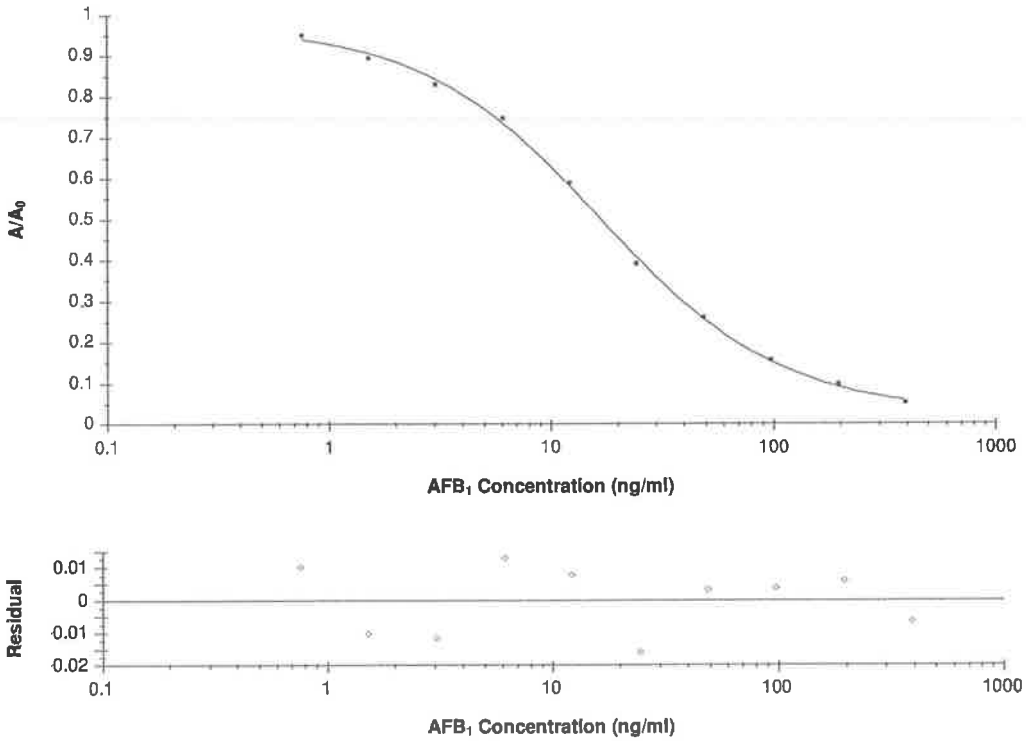


Figure 4.23. *Inter-day calibration curve for inhibition ELISA for the detection of AFB_1 in PBS using the G6 Fab fragment. AFB_1-BSA was coated at a concentration of 6.25µg/ml and antibody at a final dilution of 1/40,000 was used. Bound antibody was detected using a HRP-labelled anti-HA antibody followed by addition of a substrate TMB. Data was normalised by dividing the mean absorbance obtained at each AFB_1 concentration (A) by the absorbance values determined in the presence of zero toxin (A_0). Normalised absorbance values (A/A_0) were plotted against the logarithm of aflatoxin B_1 concentration using BIAevaluation 4.0.1 software and a 4-parameter equation fitted to the data. All analyses were performed in triplicate on three separate occasions and the limit of detection was found to be 1.3ng/ml.*

Table 4.2. *Inter-day assay coefficient of variation (CVs) for the detection of AFB_1 using the G6 Fab fragment. Each standard was analysed in triplicate over three different days and the CVs were calculated as the standard deviation (SD) expressed as a percentage of the mean value for each standard. Measured (i.e. back-calculated concentrations) were calculated from the four-parameter model produced using BIAevaluation 4.0.1 software and the percentage recovery expressed as a ratio between measured and added concentrations.*

Concentration (ng/ml)	**$A/A_0 \pm SD$**	**CV (%)**	**Back calculated concentration (ng/ml)**	**Recovery (%)**
390.50	0.052 ± 0.003	5.77	429.01	110
195.25	0.097 ± 0.008	8.25	174.97	90
97.63	0.156 ± 0.010	6.41	94.76	97
48.81	0.260 ± 0.017	6.54	48.36	99
24.40	0.393 ± 0.016	4.07	26.18	107
12.20	0.590 ± 0.033	5.60	11.79	97
6.10	0.748 ± 0.046	6.15	5.65	93
3.05	0.831 ± 0.038	4.57	3.33	109
1.53	0.896 ± 0.038	4.24	1.78	117
0.76	0.950 ± 0.006	0.63	0.60	79

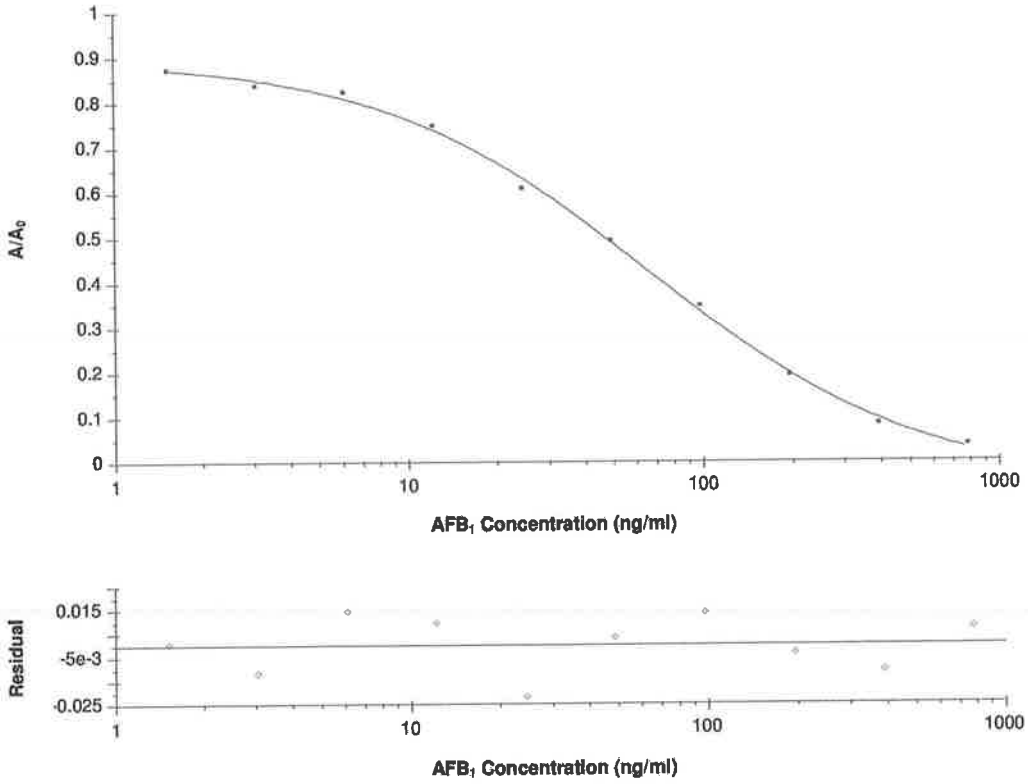


Figure 4.24. *Inter-day calibration curve for inhibition ELISA for the detection of AFB_1 in PBS using the D11 Fab fragment. AFB_1-BSA was coated at a concentration of 6.25µg/ml and a final antibody dilution of 1/30,000 was used. Bound antibody was detected using a HRP-labelled anti-HA antibody followed by addition of a substrate TMB. Data was normalised by dividing the mean absorbance obtained at each AFB_1 concentration (A) by the absorbance values determined in the presence of zero toxin (A_0). Normalised absorbance values (A/A_0) were plotted against the logarithm of aflatoxin B_1 concentration using BIAevaluation 4.0.1 software and a 4-parameter equation fitted to the data. All analyses were performed in triplicate on three separate occasions and the limit of detection was found to be 4.2ng/ml.*

Table 4.3. *Inter-day assay coefficient of variation (CVs) for the detection of AFB_1 using the D11 Fab fragment. Each standard was analysed in triplicate over three different days and the CVs were calculated as the standard deviation (SD) expressed as a percentage of the mean value for each standard. Measured (i.e. back-calculated concentrations) were calculated from the four-parameter model produced using BIAevaluation 4.0.1 software and the percentage recovery expressed as a ratio between measured and added concentrations.*

Concentration (ng/ml)	**$A/A_0 \pm SD$**	**CV (%)**	**Back calculated concentration (ng/ml)**	**Recovery (%)**
390.50	0.084 ± 0.003	3.57	429.62	110
195.25	0.193 ± 0.008	4.15	199.27	102
97.63	0.348 ± 0.010	2.87	91.84	94
48.81	0.493 ± 0.017	3.45	48.16	99
24.40	0.610 ± 0.016	2.62	27.42	112
12.20	0.749 ± 0.033	4.41	11.31	93
6.10	0.824 ± 0.046	5.58	4.99	82
3.05	0.839 ± 0.038	4.53	3.86	127
1.53	0.874 ± 0.038	4.35	1.48	97

4.2.6.3 Comparison of Fab fragments and parent scFv

The results of the inhibition ELISAs employing the G6 and D11 Fab antibody fragments were compared to the parent scFv in terms of assay sensitivity. An inhibition assay was performed as described in *Section 2.4.3.11.2* employing the anti-AFB_1 scFv at the optimal dilution of 1/5,000 against 12.5µg/ml AFB_1-BSA. The range of detection for the scFv was found to be 12-781ng/ml, with an IC_{50} value of 52ng/ml). The scFv displayed similar sensitivity to the D11 Fab, which had an assay IC_{50} value of 43ng/ml. However, the G6 Fab (IC_{50} value of 14.8ng/ml) demonstrated 3 to 3.5-fold increase in assay sensitivity when compared to both the D11 Fab and scFv, respectively. ELISA inhibition curves for all three antibodies are shown in *Figure 4.25*. Absorbance values were normalised by the transformation to $\%\ B/B_0$ according to:

$$\%\ B/B_0 = \frac{A - A_{excess}}{A_0 - A_{excess}}$$

where A is the absorption, A_0 is the absorption at zero dose of analyte and A_{excess} is the absorption at excess of analyte.

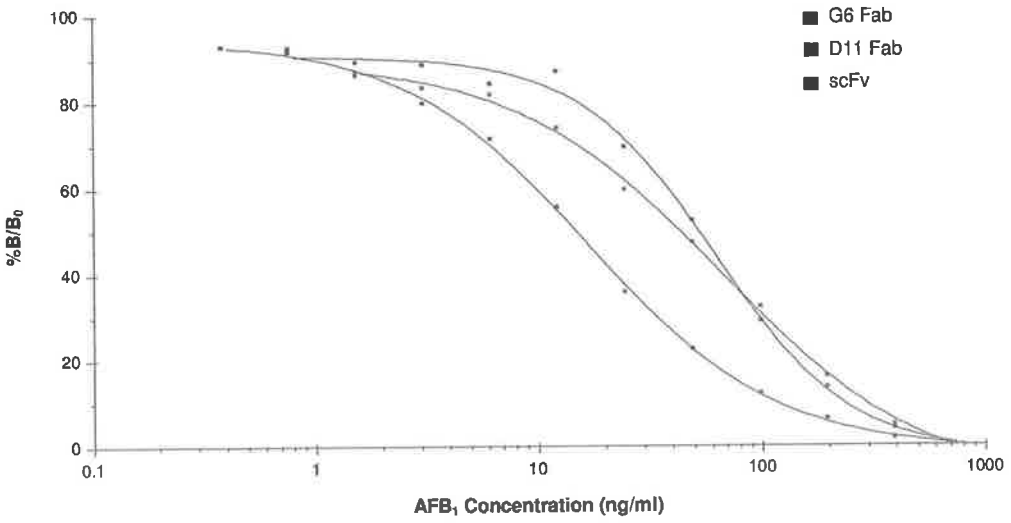


Figure 4.25. *Inhibition ELISA for the detection of AFB_1 in PBS using the scFv and G6 and D11 Fab antibody fragments. Normalised absorbance values (expressed as $\%B/B_0$) were plotted against the logarithm of AFB_1 concentration using BIAevaluation 4.0.1 software and a 4-parameter equation fitted to the data. The IC_{50} values for the scFv, D11 Fab and G6 Fab were found to be 52ng/ml, 43ng/ml and 14.8ng/ml, respectively. The Fab recombinant antibodies demonstrated greater linearity in assay response than the scFv.*

4.2.6.4 Cross-reactivity studies

To determine the degree of cross-reactivity of the Fab fragments, D11 and G6, with four structurally related aflatoxins, inhibition ELISA was performed as described in *Section 2.4.3.11.3*. Standards of each potential cross-reactant, ranging from 1.5 to 25,000ng/ml, were prepared in PBS containing 5% (v/v) methanol and mixed with an equal volume of antibody at the optimal concentration. Samples were incubated for 30 minutes at room temperature to allow equilibration and added to the conjugate coated wells of a microtitre plate. Normalised absorbance values (expressed as % B/B_0) were plotted against the log of aflatoxin concentration using BIAevaluation software 4.0.1.

Percentage cross-reactivities (% CR_{50}) were estimated by comparing the relevant IC_{50} values of the potential cross-reactants to those values of the antigen (*i.e.* AFB_1), where the IC_{50} value is the analyte concentration that results in 50% inhibition (Hennion and Barcelo, 1998). Results, listed in *Table 4.4*, indicate the D11 Fab displayed approximately the same degree of cross-reactivity with the aflatoxins B_2, G_1, G_2 and M_1 (24.5, 21.5, 15.6 and less than 0.04%, respectively) as the parent scFv. Dunne (2004) reported the scFv displayed percentage cross-reactivities (%CR_{50}) of 19% with the aflatoxins B_2, G_1 and G_2. Lower percentage cross-reactivities were reported against aflatoxin M_1 (2.4%). Cross-reactivity studies carried out on the G6 Fab fragment revealed the antibody offered greater specificity towards B_1, in comparison to the D11 Fab. The G6 antibody displayed lower levels of cross-reactivity towards B_2 and G_2, with % CR_{50} of 1.9 and 0.93, respectively (*Table 4.5*). A higher level of cross-reactivity against aflatoxin G_1 (14.8%) was detected. The antibody showed minimal cross-reactivity with aflatoxin M_1 (<0.01%).

Table 4.4. *Percentage cross-reactivity values of the D11 Fab antibody fragment to various potential cross-reactants. The cross-reactivity potential was approximated at the IC_{50} value, which was estimated at 50% B/B_0. The percentage cross-reactivity determined at IC_{50} ($\%CR_{50}$) was expressed as 100-fold the ratio of the antigen (B_1) and of the cross-reactant.*

Aflatoxin compound	IC_{50} (ng/ml)	CR_{50} (%)
B_1	42.9	100
B_2	175	24.5
G_1	200	21.5
G_2	275	15.6
M_1	>100,000	<0.04

Table 4.5. *Percentage cross-reactivity values of the G6 Fab antibody fragment to various potential cross-reactants. The cross-reactivity potential was approximated at the IC_{50} value, which was estimated at 50% B/B_0. The percentage cross-reactivity determined at IC_{50} ($\%CR_{50}$) was expressed as 100-fold the ratio of the antigen (B_1) and of the cross-reactant.*

Aflatoxin compound	IC_{50} (ng/ml)	CR_{50} (%)
B_1	14.8	100
B_2	750	1.9
G_1	100	14.8
G_2	1,600	0.93
M_1	>100,000	<0.01

4.2.7 Development of a Biacore inhibition assays for the detection of AFB_1

Assays for the detection of AFB_1 were developed employing a CM5 chip immobilised with an AFB_1 derivative, which was kindly donated, by Claire Jones of Xenosense Ltd., Queens University, Belfast. For successful development of such assays, optimisation of a number of parameters was required, including non-specific binding and sensor surface regeneration studies.

4.2.7.1 Non-specific binding studies

The three anti-AFB_1 recombinant antibodies were passed over the directly immobilised AFB_1 chip surface to investigate specific binding. Each antibody was injected simultaneously over the AFB_1 and unactivated dextran surface for four minutes at a flowrate of 5µl/minute. It can clearly be seen from *Figure 4.26 – 4.28* that negligible non-specific binding (NSB) to the CM dextran surface was observed with each purified antibody.

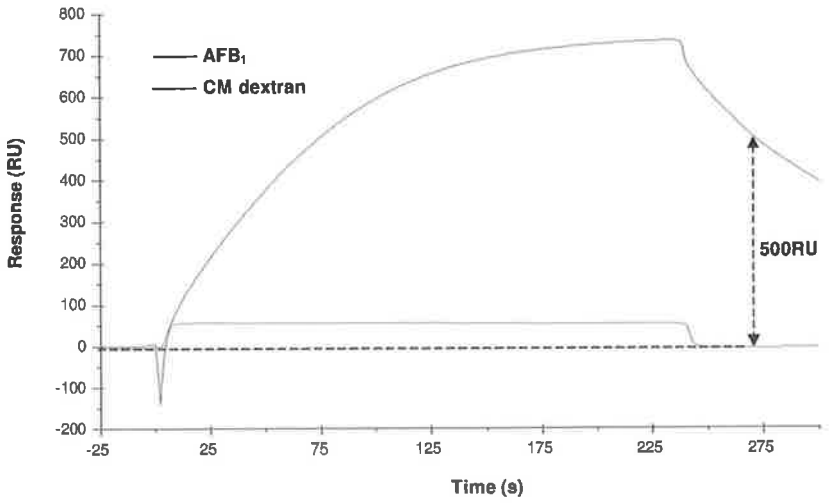


Figure 4.26. *Overlay sensogram plots of the G6 Fab fragment binding to the immobilised AFB_1 surface and an unactivated CM dextran surface. Antibody was passed over each surface simultaneously at a flow rate of 5µl/min for four minutes and the response recorded 30 seconds after the end of injection. Approximately 500RU of the G6 Fab bound to the directly immobilised AFB_1 surface, while negligible binding to the control dextran surface was observed.*

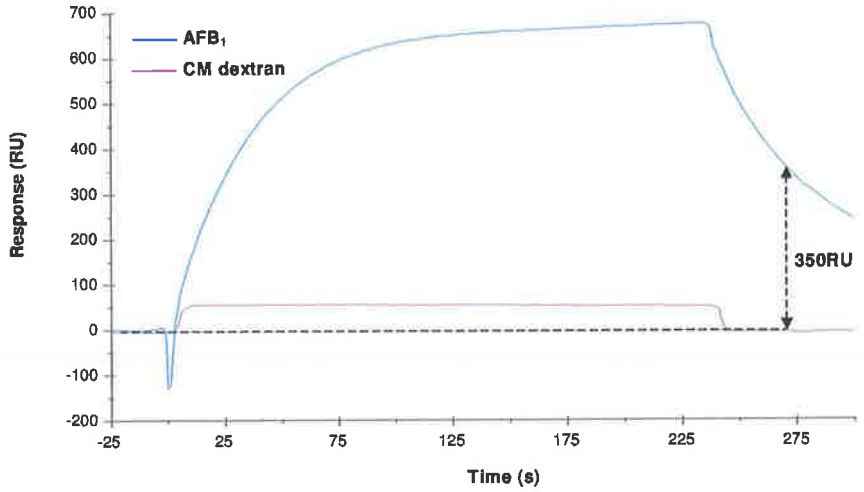


Figure 4.27. *Overlay sensogram plots of the D11 Fab fragment binding to the immobilised AFB_1 surface and an unactivated CM dextran surface. Antibody was passed over each surface simultaneously at a flow rate of 5µl/min for four minutes and the response recorded 30 seconds after the end of injection. Approximately 350RU of the D11 Fab bound to the directly immobilised AFB_1 surface, while negligible binding to the control dextran surface was observed.*

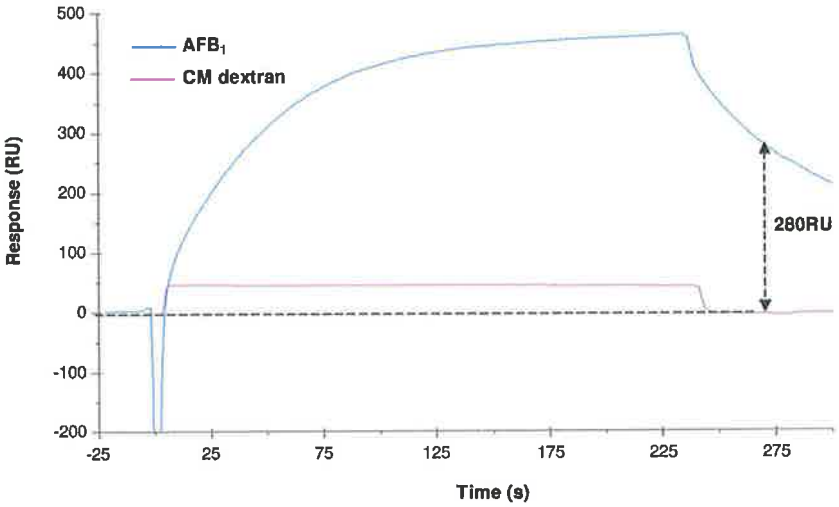


Figure 4.28. *Overlay sensogram plots of the scFv binding to the immobilised AFB_1 surface and an unactivated CM dextran surface. Antibody was passed over each surface simultaneously at a flow rate of 5µl/min for four minutes and the response recorded 30 seconds after the end of injection. Approximately 280RU of the scFv bound to the directly immobilised AFB_1 surface, while negligible binding to the control dextran surface was observed.*

4.2.7.2 Regeneration studies

For a Biacore-based assay to be cost effective, each immobilised flow cell should be capable of analysing a large number of samples. Hence for multiple analyses, a stable surface and an efficient regeneration process are imperative. Antibodies were passed over the sensor chip surface at a flowrate of 10µl/min for two minutes and the response recorded 30 seconds after the end of injection. This was followed by a 5µl injection of 10mM sodium hydroxide, which enabled complete regeneration of the AFB_1 chip surface, as seen in *Figure 4.29.*

The efficiency of the regeneration process was then evaluated by performing multiple (*i.e.* 50) binding-regeneration cycles on the directly immobilised AFB_1 surface, as shown in *Figure 4.30 and 4.31.* The G6 and D11 Fab antibodies, diluted 1/3,900 and 1/3,000, were injected over the sensor surface at a flowrate of 10µl/min for 2 minutes and specific bound antibodies removed with a 30 second pulse of 10mM NaOH at a flowrate of 10µl/min. Over the fifty binding-regeneration cycles, the binding of the antibody to the AFB_1 decreased significantly. A decrease in binding response of 8% and 12% for the G6 and D11 Fab was observed, as illustrated in *Figure 4.30* and *4.31.*

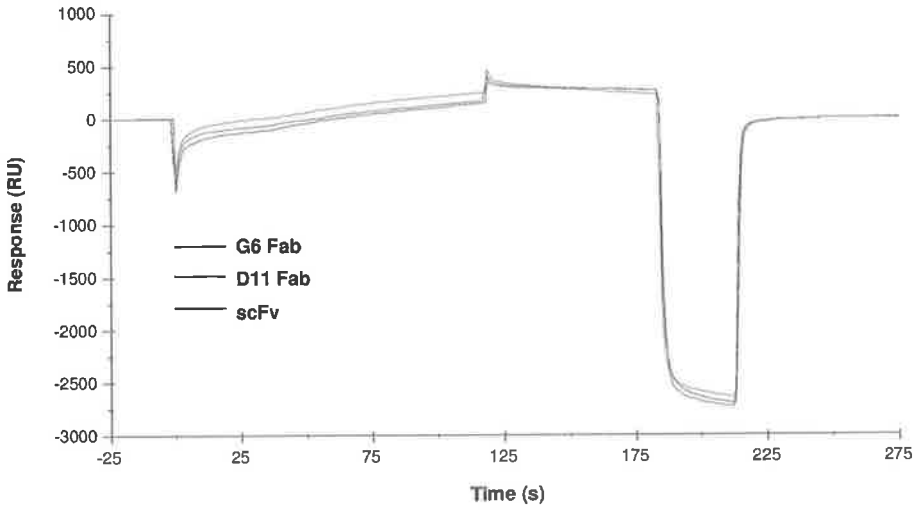


Figure 4.29. *Overlay sensogram plots of the Fab fragments (clone G6 and D11) and scFv binding to the directly immobilised AFB_1 surface. The antibodies, prepared in PBS containing 5% (v/v) methanol, were passed over the surface at a flowrate of 10µl/min for 2 minutes and the response recorded. Approximately 300RU of each antibody bound to the surface, which could be fully regenerated following a 30 second injection of 10mM NaOH.*

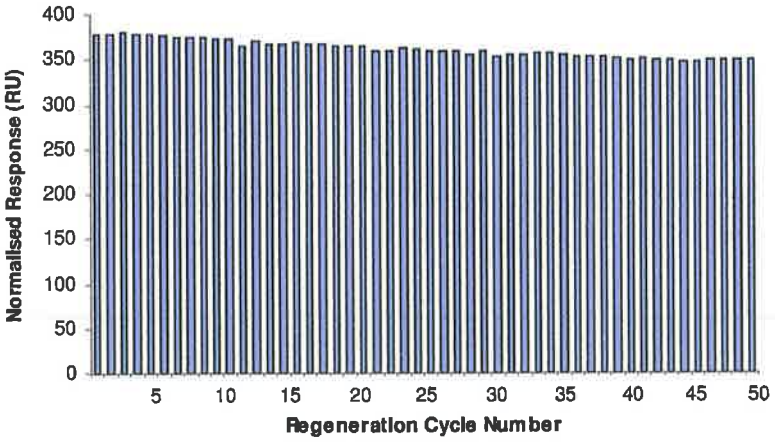


Figure 4.30. *Typical regeneration profile for fifty consecutive binding-regeneration cycles of a 2 minute pulse of purified G6 Fab antibody in HBS to the surface of a chip immobilised with an AFB_1 derivative. Antibody, at a dilution of 1/3,900, was used and the surface regenerated with a 30 second pulses of 10mM NaOH. There was a 8% decrease in binding activity observed over the fifty consecutive cycles.*

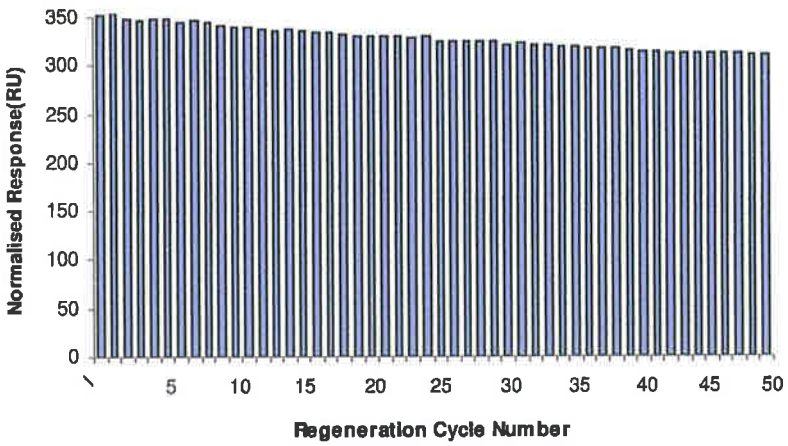


Figure 4.31. *Typical regeneration profile for fifty consecutive binding-regeneration cycles of a 2 minute pulse of purified D11 Fab antibody in HBS to the surface of a chip immobilised with an AFB1 derivative. Antibody, at a dilution of 1/3,000, was used and the surface regenerated with a 30 second pulses of 10mM NaOH. There was a 12% decrease in binding activity observed over the fifty consecutive cycles.*

4.2.7.3 Biacore inhibition assays for the detection of AFB_1

PBS containing 5%(v/v) methanol was spiked with known concentrations of AFB_1, ranging from 48 to 98,000pg/ml, as described in *Section 2.4.3.12.1,* and mixed with an equal volume of Fab antibody, at a final dilution of approximately 1/3,000. Samples were incubated for 30 minutes at room temperature to allow equilibration and then injected over the AFB_1 immobilised surface at a flowrate or 10µl/minute for 2 minutes. This was followed by a 5µl injection of 10mM NaOH, which allowed complete regeneration of the chip surface. All samples were analysed in triplicate and their respective response units (RU) normalised by dividing the mean response obtained at each AFB_1 concentration (RU) by the mean response determined in the presence of zero toxin (RU_0).

4.2.7.3.1 *Intra- and inter-assay variability studies*

To demonstrate the reproducibility of the Biacore inhibition assays both intra- and inter-variability studies were performed. Intra- and inter-day assay CV's for the G6 Fab-based assay ranged from 0.04% to 1.18% and 0.31% to 3.51%, respectively, indicating excellent reproducibility. While, intra- and inter-day assay CV's for the D11 Fab-based assay ranged from 0.09% to 10.51% and 0.83% to 8.96%, respectively. Percentage recovery values indicated reasonable accuracy, with majority of values within ±10%, with the exception of values at 381pg/ml of AFB_1, which were 127% and 125% for the G6 and D11 Fab-based assays, respectively. CV's and percentage recovery values are shown in *Table 4.6 and 4.7.*

Normalised response values (RU/RU_0) were plotted against the log of AFB_1 concentration to generate a calibration curves using BIAevaluation 4.0.1 software. The limit of detection (LOD), which is the smallest concentration of the analyte that produces a response that can be significantly distinguished from zero analyte, was calculated for both the G6 and D11 Fab-based assays. *Figure 4.32* shows the inter-day calibration curve for the assay employing the G6 Fab antibody, where the range of detection was found to be between 95 and 24,400pg/ml. The LOD of the assay, which was determined by selecting the mean normalised response minus three standard deviations for the negative standard (*i.e.* no AFB_1), was found to be 117pg/ml. The inter-day calibration curve employing the D11 Fab antibody is shown in *Figure 4.31.*

The range of detection was found to be between 191 and 48,800pg/ml for AFB_1 in the D11 Fab-based assay with a detection limit of 290pg/ml.

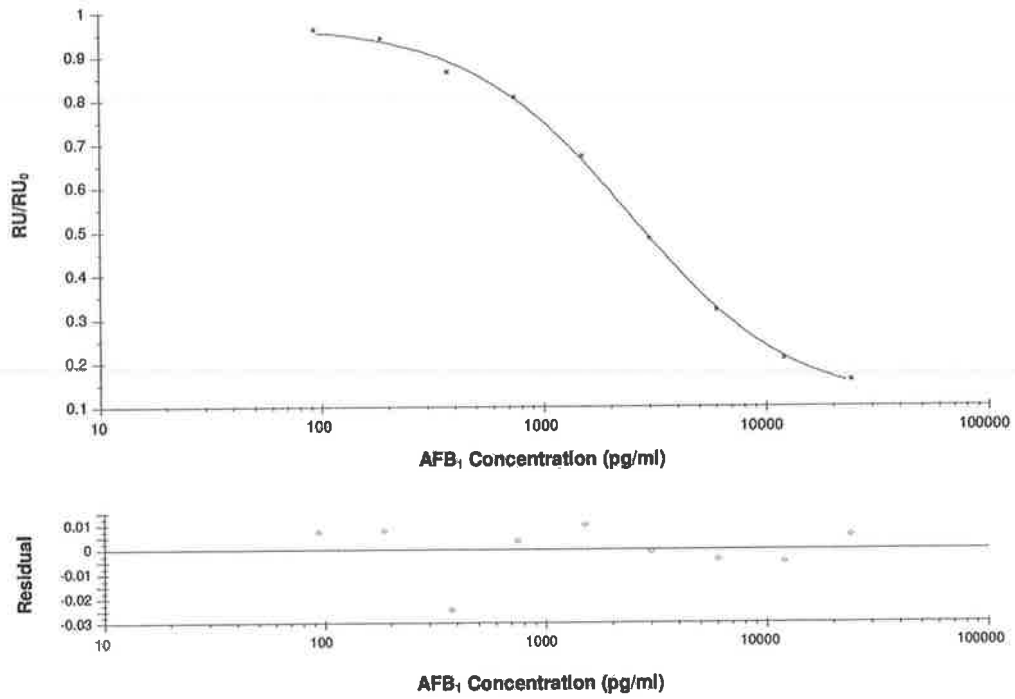


Figure 4.32. *Inter-day calibration curve for the inhibition Biacore assay for the detection of AFB_1 using the G6 Fab fragment. A calibration curve was constructing by plotting the normalised response values (RU/RU_0) against the log of AFB_1 concentration using BIAevaluation 4.0.1 software. All analyses were performed in triplicate on three separate days. The assay was found to have a range of detection of 95 to 24,400pg/ml and a limit of detection of 117pg/ml.*

Table 4.6. *Biacore inhibition assay inter-day coefficient of variation (CVs) for the detection of free AFB_1 employing the G6 Fab antibody. Each standard was assayed in triplicate over three days and the CVs were calculated as the standard deviation (SD) expressed as a percentage of the mean value for each standard. Measured concentrations (i.e. back-calculated concentrations) were calculated from the four-parameter model fitted to the assay data using BIA evaluation 4.01 software and percentage recoveries expressed a ratio between measured and added concentrations.*

Concentration (pg/ml)	**$RU/RU_0 \pm SD$**	**CV (%)**	**Back calculated concentration (ng/ml)**	**Recovery (%)**
24,406.25	0.160 ± 0.004	2.50	21,980.44	90
12,203.13	0.209 ± 0.006	2.87	12,805.43	105
6,101.56	0.320 ± 0.006	1.88	6,276.28	103
3,050.78	0.484 ± 0.017	3.51	3,062.38	100
1,525.39	0.672 ± 0.020	2.98	1,450.15	95
762.70	0.807 ± 0.007	0.87	740.29	97
381.35	0.943 ± 0.014	1.48	485.35	127
190.67	0.963 ± 0.003	0.31	172.48	90
95.34	0.979 ± 0.023	2.35	86.10	90

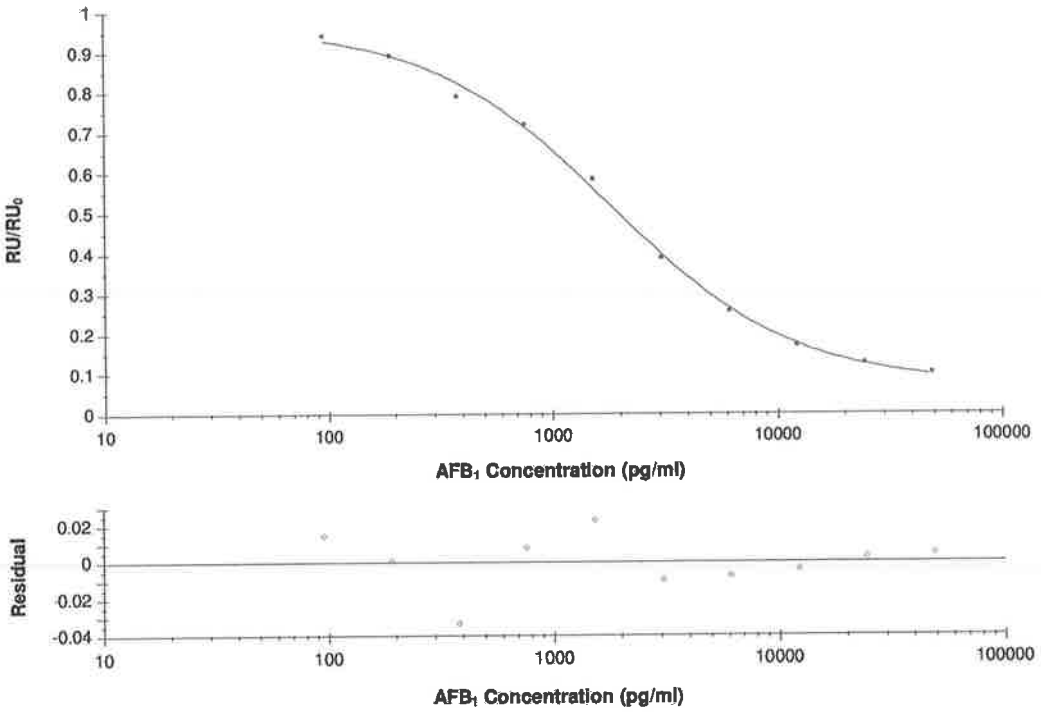


Figure 4.33. *Inter-day calibration curve for the inhibition Biacore assay for the detection of AFB_1 using the D11 Fab fragment. A calibration curve was constructing by plotting the normalised response values (RU/RU_0) against the log of AFB_1 concentration using BIAevaluation 4.0.1 software. All analyses were performed in triplicate on three separate days. The assay was found to have a range of detection of 191 to 48,800pg/ml and a limit of detection of 290pg/ml.*

Table 4.7. *Biacore inhibition assay inter-day coefficient of variation (CVs) for the detection of free AFB_1 employing the D11 Fab antibody. Each standard was assayed in triplicate over three days and the CVs were calculated as the standard deviation (SD) expressed as a percentage of the mean value for each standard. Measured concentrations (i.e. back-calculated concentrations) were calculated from the four-parameter model fitted to the assay data using BIA evaluation 4.01 software and percentage recoveries expressed a ratio between measured and added concentrations.*

Concentration (pg/ml)	**$RU/RU_0 \pm SD$**	**CV (%)**	**Back calculated concentration (ng/ml)**	**Recovery (%)**
48,812.50	0.101± 0.009	8.91	45,853.99	94
24,406.25	0.126 ± 0.004	3.17	21,299.64	87
12,203.13	0.170 ± 0.009	5.29	12,950.15	106
6,101.56	0.256 ± 0.002	0.78	6,426.61	105
3,050.78	0.389 ± 0.024	6.17	3,153.60	103
1,525.39	0.585 ± 0.027	4.62	1,418.31	93
762.70	0.722 ± 0.006	0.83	715.45	94
381.35	0.792 ± 0.020	2.53	477.15	125
190.67	0.893 ± 0.080	8.96	181.63	95
95.34	0.943 ± 0.042	4.50	71.61	75

4.2.7.4 Comparison of Fab fragments and parent scFv

The results of the inhibition Biacore assays employing the G6 and D11 Fab antibody fragments were compared to the parent scFv in terms of assay sensitivity. An inhibition Biacore assay was performed on the AFB_1 derivative sensor surface, as described in *Section 2.4.3.12.1* employing the anti-AFB_1 scFv at the optimal dilution of 1/4,000. The range of detection for the scFv was found to be 0.38-24.4ng/ml, with an IC_{50} value of 2.4ng/ml. The scFv displayed similar sensitivity to the G6 Fab, which had an assay IC_{50} value of 2.2ng/ml. However, the D11 Fab (IC_{50} value of 1.6ng/ml) demonstrated ~1.5-fold increase in assay sensitivity when compared to the parent scFv. Biacore inhibition curves for all three antibodies are shown in *Figure 4.34* and response values were normalised by expressing data as $\%B/B_0$ (as before).

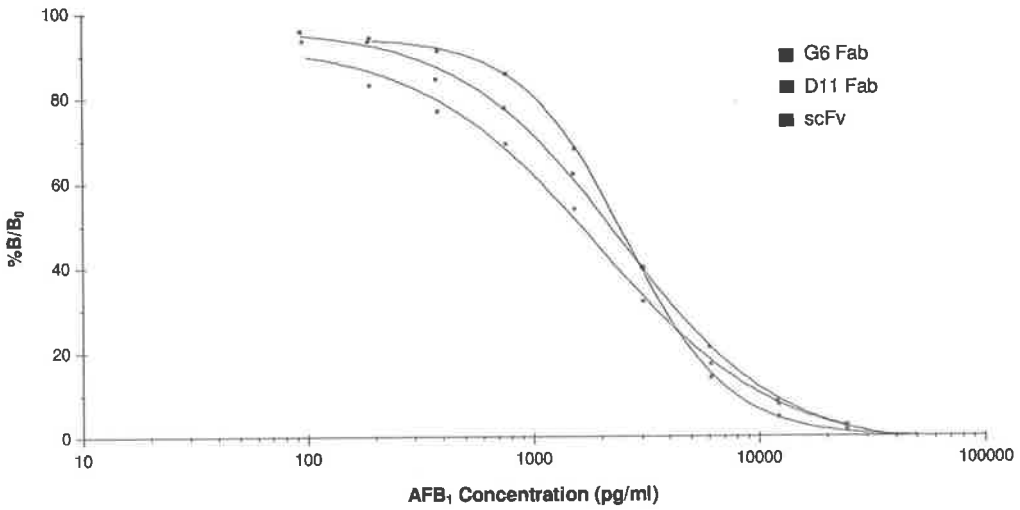


Figure 4.34. *Inhibition Biacore assay for the detection of AFB_1 in PBS using the scFv and G6 and D11 Fab antibody fragments. Normalised response values (expressed as $\%B/B_0$) were plotted against the logarithm of AFB_1 concentration using BIAevaluation 4.0.1 software and a 4-parameter equation fitted to the data. The IC_{50} values for the scFv, D11 Fab and G6 Fab were found to be 2.4ng/ml, 1.6ng/ml and 2.2ng/ml, respectively. The Fab recombinant antibodies demonstrated greater linearity in assay response than the scFv.*

4.2.8 *In vivo* biotinylation of anti-AFB_1 Fab and scFv fragments

The pAK400Bio vector, kindly donated by Dr. Warren of the Central Laboratory, The Norwegian Radium Hospital, Oslo, Norway, was used for *in vivo* biotinylation of the scFv and Fab fragments. The pAK400Bio vector was constructed by replacing the carboxy-terminal $(His)_6$ tag in the original pAK400 vector (Krebber *et al.*, 1997) with a sequence encoding a biotin acceptor peptide, GGGLNDIFEAQKIEWHE (Warren *et al.*, 2005). This vector facilitates *in vivo* biotinylation of antibody fragments by expressing them as a fusion protein with the biotin acceptor in an *E. coli* K12 strain (AVB100) that overproduces the biotin holoenzyme BirA. The *in vivo* biotinylation method allows each fragment to be labelled with only one biotin molecule distal of the antigen-binding site, thus, making it advantageous over random chemical biotinylation. The method would facilitate immobilisation of fragments onto avidin surfaces in an orientated manner and could be potential useful for future antibody array applications.

4.2.8.1 Subcloning of scFv gene into *in vivo* biotinylation vector pAK400Bio

The pAK400Bio and pAK400 expression vector (harbouring the anti-AFB_1 scFv insert) were both digested with *Sfi*I as described in *Section 2.4.4.1*. The scFv fragment and linearised pAK400Bio vector (*Figure 4.35 (A)*) were purified by agarose gel electrophoresis and the purified DNA quantified (*Section 2.4.4.1)*. The AFB_1-specific scFv was ligated into the pAK400Bio vector and transformed into *E. coli* AVB100 cells (Avidity Inc.) cells, which contain a chromosomal copy of the biotin holoenzyme synthesase (*bir*A) gene (*Section 2.4.4.3* and *2.4.4.4*).

4.2.8.2 Subcloning of Fab fragments into *in vivo* biotinylation vector pAK400Bio

The restriction sites in the pComb3X vector (5′GGCCCAGGCGGCC and 3′GGCCAGGCCGGCC) differ from those in the pAK400Bio vector (5′GGCCCAGCCGGCC and 3′GGCCTCGGGGGCC). Therefore, primers were designed for amplification of the Fab fragments from the pComb3X vector that introduced the appropriate *Sfi*I sites for pAK400Bio. The resulting Fab fragment sequences could then be directly cloned into the biotinylation vector pAK400Bio with the rare-cutting restriction enzyme *Sfi*I.

The G6 and D11 were amplified from the pComb3X vector using the primers designed as per *Section 2.4.4.2.1.* A standard PCR reaction was used to amplify the DNA sequences and the optimum annealing temperature was determined to be 43°C (*Section 2.4.4.2.2* and *2.4.4.2.3*). *Figure 4.36 (A)* shows agarose gel analysis of the amplified G6 and D11 DNA fragments at approximately 1,500bp. The pAK400Bio and Fab fragments were both digested with *Sfi*I, as described in *Section 2.4.4.1*, and digested Fab and vector DNA were purified by agarose gel electrophoresis and quantified (*Section 2.4.2.1.5). Figure 4.36 (B)* shows the agarose gel analysis on both the digested Fab fragments and vector DNA. The anti-AFB_1 Fab fragments was then ligated into the pAK400Bio vector (*Section 2.4.4.3*) and transformed into *E. coli* AVB100 cells (Avidity Inc.) (*Section 2.4.4.4*) for subsequent expression of biotinylated Fab fragments. The pAK400Bio vector containing both the scFv and Fab fragments can be seen in *Figure 4.35 (B)* and *4.37*, respectively.

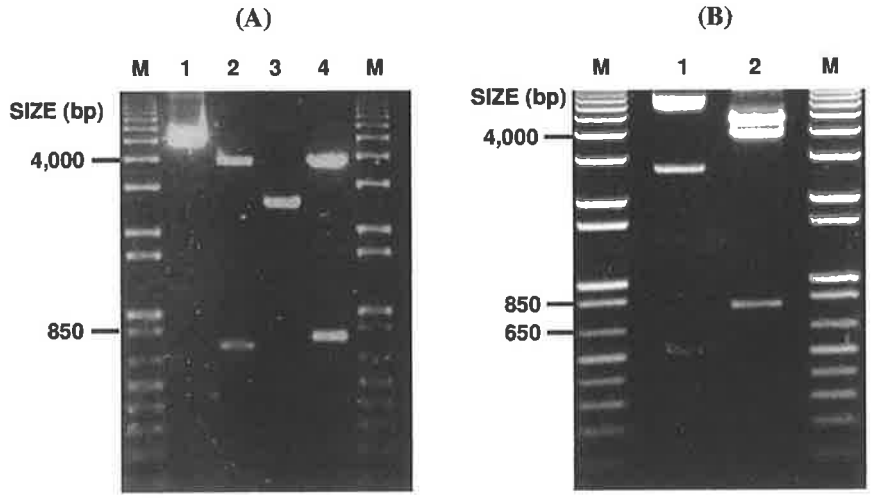


Figure 4.35. *(A) SfiI digestion of pAK400Bio and pAK400 vectors. Lanes: (M) 1Kb plus DNA ladder (Invitrogen); (1) undigested pAK400Bio vector containing stuffer fragment; (2) SfiI digested pAK400Bio (~4,000bp) with the stuffer gene removed (~800bp); (3) undigested pAK400 vector containing the AFB_1-specific scFv; (4) SfiI digested pAK400 vector (~4,000bp) with anti-AFB_1 scFv fragment (~800bp) removed. (B) SfiI digestion of pAK400Bio vector containing the AFB_1-specific scFv. Lanes: (M) 1Kb plus DNA ladder (Invitrogen); (1) undigested pAK400Bio vector containing the anti-AFB_1 scFv; (2) SfiI digested pAK400Bio. Linearised vector DNA (~4,800bp; cut only once) and double cut DNA (~4,000bp) with the anti-AFB_1 scFv gene removed (~800bp).*

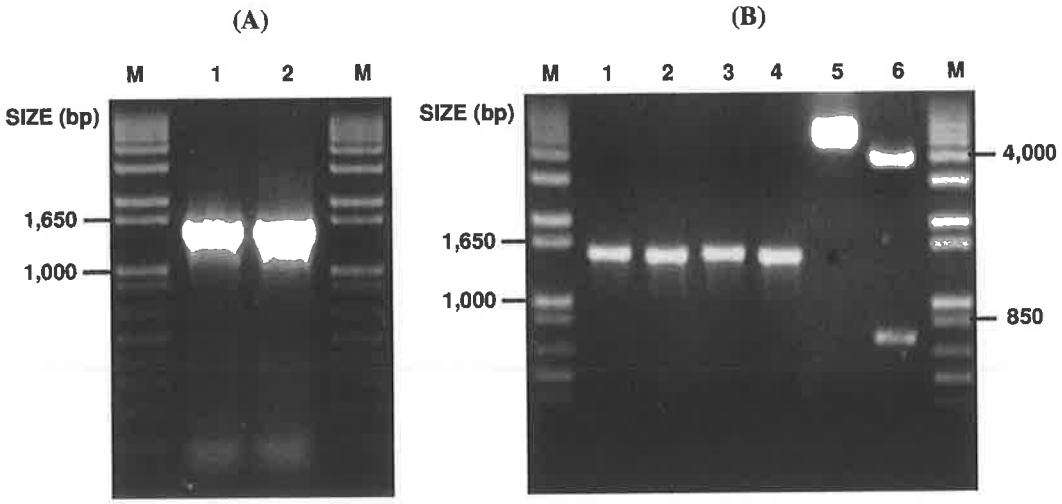


Figure 4.36. *(A) Amplification of Fab fragments. Lanes: (M) 1Kb plus DNA ladder (Invitrogen); (1) amplified D11 Fab DNA represented by a band at approximately 1,500bp; (2) amplified G6 Fab DNA represented by a band at approximately 1,500bp. (B) SfiI digestion of pAK400Bio vector containing the stuffer gene and Fab fragments. Lanes: (M) 1Kb plus DNA ladder (Invitrogen); (1) undigested D11 Fab; (2) SfiI digested D11 Fab; (3) undigested G6 Fab; (4) SfiI digested G6 Fab; (5) undigested pAK400Bio vector; (6) digested pAK400Bio vector (~4,000bp) with stuffer gene (~800bp) removed.*

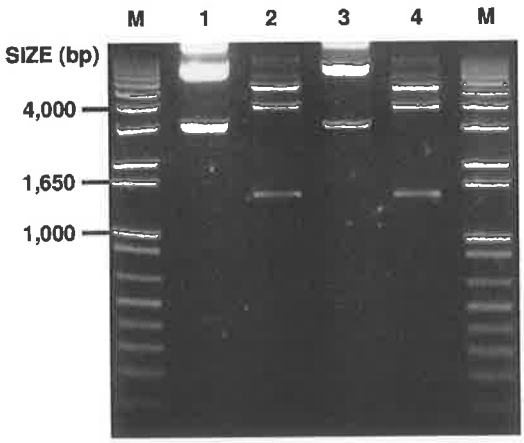


Figure 4.37. *SfiI digestion of pAK400Bio vector containing the AFB_1-specific Fab fragments. Lanes: (M) 1Kb plus DNA ladder (Invitrogen); (1) undigested pAK400Bio vector containing the anti-AFB_1 D11 Fab; (2) SfiI digested pAK400Bio. Linearised vector DNA (~5,500bp; cut only once) and double cut DNA (~4,000bp) with the AFB_1-specific D11 Fab fragment removed (~1,500bp). (3) undigested pAK400Bio vector containing the G6 Fab; (4) SfiI digested pAK400Bio. Linearised vector DNA (~5,500bp; cut only once) and double cut DNA (~4,000bp) with the anti-AFB_1 G6 Fab fragment removed (~1,500bp).*

4.2.8.3 Sequence analysis on AFB_1-specific recombinant antibodies

The pAK400Bio bearing the anti-AFB_1 Fab and scFv were isolated from AVB100 cells as described in *Section 2.3.1.1*. The plasmids were sent to Qiagen (Germany) Ltd for sequencing. Sequence analysis of the scFv was performed with the oligonucleotide primers, pAKfor and pAKback. The primers, kpel and pelseq, were used for sequence analyses of the Fab fragments. These primers are listed in *Table 2.2* (*Section 2.4.3.9.2*).

The nucleotide sequences were translated to amino acid sequences using Expasy translate tool and aligned using PBIL (Table, *Section 2.4.3.9*). *Figure 4.38* shows the alignment of the sequences of the scFv from pAK400 and the scFv-bio from pAK400Bio. The two sequences displayed 100% homology and confirmed the successful cloning of the entire scFv gene into the pAK400Bio vector. As illustrated in *Figure 4.38*, the $(His)_6$ tag encoded by the pAK400 vector (Krebber *et al.*, 1997) at the carboxy-terminal of the scFv gene sequence is replaced with the biotinylation acceptor peptide encoded by the pAK400Bio.

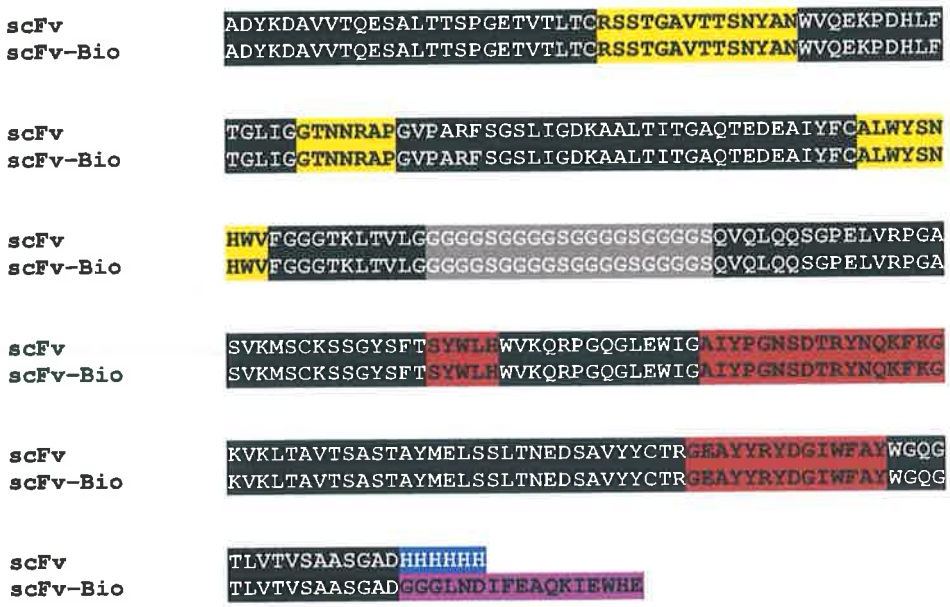


Figure 4.38. *The anti-AFB_1 scFv gene sequences from pAK400 (scFv) and pAK400Bio (scFv-bio). Nucleotide sequences were translated into amino acid sequences using the Expasy translate tool and the amino acid sequences aligned using the PBIL protein alignment tool (Section 2.4.3.9). The two sequences were found to be identical. The light and heavy chain complementarity determining regions (CDRs) were defined according to Kabat et al. (1991) and are highlighted in yellow and red, respectively. The variable regions are linked together by a flexible 20-amino acid peptide linker ($(Gly_4Ser)_4$),which is highlighted in grey. The C-terminal $(His)_6$ tag encoded by the pAK400 vector (highlighted in blue) is replaced with the biotinylation acceptor peptide (highlighted in pink) in the in vivo biotinylation vector, pAk400Bio.*

4.2.8.4 Production and purification of *in vivo* biotinylated antibodies

The scFv and Fab fragments were introduced into *E. coli* AVB100 cells (Avidity Inc.) by electroporation (*Section 2.4.4.4*) for subsequent expression of biotinylated antibody fragments. The *E. coli* AVB100 cells contain a chromosomal copy of the biotin holoenzyme synthesase (*bir*A) gene, allowing overexpression of the BirA protein by induction with L-arabinose. This *bir*A enzyme catalyses the site-specific biotinylation reaction of the lysine residue in the biotinylation acceptor domain encoded by the pAk400Bio vector. The AVB100 cells can be used with IPTG-inducible vectors (*i.e.* pAK400Bio vector), allowing independent control over the expressed antibody genes and the *bir*A levels.

The method for expression of *in vivo* biotinylated scFv described by Warren *et al.* (2005) was first used for biotinylated scFv production. However, the use of 2XTY broth rather than TB yielded poor expression of the scFv antibody (*Figure 4.39*). Therefore, for high levels of antibody expression, both Fab and scFv fragments were expressed in SB and TB media, as per *Section 2.4.3.10.1 and 2.4.3.10.2*, respectively, with the following amendments. Expression of biotin-tagged antibodies was induced with the addition of isopropyl β-D-thiogalactopyranoside (1mM) and L-arabinose (1.5μM) in the presence of D-biotin (100μM). Following overnight induction the cells were pelleted by centrifugation at 3,000*g* at 4°C for 30 minutes (Eppendorf 5810R). Each pellet was washed three times in PBS to remove free biotin and the antibodies were isolated from the host cell by sonication. The resulting cell lysates were analysed by ELISA in order to determine if the antibody fragments were successfully biotinylated during expression. The ELISA was performed as per *Section 2.4.3.11.1* with the following amendments. Plates were coated with 6.25μg/ml of the AFB_1-BSA conjugate and blocked with PBS containing 1% (w/v) BSA. Cell lysates, diluted 2 to 16-fold in PBS containing 0.1% (w/v) BSA, were then added to the coated and blocked microtitre plate wells. Bound biotinylated antibodies were detected following the addition of a 1/1,000 dilution of extravidin peroxidase (Sigma) and TMB substrate. Absorbances were read at 450nm on a Tecan Safire^{2TM} and the values plotted on bar charts, as illustrated in *Figure 4.40 - 4.42*.

The scFv displayed specific binding to AFB_1 and was detected with the HRP-labelled extravidin, indicating the functionality of the antibody was retained and it was

successfully biotinylated during expression. Minimal non-specific binding (NSB) was observed between the extravidin peroxidase conjugate and the blocking solution (*i.e.* BSA) or cell lysate. However, a much higher degree of NSB was observed between the extravidin label and the crude cell lysate preparations containing the G6 and D11 Fab fragments when analysed by ELISA (*Figure 4.41* and *4.42*). These results suggest that more stringent washing of the Fab antibody preparations was required to remove excess free biotin prior to sonication. In order to remove excess free biotin and reduce any interference caused by any contaminating proteins in subsequent assay development, cell lysates were applied to a monovalent avidin column for the purification of antibody fragments, as described in *Section 2.4.4.5.*

However, following purification only the scFv fragments could be detected using the extravidin peroxidase (*Figure 4.43*). The G6 and D11 fragments were not detected following purification, suggesting they were unsuccessfully biotinylated during expression or the biotin tag was not accessible.

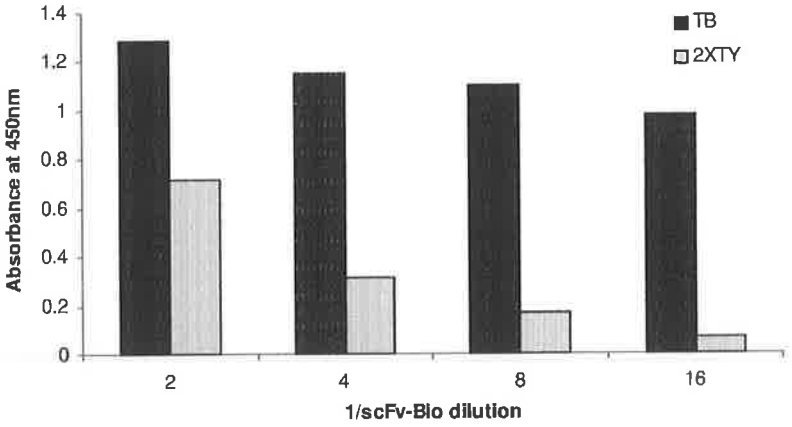


Figure 4.39. *Indirect ELISA for the detection of <u>in vivo</u> biotinylated scFv (scFv-Bio) following expression in TB and 2xTY media. Maxisorp™ plates (Nunc) were coated with 6.25µg/ml AFB_1-BSA and blocked with 1% (w/v) BSA. Cell lysates (containing antibody), diluted 2-16-fold were added to the plate in triplicate and bound scFv-Bio antibody was detected using extravidin peroxidase (Sigma). Increased levels of biotinylated scFv were observed following expression in TB media.*

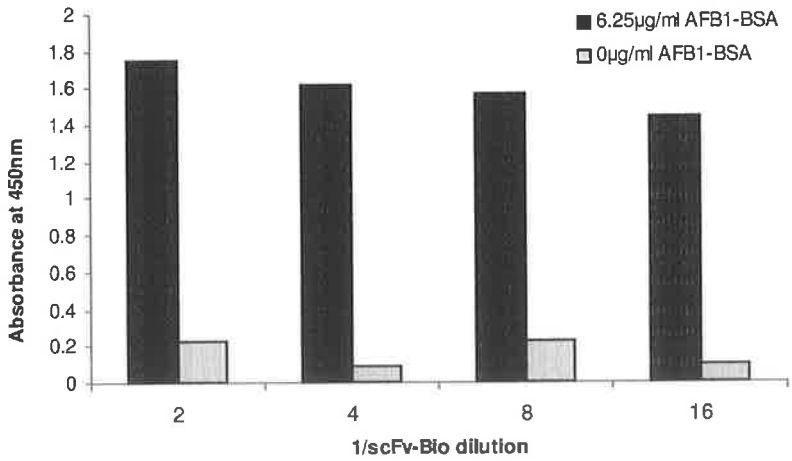


Figure 4.40. *Indirect ELISA for the detection of <u>in vivo</u> biotinylated scFv (scFv-Bio) following expression in TB media. Maxisorp™ plates (Nunc) were coated with 6.25µg/ml and 0µg/ml (control) AFB_1-BSA and blocked with 1% (w/v) BSA. Cell lysates (containing scFv antibody), diluted 2-16-fold were added to the plate in triplicate and bound scFv-Bio antibody was detected using extravidin peroxidase (Sigma). The <u>in vivo</u> biotinylated scFv were shown to bind AFB_1.*

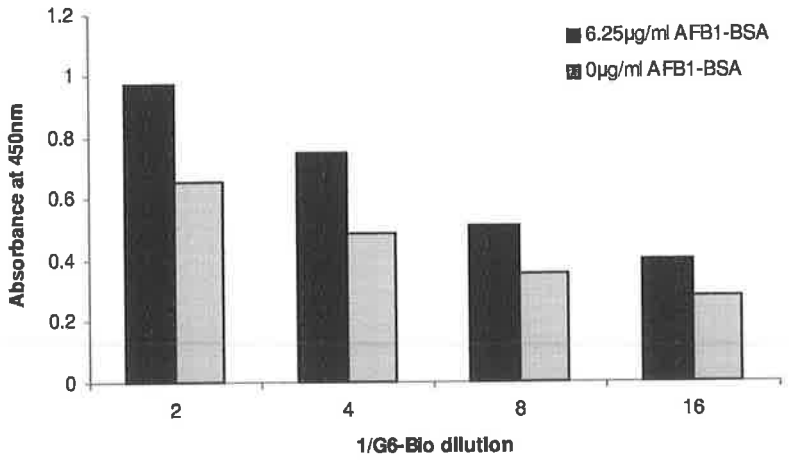


Figure 4.41. *Indirect ELISA for the detection of <u>in vivo</u> biotinylated G6 Fab (G6-Bio) following expression in SB media. Maxisorp™ plates (Nunc) were coated with 6.25µg/ml and 0µg/ml (control) AFB_1-BSA and blocked with 1% (w/v) BSA. Cell lysates (containing G6 Fab antibody), diluted 2-16-fold were added to the plate in triplicate and bound G6-Bio antibody was detected using extravidin peroxidase (Sigma). The <u>in vivo</u> biotinylated G6 Fab displayed binding to AFB_1-BSA conjugate but a high degree of non-specific binding was observed.*

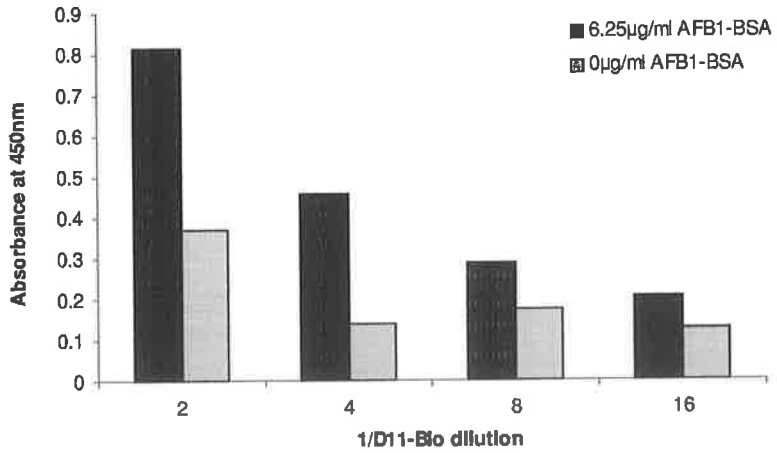


Figure 4.42. *Indirect ELISA for the detection of <u>in vivo</u> biotinylated D11 Fab (D11-Bio) following expression in SB media. Maxisorp™ plates (Nunc) were coated with 6.25µg/ml and 0µg/ml (control) AFB_1-BSA and blocked with 1% (w/v) BSA. Cell lysates (containing antibody), diluted 2-16-fold were added to the plate in triplicate and bound D11-Bio antibody was detected using extravidin peroxidase (Sigma). The <u>in vivo</u> biotinylated D11 Fab displayed binding to AFB_1-BSA conjugate but a high degree of non-specific binding was observed.*

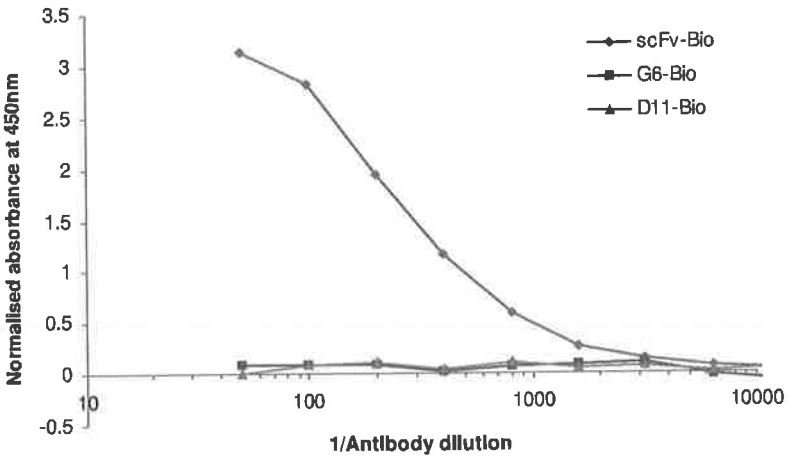


Figure 4.43. *Titres of the expressed scFv, G6 and D11 Fab fragments against 6.25μg/ml AFB_1-BSA following monomeric avidin-purification. The titre recorded for the biotinylated scFv was in excess of 1/100,000. The G6 and D11 Fab fragments were not detected following purification.*

4.2.8.5 Inhibition ELISA incorporating the *in vivo* biotinylated scFv

An inhibition ELISA was performed employing the biotinylated scFv to determine its range of detection for AFB_1 in buffer and to determine whether the biotinylation reaction would result in decreased assay performance. PBS containing 5% (v/v) methanol was spiked with known concentrations of AFB_1, ranging from 0.38 to 781ng/ml, as described in *Section 2.4.3.11.2,* and mixed with an equal volume of biotinylated scFv, at a dilution of approx 1/250. Samples were incubated for 30 minutes at room temperature to allow equilibration before they were added to plates coated with 12.5μg/ml AFB_1-BSA and blocked with PBS containing 1%(w/v) BSA.

To demonstrate the reproducibility of the assay both intra- and inter-variability studies were performed. Inter-day assay CV's employing the *in vivo* biotinylated scFv ranged from 2.08% to 12.28%, respectively, indicating adequate reproducibility. Percentage recovery values indicated reasonable accuracy, with majority of values within ±10%. CV's and percentage recovery values are shown in *Table 4.8.*

Normalised absorbance values (A/A_0) were plotted against the log of AFB_1 concentration to generate a calibration curves using BIAevaluation 4.0.1 software. *Figure 4.44* shows the inter-day calibration curve employing the biotinylated scFv antibody fragment, where the range of detection was found to be between 6 and 391ng/ml. The limit of detection (LOD) was determined by selecting the mean normalised response minus three standard deviations for the negative standard (no AFB_1). This value was found to be 3.4ng/ml.

The results of the inhibition ELISAs employing both the scFv and biotinylated scFv were compared in terms of sensitivity. The overlay of their respective calibration curves is shown in *Figure 4.45*. Absorbance values were normalised by expressing them as a percentage of B/B_0. The biotinylated scFv displayed a greater range of linear detection when compared to its non-biotinylated counterpart. The differences in direct detection (*i.e.* through use of extravidin peroxidase) and indirect detection (*i.e.* through use of a secondary antibody, anti-histidine-HRP) are represented in their respective assay curves (*Figure 4.45*). The assay, with an IC_{50} of 31ng/ml, displayed similar assay sensitivity with the assay developed with the non-biotinylated scFv (IC_{50} of 52ng/ml).

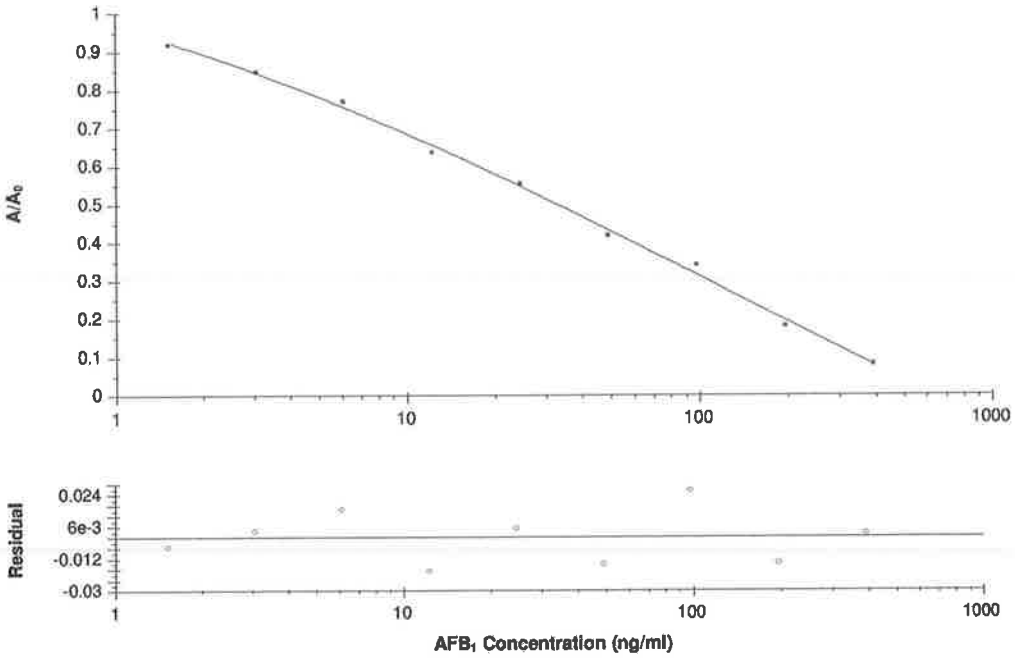


Figure 4.44. *Inter-day calibration curve for inhibition ELISA for the detection of AFB_1 in PBS using the biotinylated scFv fragment. AFB_1-BSA was coated at a concentration of 12.5µg/ml and antibody at a final dilution of 1/500 was used. Bound antibody was detected using an extravidin-peroxidase conjugate followed by addition of a TMB substrate. Data was normalised by dividing the mean absorbance obtained at each AFB_1 concentration (A) by the absorbance values determined in the presence of zero toxin (A_0). Normalised absorbance values (A/A_0) were plotted against the logarithm of aflatoxin B_1 concentration using BIAevaluation 4.0.1 software and a 4-parameter equation fitted to the data. All analyses were performed in triplicate on three separate occasions.*

Table 4.8. *Inter-day assay coefficient of variation (CVs) for the detection of AFB_1 employing the biotinylated scFv fragment. Each standard was analysed in triplicate over three different days and the CVs were calculated as the standard deviation (SD) expressed as a percentage of the mean value for each standard. Measured (i.e. back-calculated concentrations) were calculated from the four-parameter model produced using BIAevaluation 4.0.1 software and the percentage recovery expressed as a ratio between measured and added concentrations.*

Concentration (ng/ml)	**$A/A_0 \pm SD$**	**CV (%)**	**Back calculated concentration (ng/ml)**	**Recovery (%)**
390.50	0.083 ± 0.006	7.23	385.38	99
195.25	0.183 ± 0.013	7.10	212.39	109
97.63	0.342 ± 0.042	12.28	83.713	86
48.81	0.419 ± 0.019	4.53	53.33	109
24.40	0.553 ± 0.023	4.16	23.62	97
12.20	0.637 ± 0.048	7.54	13.84	113
6.10	0.771 ± 0.016	2.08	5.43	89
3.05	0.849 ± 0.076	8.95	2.96	87
1.53	0.919 ± 0.071	7.73	1.60	105

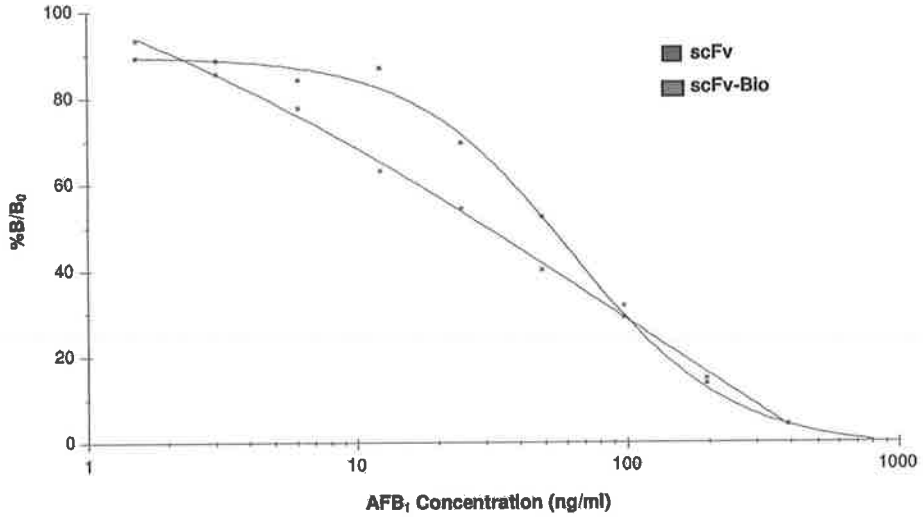


Figure 4.45. *Inhibition ELISA for the detection of AFB_1 in PBS using the scFv and biotinylated scFv antibody fragments. Normalised absorbance values (expressed as $\%B/B_0$) were plotted against the logarithm of AFB_1 concentration using BIAevaluation 4.0.1 software and a 4-parameter equation fitted to the data. The IC_{50} values for the scFv and scFv-Bio were found to be 52ng/ml and 31ng/ml, respectively.*

4.3 Discussion

In this chapter the library construction and selection of phage displaying-Fab antibodies derived from a parent scFv is presented. The purpose of the study was to examine the advantages of Fab fragments in terms of stability, sensitivity and specificity over smaller scFv fragments. A previously generated murine scFv specific for AFB_1 (Dunne *et al.*, 2005) was converted to a chimeric Fab format by the addition of human constant regions using a number of overlap extension polymerase chain reactions (PCRs), as described by Barbas *et al.* (2001). Electroporation of *E. coli* ER2738 cells with pComb3X (bearing the anti-AFB_1 Fab constructs) yielded a library size of 6.3 x 10^7 cells transformants and following three rounds of panning, two clones (D11 and G6) were isolated. These clones displayed specific hapten-binding activity when analysed by ELISA and their respective plasmids were thus transformed into *E. coli* Top10F' by electroporation to facilitate soluble expression of the antibodies and further analysis.

Sequence analysis of variable regions revealed that both the D11 Fab and parent scFv displayed 100% homology, as expected. However, a single point mutation was identified in the CDRH3 when comparing the G6 Fab variable regions with the corresponding scFv sequence. Therefore, to investigate whether the amino acid substitution would result in varied antigen binding ability, the functional characteristics of the G6 Fab fragment were examined (in parallel with the D11 Fab) by ELISA and Biacore and compared to the parent scFv.

The presence of functional, accessible peptide tags ((His_6) and HA) at the C-terminal of the antibody fragments was confirmed by Western blotting. Following SDS-PAGE analysis, a band at approximately 30kDa was observed for the scFv fragment, which corresponds with the size reported by Dunne (2004). SDS-PAGE analysis on the Fab fragments revealed two bands at approximately 25 to 30kDa representing the heavy and light chains of each Fab fragment following reduction of the disulfide bonds with β-mercaptoethanol prior to gel electrophoresis. When SDS-PAGE analysis was performed on the non-reduced (*i.e.* no addition of β-mercaptoethanol in the sample buffer) Fab fragments, bands at approx 45kDa were observed. This size corresponds

with the molecular weight of Fab fragments reported in published research (Better *et al.*, 1988; Kramer *et al.*, 2002; Röthlisberger *et al.*, 2005; Townsend *et al.*, 2006).

The use of the chimeric system allows a compromise between high expression levels of humanised antibodies and the low levels observed for murine scFv fragments, while avoiding loss of antigen binding activity of the variable regions. The potential stability benefits of converting an scFv to a Fab format and both the influence of the additional constant regions and the presence of the interchain disulphide bond in the Fab fragment was previously highlighted in published research. The presence of human constant regions was shown to increase the expression yields of resulting Fab fragments in *E. coli* expression systems (Carter *et al.*, 1992; Ulrich *et al.*, 1995) and it was suggested that chimeric Fabs with human constant regions lead to higher expression in *E. coli* than murine antibody fragments (Barbas *et al.*, 2001). Röthlisberger *et al.* (2005) found that the constant regions allow stabilisation of variable domains with weak or medium stability. However, in the case of very stable variable domains, the addition of disulphide-linked constant domains yields a Fab fragment with similar stability to its scFv counterpart. In this chapter the influence of additional human constant regions on the bacterial expression of murine immunoglobulin variable domains was assessed. The yields of both scFv and Fab fragments were found to be equivalent after optimisation of expression conditions for both antibody constructs and milligram quantities of functional antibodies were produced in standard lab shake-flask cultures. These findings suggest that different constructs require different expression conditions (*i.e.* different expression media, expression temperature and length of time following induction) and conditions must be optimised accordingly.

Following successful expression and purification of anti-AFB_1 antibody fragments, they were applied to the development of several immunoassay formats, including ELISA and Biacore-based assays. Their assay performance was investigated and compared to the parent scFv in terms of sensitivity and specificity.

ELISA conditions were optimised to offer maximum assay sensitivity. The D11 Fab antibody was found to have a range of detection of 6 to 391ng/ml (IC_{50} value of 43ng/ml) and a limit of detection of 4.1ng/ml for AFB_1. When compared to the parent

scFv, the D11 displayed similar sensitivity taking into account experimental error. The scFv exhibited a range of detection of 12 to 781ng/ml (IC_{50} of 52ng/ml) for AFB_1. However, the G6 Fab demonstrated a 3 to 3.5-fold increase in assay sensitivity when compared to both the D11 Fab and scFv, respectively. The G6 Fab antibody fragment displayed a range of detection between 0.76 and 391ng/ml (IC_{50} value of 14.8ng/ml) for AFB_1 with a limit of detection of 1.3ng/ml for AFB_1. All ELISA-based assays proved to be reasonably accurate with the majority of percentage recovery values within ±10%. Assay variability studies also demonstrated acceptable reproducibility of the Fab-based ELISAs. Inter-assay CV's ranged from 0.63 to 8.25% and 2.62 to 5.58%, for the G6 and D11 Fab fragments, respectively. These results, in terms of assay precision, were found to be comparable to the ELISA employing the AFB_1-specific scFv developed by Dunne (2004). The author reported ELISA inter-assay CVs of below 11.35% for the scFv fragment. However, in this study the scFv was found to be more sensitive in an ELISA format than that reported by Dunne (2004). The author reported a similar range of detection (12-781ng/ml for AFB_1) for the scFv-based ELISA. However, an IC_{50} of 150ng/ml was determined for AFB_1 in the scFv-based ELISA, representing a 3-fold decrease in sensitivity observed in this study (IC_{50} of 52ng/ml). This increase in sensitivity was possibly due to use of purified antibody (over a crude lysate preparation used by Dunne (2004)) and the use of a different secondary antibody (*i.e.* anti-his-HRP as opposed to a mouse anti-FLAG antibody which required a tertiary HRP-labelled anti-mouse antibody for detection).

Comparison of cross-reactivities with structurally related aflatoxins revealed that both the D11 Fab and parent scFv exhibited no significant difference, indicating that the functional characteristics of the scFv were retained in the Fab construct. Cross-reactivity studies carried out on the G6 Fab fragment revealed the antibody offered greater specificity towards B_1, in comparison to the D11 Fab and scFv. The G6 antibody displayed lower levels of cross-reactivity towards B_2 and G_2, with % CR_{50} of 1.9 and 0.93, respectively. A higher level of cross-reactivity against aflatoxin G_1 (14.8%) and minimal cross-reactivity with aflatoxin M_1 (<0.01%) was observed.

The Fab fragments were also applied to the Biacore™ 3000 instrument for development of assays for the detection of AFB_1. Problems, such as regeneration, have been encountered with the use of hapten-protein immobilised sensor surfaces

(Daly *et al.*, 2000) and directly immobilised hapten surfaces have often resulted in increased assay sensitivity and reduction of non-specific interactions (Darmanin Sheehan *et al.*, 2006). Therefore, Biacore inhibition assay development was performed on a CM5 chip directly immobilised with an AFB_1 derivative (kindly donated by Xenosense Ltd., Belfast). All anti-AFB_1 recombinant antibodies displayed non-specific binding (NSB) to the CM dextran surface, eliminating the need for pre-incubation steps with CM dextran salt. The immobilised surface was also found to be quite stable facilitating fifty binding-regeneration cycles to be performed before a decrease of 8% and 12% in binding capacity for the G6 and D11 Fab antibodies, respectively, was observed. It also should be noted that all Biacore assay development was performed on a single chip, which allowed for over 600 regenerations to be successfully carried out on each flow cell without significant loss of binding activity, making it suitable surface for high throughput sample analysis.

The results of the inhibition Biacore assays employing the G6 and D11 Fab antibody fragments were compared to the parent scFv in terms of assay sensitivity. The assay employing the G6 Fab antibody was found to have a range of detection between 0.095 and 24.4ng/ml (IC_{50} value of 2.2ng/ml) and a limit of detection of 0.12ng/ml for AFB_1. The D11 Fab fragment with a range of detection of 0.19 to 48.8ng/ml (IC_{50} value of 1.6ng/ml) and limit of detection determined as 0.29ng/ml for AFB_1. The scFv displayed similar sensitivity (IC_{50} value of 2.4ng/ml) to both Fab fragments within experimental error. The range of detection for the scFv was found to be 0.38-24.4ng/ml. A similar range of detection (0.375 to 12ng/ml) for the scFv was reported by Dunne *et al.* (2005). Although no significant increases were observed with the Biacore-based Fab assays comparative to the scFv, the assay employing the G6 Fab proved more reproducible with inter-day assay CV's below 3.5%. Inter-assay CV's for the D11 Fab-based assay were found to be up to 8.96%.

Finally, the advantages of an *in vivo* biotinylation strategy were highlighted. The three antibody fragments were subcloned into the pAK400Bio vector for the subsequent expression of *in vivo* biotinylated antibodies in *E. coli* AVB100 cells (Avidity Inc.). This system allows the coexpression of the target antibody linked to a 17-amino acid C-terminal biotin acceptor domain, and the enzyme biotin holoenzyme synthesase, BirA. The lysine residue in the biotin receptor domain is site-specifically biotinylated

during expression in *E. coli* in the presence of free biotin through a reaction catalysed by the biotinylating enzyme (BirA). The technique allows each antibody fragment to be labelled with only one biotin molecule distal of the antigen-binding site. The method is advantageous over random biotinylation by conventional *in vitro* chemical means, which can lead to the obstruction of the antigen-binding site. The scFv was successfully biotinylated *in vivo* during expression and applied to an ELISA format to assess whether biotinylation had any adverse affects on assay performance. The assay employing the biotinylated scFv enabled assay sensitivity comparable to that of its non-biotinylated counterpart. The biotinylated scFv-based assay also exhibited greater linearity in response. The assay demonstrated almost a two-fold increase in sensitivity compared to the assay developed employing an anti-histidine secondary antibody and facilitated direct detection with an extravidin conjugate, with a reduction assay time and cost. It could also allow for future detection with other highly sensitive extravidin/streptavidin labels (*e.g.* fluorophores, nano-particles etc.), which could possibly lead to further increases in assay sensitivity.

This biotinylated scFv has the potential to be very useful in other applications (*e.g.* arrays and lateral-flow immunoassays) as the biotin tag could facilitate immobilisation on avidin surfaces in an orientated manner. Immobilisation of antibodies onto chip surfaces is one of the major challenges in the development of antibody microarrays. Non-specific adsorption or covalent coupling of antibodies onto solid supports can lead to denaturation and hence, loss of functionality. The production of biotinylated Fab fragments was unsuccessful. This suggests that further optimisation of conditions for coexpression of the BirA enzyme and the Fab fragments in the *E. coli* strain AVB100 is required. Successful biotinylation of only 3-15% of the total Fab products expressed in *E. coli* has been reported by Weiss *et al.* (1994), indicating the importance of the optimisation of expression conditions for satisfactory yields. The other possibility that fragments were not detected with the extravidin conjugate is that the biotin tag was not accessible following folding of the Fab construct. The incorporation of a flexible linker (*i.e.* $(Gly_4Ser)_4$) at the junction of the Fab sequence and the biotin tag could overcome this problem.

In summary, in terms of sensitivity and specificity, the D11 Fab fragment and scFv yielded the similar results within experimental error in an ELISA-based assay. The

mutant G6 Fab fragment displayed increased sensitivity (~3.5 fold) in ELISA sensitivity when compared to the parent scFv and reduced cross-reactivity with AFB_2 and AFG_2 molecules. When the three antibodies were applied to the development of a Biacore inhibition assay, similar sensitivities were obtained for each antibody. However, it should be noted that the SPR-based biosensor enabled a 22 to 27-fold increase in sensitivity over the ELISA assay employing the D11 Fab and scFv fragments, respectively. A 7-fold increase in assay sensitivity was observed for the G6 Fab-based Biacore assay over the ELISA. These results highlight the advantages of the use of the SPR-based biosensor over the conventional ELISA assay. In terms of stability, the Fab fragments were found to be more stable than the scFv. Over the course of experimental analysis the Fab fragments could be stored at 4°C for longer periods of time than the scFv with no significant effects on binding activity observed. Similar results were also observed by Kramer *et al.* (2002).

Finally, the G6 Fab fragment would prove useful in a range of future applications. The antibody enabled the development of an accurate and highly reproducible Biacore inhibition assay for the detection of AFB_1. Cross-reactivity studies also indicated the G6 Fab had a high level of specificity for AFB_1. It also provided excellent sensitivity (LOD = 117pg/ml for AFB_1) and could detect AFB_1 in buffer below the current EU maximum limits (with the exception of limits set for infants at 0.1ng/ml). This assay sensitivity compares favourably with the published literature. Other immunoassays including an ELISA format developed by Candlish *et al.* (1985) and a dipstick assay developed by Aldao *et al.* (1995) reported limit of detection of 0.2ng/ml and 0.25ng/g for AFB_1. The sensitivity also compares favourably with other Biacore assays developed. Daly *et al.* (2002) reported a Biacore assay with limits of detection at 3 and 0.75ng/ml for AFB_1 in spiked PBS and grain, respectively. Van der Gaag *et al.* (2003) employed a monoclonal antibody in a Biacore-based assay was reported to have a detection limit of 0.2ng/g for AFB_1 in spiked grain samples. The recombinant G6 Fab antibody could also be further engineered to improve sensitivity and to generate future novel functionalities.

Chapter 5

Development of biosensor-based assays for the detection of ampicillin in milk

5.1 Introduction

5.1.1 The beta-lactam antibiotics

The antibacterial effect of penicillins was first discovered in 1928 by Alexander Fleming. The original natural penicillins obtained from *Penicillium notatum* or the closely related species *P. chrysogenum* were produced by fermentation, and were often mixtures of various β-lactams such as penicillins G and V. These penicillins were only active against Gram-positive bacteria and not against Gram-negative species. However, it was later discovered that the natural penicillins could be modified chemically by removing the acyl group to leave 6-aminopenicillanic acid (6-APA). With the availability of 6-APA, hundreds of synthetic and semi-synthetic penicillins have been created. These antibiotics offer a number of advantages, including resistance to degradation by stomach acids so can be taken orally and an extended range of activity against some Gram-negative bacteria.

The β-lactam group comprises of the penicillin, cephalosporin, penem, carbapenem and monobactam antibiotics. The group share a four-membered ring, which makes the molecule thermodynamically unstable, and is easily hydrolysed in the presence of acid. In addition to chemical means of degradation, many bacteria produce a group of enzymes, known as penicillinases that are designed to degrade and inactivate β-lactam antibiotics. *Figure 5.1* illustrates the chemical and enzymatic hydrolysis of penicillins. Opening of the β-lactam ring by enzymatic or basic hydrolysis produces an inactive penicilloic acid, whereas acid hydrolysis results in the inactive derivative, penicillenic acid (Gaudin *et al.*, 2001).

5.1.2 Mechanism of antimicrobial activity

Beta-lactam antibiotics exhibit their antibacterial effect by binding to and inactivating membrane bound enzymes, referred to as penicillin binding proteins (PBPs) (Cacciatore *et al.*, 2004). The cell wall of Gram-positive bacteria consists of peptidoglycans, a network of *N*-acetylmuramyl and *N*-acetylglusosaminyl residues that are cross-linked by short peptides. The transpeptidase enzymes (PBPs) are involved in the cross-linking of the small peptide chains in the peptidoglycan cell wall layer of growing bacterial cells (Park and Strominger, 1957). β-lactams bind to these

enzymes, inhibiting bacterial wall synthesis and thus, cause structural weakness of the cell wall and ultimately cell death (Miller, 2002).

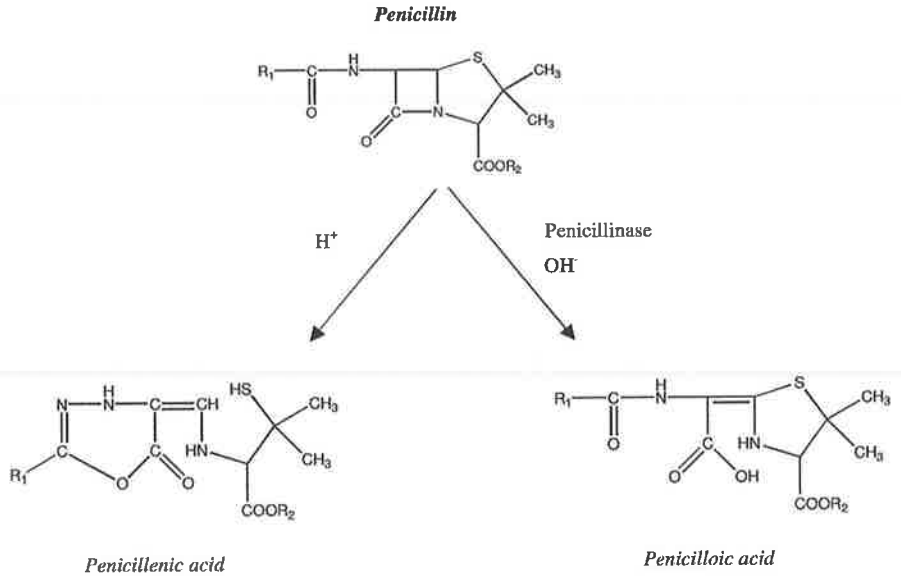


Figure 5.1. *Chemical and enzymatic hydrolysis of penicillins. Opening of the β-lactam ring by enzymatic or basic hydrolysis produces an inactive penicilloic acid, while acid hydrolysis results in the inactive derivative, penicillenic acid (adapted from Gaudin et al., 2001).*

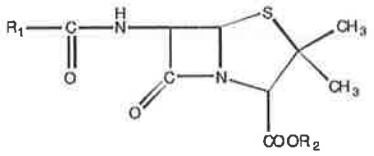
5.1.3 Problems associated with antibiotic residues in milk

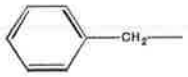
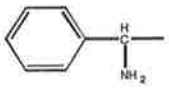
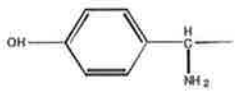
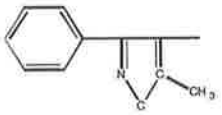
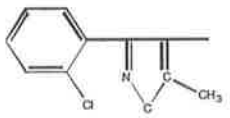
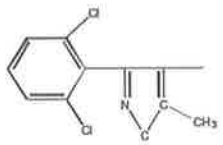
Beta-lactam antibiotics are the most commonly used antimicrobials for the treatment of bacterial infections in dairy cows, such as mastitis, and thus, are the most common antimicrobial residue found in milk (Setford *et al.*, 1999). The presence of these residues in milk can result from overuse or the lack of sufficient withdrawal-time elapsing following administration. Antibiotic contamination in milk or milk products can pose serious economical, environmental and health problems. Milk and its associated food products are of critical importance to Irish industry generating in excess of €2.5 billion in exports every year. Thus, contamination can cause major economic losses for food producers, processors and industries if large quantities of milk require disposal. The presence of antibiotic residues can also cause delays in the

production of fermented milk products, by inhibiting starter cultures, and flavours may also be affected. Finally, there may be major implications for consumers' health, including the development of resistant strains of microorganisms and allergic reactions. To ensure that food of animal origin is free from antibiotic residues, the EU has set maximum residues limits (MRLs) for each of these veterinary drugs, where MRL is defined as "the maximum concentration of residues resulting from the use of a veterinary medicinal product, which may be legally permitted or recognised as acceptable on or on a food" (FSAI, 2002). The MRLs for some of the penicillins are outlined in *Table 1* (2377/90/EC).

Therefore, to comply with EU council regulations and ensure the quality of milk and milk products, a sensitive, rapid, specific assay capable of detecting antibiotic residues in diverse sample matrices is essential.

Table 5.1. *The chemical structure of some of the penicillin antibiotics and their relevant EU maximum residue limits (2377/90/EC). Penicillins share a common 6-APA structure, consisting of a β-lactam ring coupled to a five-membered thiazolidine ring. Variability in the structure, caused by two side chain groups (R_1 and R_2), alters stability, adsorption, allergenicity and spectrum of activity.*



Compound	R_1	R_2	MRL (µg/kg)
Penicillin G		H	4
Ampicillin		H	4
Amoxicillin		H	4
Oxacillin		H	4
Cloxacillin		H	4
Dicloxacillin		H	4

5.1.4 Current methods of detection for antibiotics in milk

Microbial inhibition tests, based on the growth of microorganisms in the presence of antibiotics, were most commonly used for the detection of ampicillin residues in meat and milk products. The Delvotest® SP kit (Gist-brocade/DSM) developed in the Netherlands was initially the only test listed in the Joint Committee of Practice for the assessment of milk quality. It is currently the standard test method used for the detection of antibiotics residues in milk at pasteurizing plants throughout Ireland (FSAI, 2002). This microbial inhibitor test, dependent on the growth and acid production of *Bacillus stearothermophilus* subsp. *calidolactis*, is particularly sensitive to penicillin G with a limit of detection of 2μg/kg in milk. Although the method has numerous advantages including sensitivity, reliability and is cost effective, the test requires an incubation time of two and a half hours before a result is available.

Other methods including high performance liquid chromatography (HPLC) and mass-spectrometry (MS)-based systems have also been developed for the detection of β-lactams. Although these detection systems are suitable for confirmation and possible quantification, they are time consuming, require highly specialised equipment and are very expensive.

A number of rapid assays for the detection of β-lactam antibiotics now exist (FSAI, 2002). These include a number of commercially available receptor protein-based assays, including the Beta s.t.a.r. (UCB Bioproducts), Delvo-X-Press® βL-II (Gist-brocades/DSM) and SNAP β-lactam (IDEXX Laboratories Inc.) test kits, where a result can be obtained in less than ten minutes. These methods exploit the specific binding of β-lactam residues to β-lactam receptor binding proteins, isolated from bacterial cell walls. Setford *et al.* (1999) also employed a penicillin-binding protein (PBP) in a rapid method for quantification of β-lactam residues in milk, using disposable, electrochemical sensor devices. Quantification was obtained through competitive binding between sample penicillin G and 7-aminocephalosporanic acid – glucose oxidase (7-ACA-Gox) conjugate for penicillin binding protein sites immobilised on the electrode surface. The assay is reported to be capable of distinguishing between milk samples containing 0 and 5μg/kg penicillin G.

A number of immunoassay-based tests have also been developed as an alternative to these techniques. The Parallux™ β-lactam (IDEXX Laboratories Inc.) detection system, a capillary-based solid-phase fluorescent immunoassay test takes approximately four minutes to complete and can detect a number of β-lactam antibiotics in bovine milk below their respective MRL. The ELISA-based Fluorophos BetaScreen® test (Advanced Instruments Inc.) can detect the presence of amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin and penicillin G in raw milk at 10, 3, 20, 30, 20 and 1μg/kg, respectively, according to the manufacturer. The test kit consists of antibody-coated test tubes to which raw milk and a β-lactam-alkaline phosphatase conjugate are added. A β-lactam standard is also included, which can be applied to one of the test tubes instead of milk. A maximum of five samples and the β-lactam standard can be run simultaneously. After addition of substrate solution, the resulting colour change is measured, compared to that of the β-lactam standard and the concentration of antibiotic residues in the milk can be determined. In 1998 this commercially available test was evaluated and compared with the Delvotest® SP by Sternesjö and Johnsson. A large number of raw milk samples (5,061) were randomly selected and analysed by the Fluorophos BetaScreen® and the Delvotest® SP. The sensitivity of the BetaScreen® for penicillin G was 1.8μg/kg, which was lower than that claimed by the manufacturer. This could be due to a difference in the method in which the limit of detection was determined, a major problem often encountered in assay development. In total, 23 samples (0.45%) were found to be positive using the BetaScreen test, compared to 13 samples (0.26%) found using the Delvotest SP.

Zhi *et al.* (2001) described an automated flow-through amperometric immunoanalysis system for the quantitation of cephalexin with a reported detection limit of 1μg/l in milk. A column reactor containing protein G-coated porous polymer beads was used as the affinity matrix for antibody immobilisation. Cephalexin-alkaline phosphatase conjugate and spiked milk sample were pumped into the immunoreactor, where they competed for binding to the immobilised antibody. Following the addition of ρ-aminophenyl phosphate substrate solution, ρ-aminophenol generated from the enzymatic reaction was detected amperometrically. Antibody-conjugated complexes were dissociated from the protein G matrix using a regeneration buffer. The method was both rapid, with the whole procedure including regeneration taking

approximately 16 minutes and reproducible, with a mean inter-assay variation of 10.9%.

Usleber *et al.* (2000) produced a number of polyclonal antibodies against penicillin, including antibodies with group specificity, antibodies with limited sub-group specificity for isoxazolyl penicillins and antibodies with specificity for penicillin G degradation products. The most sensitive antibody produced was used in the development of an ELISA and detection limits for penicillin G in the range of 2-3 ng/ml were achieved.

Cliquet *et al.* (2001) generated monoclonal antibodies specific for the common 6-APA core structure of penicillins for use in an ELISA. A number of ampicillin conjugates were designed for immunisation and various coupling strategies were employed. Two methods using a cross-linker (glutaraldehyde or a succinimide ester), one carbodiimide-mediated coupling method and a method without any cross-linker or mediator molecule (physiological binding) were all compared. Immunisations with carbodiimide-mediated ampicillin conjugates induced a specific response to ampicillin in the animal, while, only a moderate antibody response against ampicillin was observed after immunisation with conjugates prepared with cross-linking reagents. A high antibody response was obtained against the physiological ampicillin-protein conjugates. However, natural conjugation results in the opening of the β-lactam ring structure and hence, loss of antimicrobial activity. This is a major limitation associated with assay development as only the active form is covered by legislation. However, specific antibodies against intact ampicillin and other penicillins have been raised (Dietrich *et al.*, 1998; Burmester *et al.*, 2001).

The commercially available surface plasmon resonance (SPR)-based sensors, developed by Biacore™, have also proven to be useful tools for rapid and automated screening of drug residues in various matrices. Sternesjö *et al.* (1995) first demonstrated the use of Biacore™ for the detection of the sulphamethazine in milk and since then the Biacore™ system has been applied for the detection of other antibiotics (Mellgren *et al.*, 1998; Gaudin *et al.*, 2001; Fergsuon, 2002; Gustavsson *et al.* 2002 and Cacciatore *et al.*, 2004).

Gustavsson *et al.* (2002) employed the biosensor for the development of a receptor protein-based inhibition assay to detect β-lactam antibiotics in milk. The Biacore-based assay is based on the determination of the enzymatic activity of a microbial receptor protein. The milk sample is mixed with a 3-peptide and a carboxypeptidase from *Streptomyces* R39. During incubation R39 hydrolyses the 3-peptide into a 2-peptide. However, in the presence of β-lactams the enzymatic activity of R39 is inhibited and less 2-peptide is produced. The amount of 2-peptide, which is proportional to the amount of β-lactams present in the milk sample, can then be detected by an anti-2-peptide antibody. The limit of detection of the assay for penicillin G was determined as 2.6μg/kg and coefficients of variation ranged between 7 and 16% on three different days.

Antibodies previously produced against the hydrolysed form of ampicillin have been applied to the development of a biosensor-based immunoassay for detection of residues in milk (Gaudin *et al.*, 2001). The SPR biosensor, Biacore X and a commercially available anti-ampicillin monoclonal antibody, which had a much higher affinity for the open β-lactam ring than the closed ring, were used for the development of the assay. This paper reports the comparison of enzymatic antibiotic sample pre-treatment with penicillinase, and chemical pre-treatment using a basic solution of 0.1M NaOH. The resultant assay had a high specificity for all penicillins but could not detect cephalosporins. Secondly, the sensitivity of the assay was not satisfactory, as it was unable to detect ampicillin and amoxicillin below their maximum residue limits (MRLs). Finally, the major limitation of the assay was it detected the inactive β-lactam structure and only the active form is covered by legislation. The development of a Biacore-based assay for the detection of the intact β-lactam antibiotic structure poses a major problem. The direct immobilisation of ampicillin onto the surface of a sensor chip can result in hydrolysis of the β-lactam ring structure and thus, the inactivation of the antibiotic. Consequently, antibodies specific to the closed β-lactam ring would not recognise the immobilised antigen surface. A solution, as discussed by the authors, would be to work on the stabilisation of the β-lactam ring structure during and after immobilization to prevent hydrolysis. Selectivity could also be improved by mixing two antibodies, *i.e.* anti-penicillin and anti-cephalosporin antibodies, to develop a β-lactam assay, or mix two anti-penicillin antibodies with different specificities to improve the detection level for all penicillins.

5.1.5 Chapter outline

The chapter focuses on the generation of ampicillin-specific polyclonal antibodies and their subsequent use in biosensor-based assays. Ampicillin was coupled to bovine serum albumin (BSA) and the resulting conjugate used to immunise a rabbit for the production of polyclonal antibodies. Antibodies specific to ampicillin were purified from the rabbit sera and employed in the development of immunoassays for the detection of ampicillin. The performance of the antibodies was first assessed by ELISA and then employed in a Biacore inhibition assay for the detection of ampicillin in processed milk. Finally, the applicability of a novel biochip sensor system was investigated.

Naïve and immune phage display libraries were also screened against a number of ampicillin conjugates for the selection of an ampicillin-specific single chain variable fragment (scFv).

5.2 Results

5.2.1 Production and characterisation of ampicillin-protein conjugates

In contrast to the expense and expertise required for traditional hybridoma and recombinant antibody production, polyclonal antibodies can be raised quickly and at relative low cost. The first steps in polyclonal antibody production are to select the target molecule, identify the appropriate groups for conjugation and then design an appropriate hapten-carrier conjugate for immunisation. Due to its similarity in structure to penicillin, and the availability of reactive functional groups for conjugation to proteins, ampicillin was chosen as the antigen of interest for immunisation (*Figure 5.2*). The hapten, with a molecular weight of only 349.4Da, is too small to induce an immune response and therefore, required conjugation to a carrier protein to render it immunogenic.

The choice of both the site of coupling and the conjugation method are imperative in antibody generation, as the immunogen will ultimately determine the sensitivity and selectivity of the resulting antibodies. Although a variety of coupling chemistries exist, the available functional groups on the hapten generally govern the choice of method. Ampicillin has two major functional groups, a primary amine and a carboxyl group; therefore carbodiimide coupling was chosen. The EDC (*N*-ethyl-*N*-(dimethyl-aminopropyl) carbodiimide hydrochloride) mediated method is simple with a reaction time of less than two hours and the zero-length cross-linking method eliminates the problem of antibody recognition to the linker between the hapten and protein carrier.

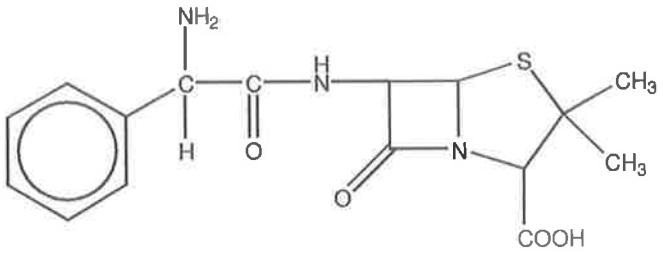


Figure 5.2. *The structure of ampicillin, which contains a primary amine group and a carboxyl group.*

5.2.1.1 Carbodiimide-mediated hapten-carrier conjugation

Carbodiimide coupling chemistry was used to conjugate ampicillin to various carrier proteins, *i.e.* bovine serum albumin (BSA), ovalbumin (OVA) and thyroglobulin (THY). Both the primary amine and carboxylic acid groups available on ampicillin were used for coupling, as described in *Section 2.5.1.1* and *2.5.1.2*. The carboxylic acid on the drug was activated and subsequently allowed to react with amine groups on the protein, as illustrated in *Figure 5.3 (B)*. The resulting immunogen had the potential to generate ampicillin-specific antibodies. However, it was reported that coupling through the carboxyl group can result in an immunogen similar to the hydrolysed form of ampicillin and can therefore yield antisera specific to the open β-lactam ring structure (Grubelnik *et al.*, 2001). Other conjugates were also produced that had the capability of generating antibodies against the common penicillin group. This was achieved using the same chemistry to couple the hapten through the primary amine group to carboxylic acid groups on the protein, as illustrated in *Figure 5.3 (A)*. This method allows conjugation distal of the β-lactam ring and utilises buffers with pH values near neutral to prevent hydrolysis.

A number of methods were employed in an attempt to characterise the ampicillin-protein conjugates. A Bicinchonic acid (BCA) protein assay reagent kit (Pierce) was first used to determine the final protein concentration of each conjugate, as described in *Section 2.5.1.3*. UV-spectroscopy was also used in an attempt to analyse the drug-protein conjugates. By comparing the UV spectra obtained for the conjugates and for the unconjugated protein and drug molecules, it is possible to see whether the coupling procedure was successful. However, the broad absorbance peak of ampicillin masked the protein maximum absorbance peak at 280nm, and thus, the method could not confirm successful conjugation.

Characterisation of conjugates was also indirectly carried out by immunisation followed by the characterisation of the specific antibody produced in serum. Balb/c mice were immunised with hapten-protein conjugates, as described in *Section 2.5.2.1*, to elicit an immune response. Blood samples were collected 10 days after immunisation and the specific antibody titre estimated by ELISA, as described in *Section 2.5.2.2*. The results shown in *Figure 5.4* indicate that the ampicillin-BSA

conjugate, produced by ampicillin activated coupling, induced a high antibody response to ampicillin in the mouse.

A competition assay was also carried out using these antibodies to determine the specificity of the antibodies to ampicillin. A competition ELISA was carried out as described in *Section 2.5.2.4*. Standards were prepared in PBS, ranging from 390 to 25,000ng/ml, from both an untreated ampicillin stock (intact molecule) and from ampicillin that had been enzymatically pre-treated with penicillinase, as described in *Section 2.5.2.5*. Both sets of standards were added to the wells of an immunoplate with a 1 in 4,000 dilution of mouse antiserum, at a ratio of 1:1 and absorbance results plotted against the logarithm of ampicillin concentration. Results shown in *Figure 5.5* indicated that as the concentration of ampicillin increased in solution, the absorbance decreased confirming that free drug displaces antibody. The presence of specific antibodies to ampicillin in mouse serum indicated the successful coupling of drug to protein. As previously discussed, the literature has reported that penicillins present a specific difficulty because antibodies can be produced against the open β-lactam ring structure if a coupling methods result in hydrolysis of the antibiotic. Only antibodies specific for the closed β-lactam structure are valuable for the development of an assay since only the active form is covered by legislation. Although polyclonal antiserum contains a mixture of antibodies with varying affinities directed for different epitopes of the immunogen, it was observed that the majority of antibodies being produced recognised the intact ring structure of the drug, as illustrated in *Figure 5.5*.

Other coupling methods, including glutaraldehyde coupling, were used to conjugate ampicillin to various proteins. However, the conjugate derived from coupling the primary amine of ampicillin to carboxylic groups of BSA induced the highest specific immune response and was thus used for subsequent polyclonal antibody production.

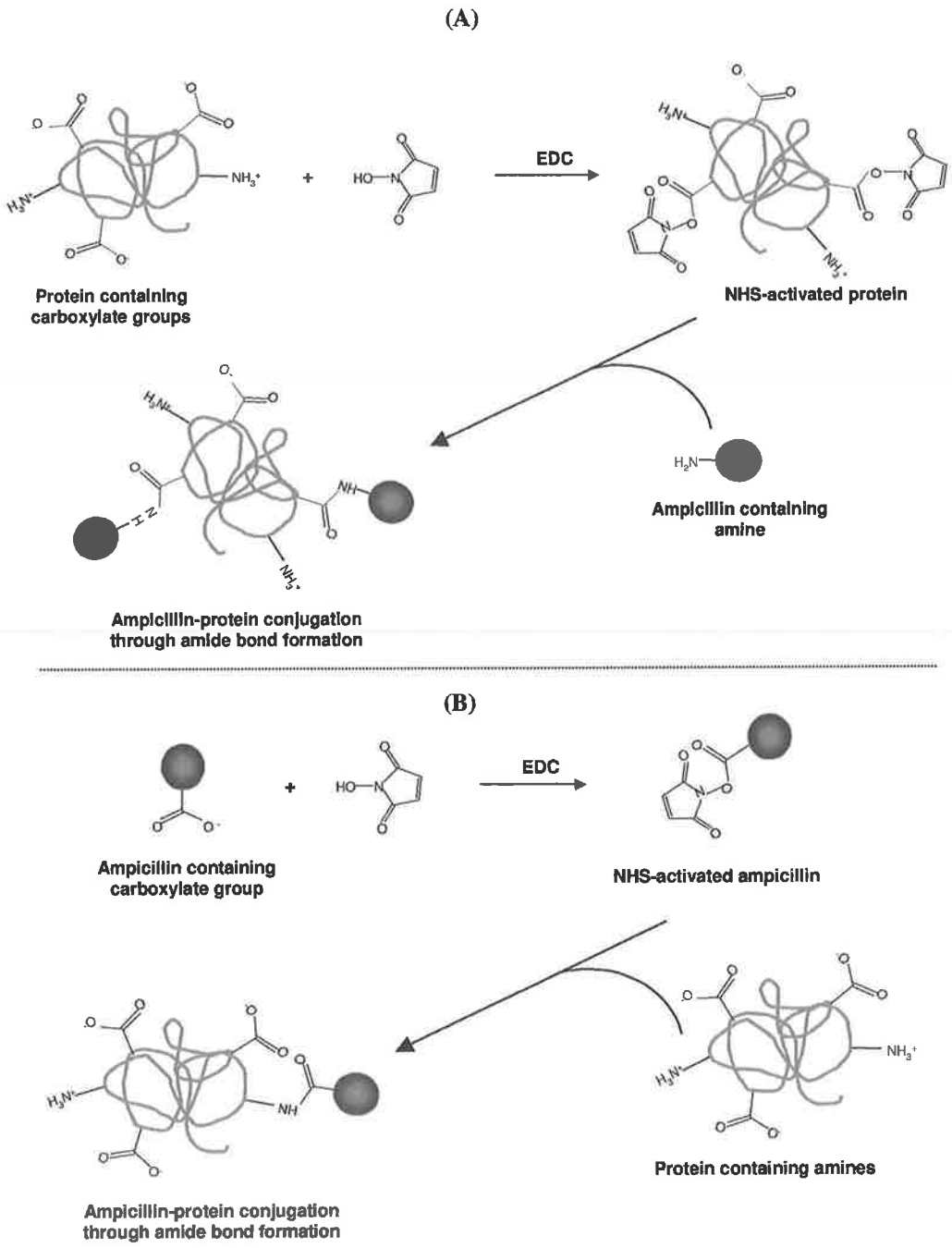


Figure 5.3. *(A) Overview of protein-activated carbodiimide and (B) ampicillin-activated carbodiimide coupling chemistry coupling chemistry. EDC, with a molecular weight of 191.7Da, reacts with carboxylate groups on the protein or ampicillin to form a highly active o-acylisourea intermediates that can then react with a amine groups on the protein or ampicillin to form an amine bond and EDC is released as a soluble isourea derivative. The active ester is subject to rapid hydrolysis in aqueous conditions, so NHS was therefore used to increase stability of the active intermediate.*

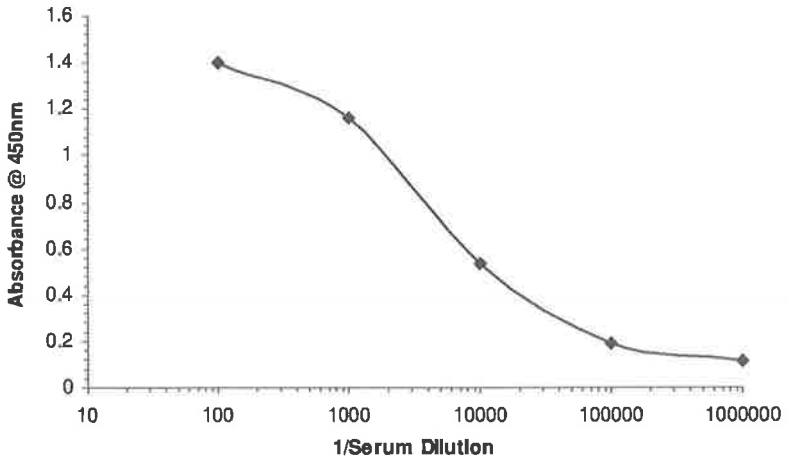


Figure 5.4. *Serum antibody titre following immunisation of a mouse with AMP-BSA. The serum was screened against AMP-THY and samples were also pre-incubated with 1% (w/v) BSA to prevent any non-specific binding of antibodies raised against the carrier molecule.*

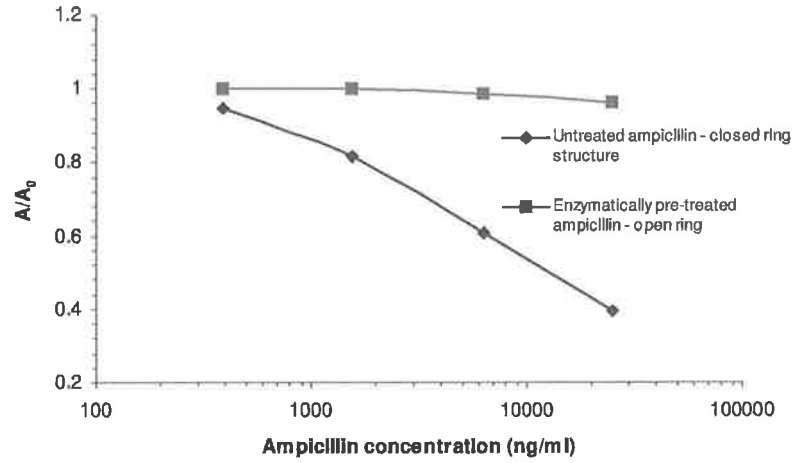


Figure 5.5. *Competitive ELISA carried out for the detection of free ampicillin (treated and untreated) at drug concentrations of 390 to 25,000ng/ml. Each ampicillin standard with an equal volume (50µl) of anti-ampicillin-BSA mouse serum was added to wells of an immunoplate coated with 10µg/ml ampicillin-thyroglobulin (AMP-THY). The mean absorbance obtained at each ampicillin concentration (A) was divided by the absorbance values determined in the presence of zero drug (A_0). Normalised absorbance values (A/A_0) were plotted against the logarithm of ampicllin concentration. It can be seen from the results that the antibodies produced recognised the intact ring structure of the drug.*

5.2.2 Production and purification of polyclonal anti-ampicillin antibodies

5.2.2.1 Immunogen preparation and immunisation

Polyclonal anti-ampicillin antibodies were produced using an AMP-BSA conjugate. A New Zealand white female rabbit was sub-cutaneously injected with an emulsion of the immunogen and Freund's Complete adjuvant and then periodically boosted using the same immunogen emulsified with Freund's Incomplete adjuvant, as described in *Section 2.5.3.1.*

5.2.2.2 Titre of polyclonal anti-serum

Blood was drawn at regular intervals to estimate the specific antibody titre according to *Section 2.5.3.2.* *Figure 5.6* shows the resulting serum antibody titres at various stages of the immunisation schedule. When an acceptable titre (~1/100,000) was obtained, the animal was sacrificed and the blood recovered by cardiac puncture.

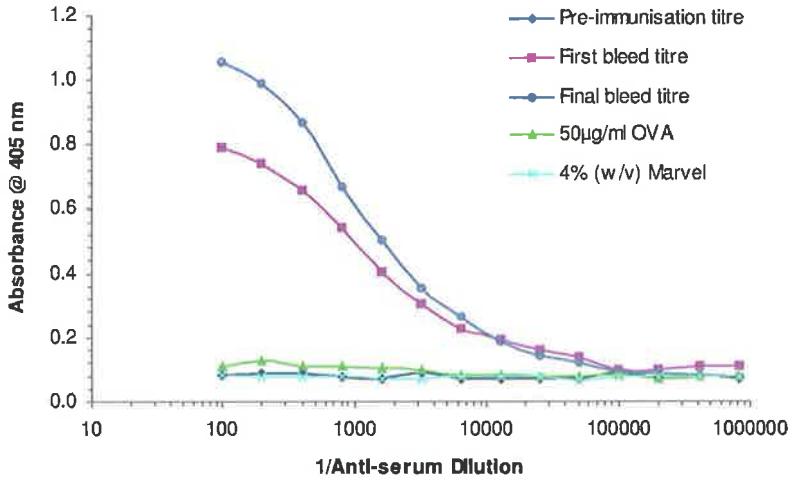


Figure 5.6. *Titres of serum obtained from a rabbit pre- and post-immunisation with AMP-BSA. Serum samples were screened against 50µg/ml AMP-OVA and also against OVA and Marvel™ to determine if there was any non-specific binding between the antibody and the carrier protein or the blocking solution. Antiserum was pre-incubated in PBS containing 1% (w/v) OVA to remove any non-specific interactions. The final antibody titre recorded was in excess of 1 in 100,000.*

5.2.2.3 Purification of polyclonal anti-serum

Purification of polyclonal anti-ampicillin antibodies was achieved using saturated ammonium sulphate precipitation followed by protein G affinity chromatography, as described in *Section 2.5.4.1* and *2.5.4.2*. Protein G affinity chromatography exploits the ability of the recombinant streptococcal protein G to bind specifically to the Fc portion of the IgG molecule. Therefore, non-specific contaminating proteins can be washed through the column leaving only IgG antibodies bound to the column, which can be subsequently eluted using 0.1M glycine buffer, pH 2.2. Fractions of the eluted affinity-purified antibody population were collected, immediately neutralised with 2M Tris, pH 8.6 and analysed. Total protein content in each fraction was estimated spectrophotometrically at 280nm and fractions containing protein were pooled and dialysed in PBS overnight at 4°C. The anti-ampicillin polyclonal antibodies were further purified by subtractive immunoaffinity chromatography. Antibodies that were raised against the carrier protein moiety of the immunogen were removed by passing the protein G purified antibodies through a BSA immobilised column, as described in *Section 2.5.4.3.*

5.2.3 Characterisation of purified polyclonal antibodies by SDS-PAGE and Western blotting

The level of purity of the antibodies was then assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (*Section 2.2.2*). *Figure 5.7 (A)* shows the resulting acrylamide gel and the increase in the level of purity from the crude serum to the affinity-purified rabbit antibodies. To confirm the presence of rabbit IgG antibody molecules, a Western blot was also performed, which can be seen in *Figure 5.7 (B).* Proteins from the polyacrylamide gel were transferred to a nitrocellulose membrane by electrophoretic blotting, as described in *Section 2.2.3.* The nitrocellulose was then probed with a horse radish peroxidase (HRP)-labelled goat anti-rabbit IgG and the presence of rabbit IgG antibodies confirmed by addition of a chromogenic substrate, *o*-phenylenediamine dihydrochloride (*o*-PD).

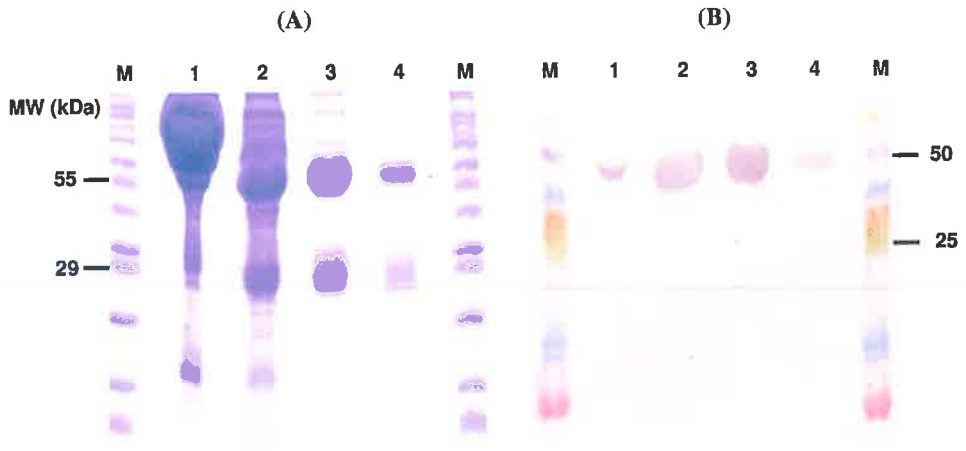


Figure 5.7. *(A) SDS-PAGE and (B) Western blot analysis on the purification of the anti-ampicillin polyclonal antibodies. (A) Lanes: (M) Sigma wide range molecular weight markers; (1) serum sample, diluted 5-fold in PBS; (2) saturated ammonium sulphate (SAS)-precipitated antibody sample, diluted 2-fold in PBS; (3) protein G affinity-purified pooled antibody fractions, applied neat; (4) standard commercial rabbit IgG (Sigma). Two distinct bands are visible, at approx. 50kDa and 25kDa, in the affinity-purified sample (lane 3), which represent the heavy and light chain, respectively. (B) Lanes: (M) Amersham Biosciences full range molecular weight markers; (1-4) same as above. The heavy chain, at approx. 50kDa, can be seen clearly in lanes 1-4. However, the light chain band is not visible. This is due to the fact that the secondary antibody was specific for only the heavy chain of the antibody (Fc-portion specific).*

5.2.4 Development of an ELISA for the detection of ampicillin in PBS using the polyclonal antibody

Following generation and purification of the polyclonal antibody, their use in a competitive immunoassay for the detection of ampicillin was investigated.

5.2.4.1 *Checkerboard ELISA for the determination of optimal coating conjugate concentration and antibody dilution*

In order for a competitive ELISA to function effectively, it is essential to optimise both the coating concentration of the conjugate and the optimal dilution of the antibody to obtain equilibrium between both free and immobilised antigen. A checkerboard ELISA was performed, as described in *Section 2.5.5.1.1,* with the affinity-purified antibodies and plates coated with AMP-OVA to determine those concentrations that would give the greatest assay sensitivity. These results were plotted, as shown in *Figure 5.8.* An optimum conjugate coating concentration of 12.5µg/ml of AMP-OVA was determined as that which gave the highest absorbance, using the most economical conjugate concentration. The optimal antibody dilution, *i.e.* which gave the greatest change in absorbance per change in antibody dilution, was chosen to be 1/20.

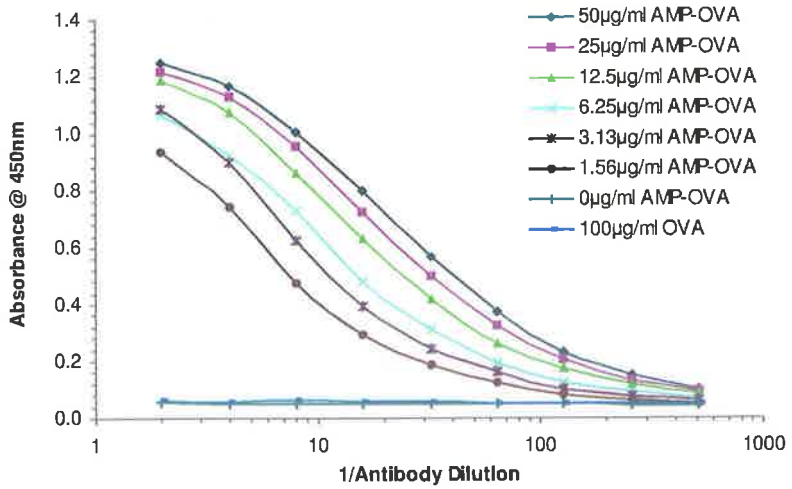


Figure 5.8. *Checkerboard ELISA for the determination of optimal conjugate coating concentration and polyclonal antibody dilution for use in a competitive ELISA. Varying ampicillin-OVA coating concentrations ranging from 0 - 50µg/ml and antibody dilutions from 1/2 - 1/512 were assayed. A 12.5µg/ml conjugate concentration and an antibody dilution of 1/20 were chosen for use in a competitive ELISA.*

5.2.4.2 *Intra- and inter-assay variability studies*

Using the optimised dilutions determined, a model competitive assay (*i.e.* free drug in solution and surface bound drug conjugate compete with one another to bind to antibody) was developed for these antibodies in PBS to determine the antibody's range of detection for ampicillin. PBS was selected as the diluent as it is cheap and easily prepared; hence its use in routine testing. Standards of ampicillin were prepared in PBS ranging from 1.5 to 100,000ng/ml and mixed with an equal volume of antibody, at the optimal dilution and added to a coated and blocked immunoplate, as described in *Section 2.5.5.1.2.*

Intra-assay (variation within an assay) and inter-assay (variation between assays) variability studies were also performed to demonstrate the reproducibility of the competitive assay. To carry out intra-day assay variation, three sets of each standard were assayed on the same day (Wong *et al.*, 1997). The coefficients of variation (CV's) were determined to assess the precision of the assay, expressing standard deviation as a percentage of the mean values. Intra- and inter-day assay CV's ranged from 0.99% to 16.49% and 1.03% to 10.74%, respectively, indicating acceptable reproducibility (*i.e* below 20%) (Findlay *et al.*, 2000). To determine whether measured concentrations agreed with theoretical values, percentage recovery values were also calculated by expressing the measured concentration as a percentage of the actual concentration. The percentage recoveries were acceptable, with values ±20%, (with the exception of values of 73% and 124% at concentrations of 390ng/ml and 90ng/ml, respectively), indicating the curve provide an accurate representation of the sigmoidal representation between the measured response and the logarithm of concentration (Findlay *et al.*, 2000). Both percentage recovery and CV values are shown in *Table 5.2.*

Figure 5.9 shows the inter-day calibration curve using the polyclonal antibody, where the range of detection was found to be between 48 and 50,000ng/ml. The mean absorbance obtained at each ampicillin concentration (A) was divided by the absorbance values determined in the presence of zero drug (A_0). Normalised absorbance values (A/A_0) were plotted against the logarithm of ampicllin concentration using BIAevaluation 4.0.1 software and a four-parameter equation was fitted to the data to generate a calibration curve.

The limit of detection (LOD), also termed the least detectable dose (LDD), is the smallest concentration of the analyte that produces a signal which can be significantly distinguished from zero for a given sample matrix (Hennion and Barcelo, 1998). This value was determined by selecting the mean normalised absorbance minus three standard deviations for the negative standard (no ampicillin) and was found to be approximately 73ng/ml. However the assay sensitivity does not comply with the current EU maximum residue limit (MRL) of 4ng/ml for ampicillin in milk.

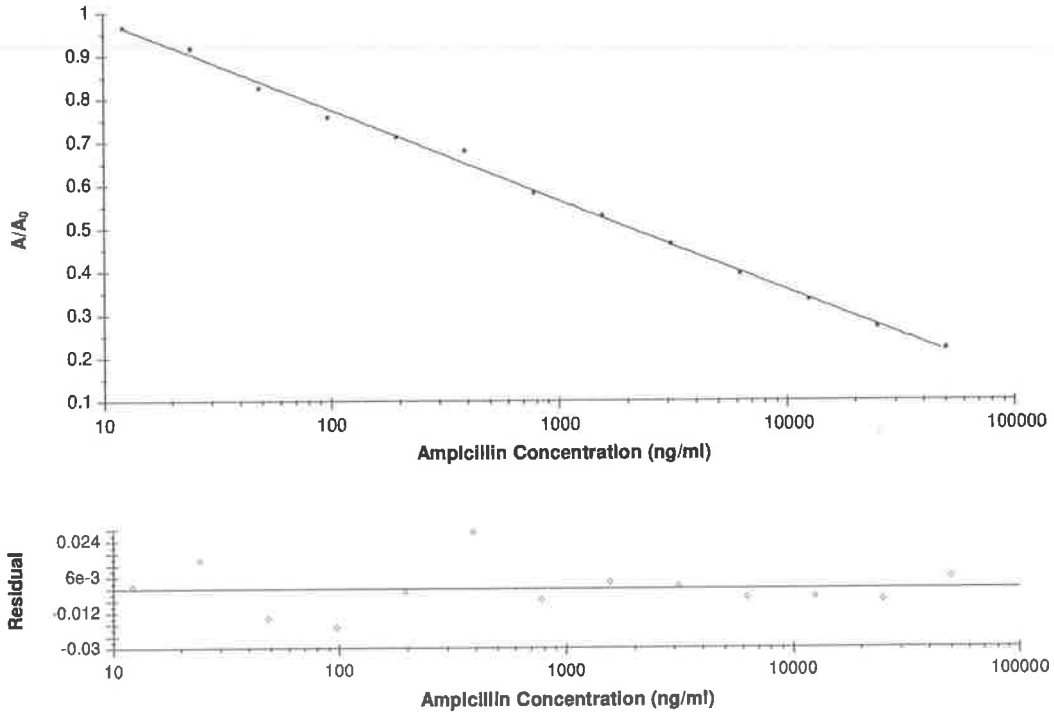


Figure 5.9. *Inter-day calibration curve for competitive ELISA for the detection of free ampicillin in PBS using the anti-ampicillin polyclonal antibody. AMP-OVA was coated at 12.5µg/ml and polyclonal antibody at a final dilution of 1/20 was used. Bound antibody was detected using an anti-rabbit HRP-labelled antibody followed by addition of a chromogenic substrate o-PD. Three replicates of each standard were analysed on three separate days and the limit of detection was found to be 73ng/ml.*

Table 5.2. *Inter-day assay coefficient of variation (CVs) for the detection of free ampicillin using the affinity-purified polyclonal antibody. Each standard was analysed in triplicate over three different days and the CVs were calculated as the standard deviation (SD) expressed as a percentage of the mean value for each standard. Measured (i.e. back-calculated concentrations) were calculated from the four-parameter model produced using BIAevaluation 4.0.1 software and the percentage recovery expressed as a ratio between measured and added concentrations.*

Concentration (ng/ml)	**$A/A_0 \pm SD$**	**CV (%)**	**Back calculated concentration (ng/ml)**	**Recovery (%)**
50,000	0.220 ± 0.009	4.09	46,812.94	94
25,000	0.270 ± 0.029	10.74	26,578.48	106
12,500	0.333 ± 0.025	7.51	13,077.64	105
6,250	0.395 ± 0.022	5.57	6,547.07	105
3,125	0.463 ± 0.017	3.67	3,082.23	99
1,562.50	0.528 ± 0.016	3.03	1,506.11	96
781.25	0.582 ± 0.006	1.03	832.47	106
390.62	0.680 ± 0.033	4.85	284.49	73
195.31	0.713 ± 0.023	3.22	198.19	101
97.66	0.758 ± 0.042	5.54	121.01	124
48.83	0.826 ± 0.036	4.36	57.32	117
24.41	0.918 ± 0.059	6.43	20.74	86
12.21	0.967 ± 0.069	7.16	12.02	100

5.2.5 Development of a Biacore-based assay using the polyclonal antibody for the detection of ampicillin in milk

The applicability of an SPR biosensor for the detection of antibiotic residues in milk and whether it would display increased assay sensitivity was investigated.

5.2.5.1 Production of a directly immobilised ampicillin CM5 sensor chip surface

Ampicillin was directly immobilised onto the surface of a CM5 chip using standard amine coupling chemistry, as described by *Section 2.5.5.2.1* and illustrated in *Figure 5.10.* The CM dextran surface of the chip was activated with a mixture of NHS and EDC and a 10mM solution of ampicillin, freshly prepared in 50mM borate buffer, pH 8.5, was injected over the surface allowing the activated NHS esters to react with the amine groups on the ampicillin molecules. Ethanolamine was then passed over the chip surface to cap any remaining NHS unreacted sites and the surface was conditioned with an injection of 100mM NaOH containing 20% (v/v) dimethylformamide (DMF) followed by an injection of 100mM HCl containing 20% (v/v) dimethylformamide (DMF). A negative flow cell was also prepared in the same way, however, ampicillin was omitted, as seen in *Figure 5.11.*

5.2.5.2 Binding studies on the directly immobilised ampicillin surface

Polyclonal anti-ampicillin antibody, at a concentration of approximately 44μg/ml in HBS, was passed over the directly immobilised ampicillin chip surface to investigate specific binding. Antibody was injected over the surface for two minutes at a flowrate of 10μl/minute. It can clearly be seen from *Figure 5.12* that there was negligible non-specific binding (NSB) to the control capped CM dextran surface.

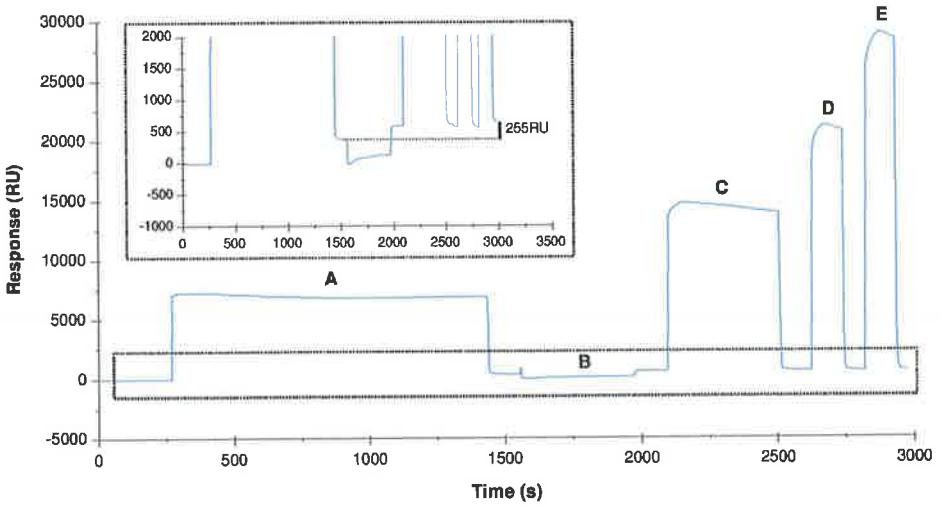


Figure 5.10. *Sensogram illustrating the direct coupling of ampicillin to a CM5 sensor chip surface.* ***(A)*** *NHS/EDC activation of the carboxyl groups on the dextran surface,* ***(B)*** *covalent coupling of ampicillin to the chip surface,* ***(C)*** *deactivation of any remaining active esters,* ***(D)*** *conditioning of surface with a 2 minute injection of injection of 100mM NaOH containing 20% (v/v) DMF followed by* ***(E)*** *an injection of 100mM HCl containing 20% (v/v) DMF. A final level of 255RU of covalently attached ampicillin was achieved.*

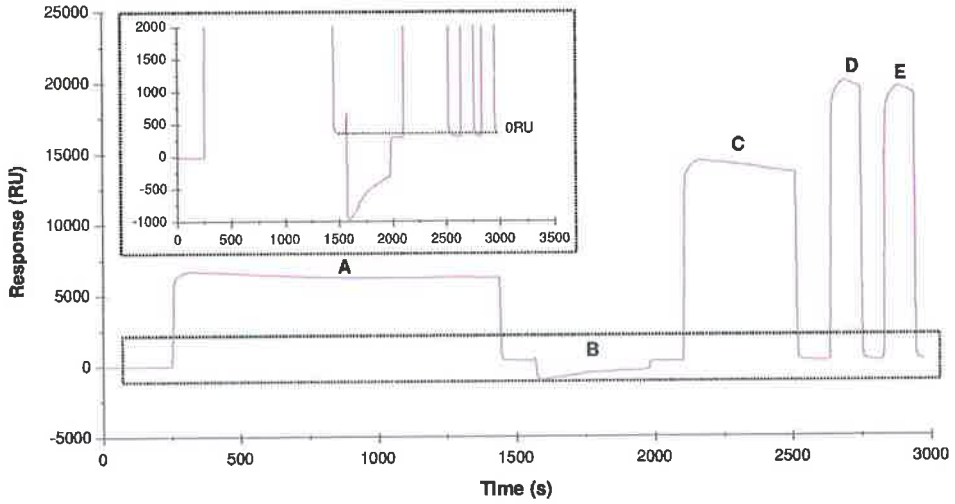


Figure 5.11. *Sensogram illustrating activation and capping of the control flow cell.* ***(A)*** *NHS/EDC activation of the carboxyl groups on the dextran surface,* ***(B)*** *injection of borate buffer containing no ampicillin,* ***(C)*** *deactivation of any remaining active esters,* ***(D)*** *&* ***(E)*** *conditioning of surface as before. A final response of approximately 0RU was achieved as expected.*

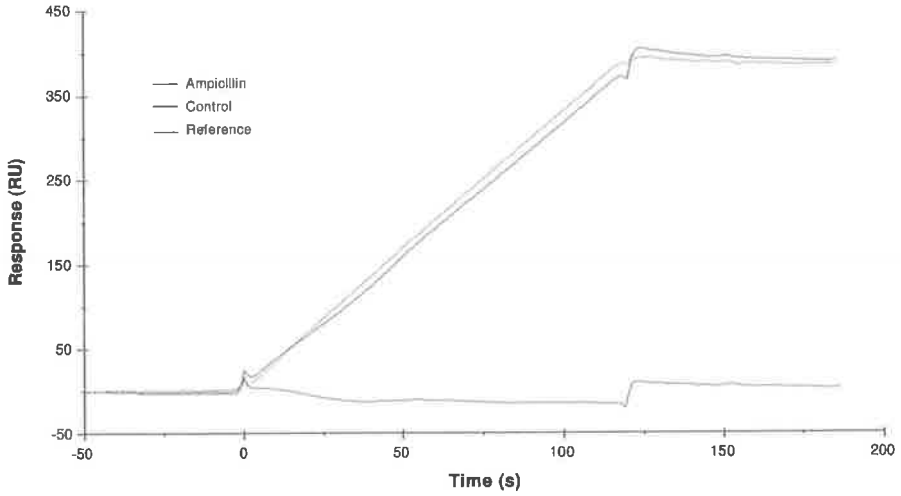


Figure 5.12. *Overlay sensogram plots of the polyclonal anti-ampicillin antibody binding to the directly immobilised ampicillin surface and the capped CM dextran surface. The resulting 'real-time' reference subtraction is also shown. Anti-ampicillin polyclonal antibody, at a concentration of 44µg/ml in HBS buffer, was passed over each surface simultaneously at a flow rate of 10µl/min for two minutes and the response units recorded. Negligible non-specific binding to the control surface was observed.*

5.2.5.3 Determination of the range of detection of ampicillin in HBS buffer using a Biacore inhibition assay format

A preliminary assay was first carried out in HBS to investigate the applicability of an SPR biosensor for the detection of ampicillin using the polyclonal antibody, as described *Section 2.5.5.2.2.* Different antibody concentrations were tested on the surface and the percentage inhibition at 100ng/ml compared. An optimal antibody concentration of approximately 44µg/ml was chosen as compromise between sensitivity and antibody consumption (data not shown). Varying concentrations of ampicillin ranging from 6 to 400,000ng/ml were prepared in HBS and mixed with an equal volume of antibody at the optimal concentration. Samples were incubated for 30 minutes at room temperature to allow equilibration before they were each injected simultaneously over the ampicillin immobilised and control surface. Each standard was injected in triplicate for 2 minutes at a flowrate of 10µl/minute followed by two 30 second pulses of 100mM NaOH, which allowed complete regeneration of the chip

surface. The response, which is proportional to the mass of bound analyte, was recorded after each injection.

To demonstrate the reproducibility of the assay three replicates of each concentration were analysed to determine intra-assay variability and analysis repeated on three different days to determine inter-assay variability. The coefficients of variation (CV's) were determined to assess the assay precision, expressing standard deviation as a percentage of the mean values. Intra- and inter-day assay CV's ranged from 0.08% to 1.5% and 2.90% to 9.92%, respectively, indicating excellent reproducibility. Percentage recovery values were also estimated and values were found to be more accurate at lower concentrations of ampicillin. CV's and percentage recovery values are shown in *Table 5.3.*

The mean response obtained at each ampicillin concentration (RU) was divided by the response determined in the presence of zero drug (RU_0). The normalised responses (RU/RU_0) were plotted against the logarithm of ampicllin concentration using BIAevaluation 4.0.1 software and a four-parameter equation fitted to the data. *Figure 5.13* shows the inter-day calibration curve using the polyclonal antibody, where the range of detection was found to be between 24.4 and 400,000ng/ml. The limit of detection (LOD) determined as before and was found to be 11.5ng/ml, representing a 6-fold increase in sensitivity compared to the ELISA.

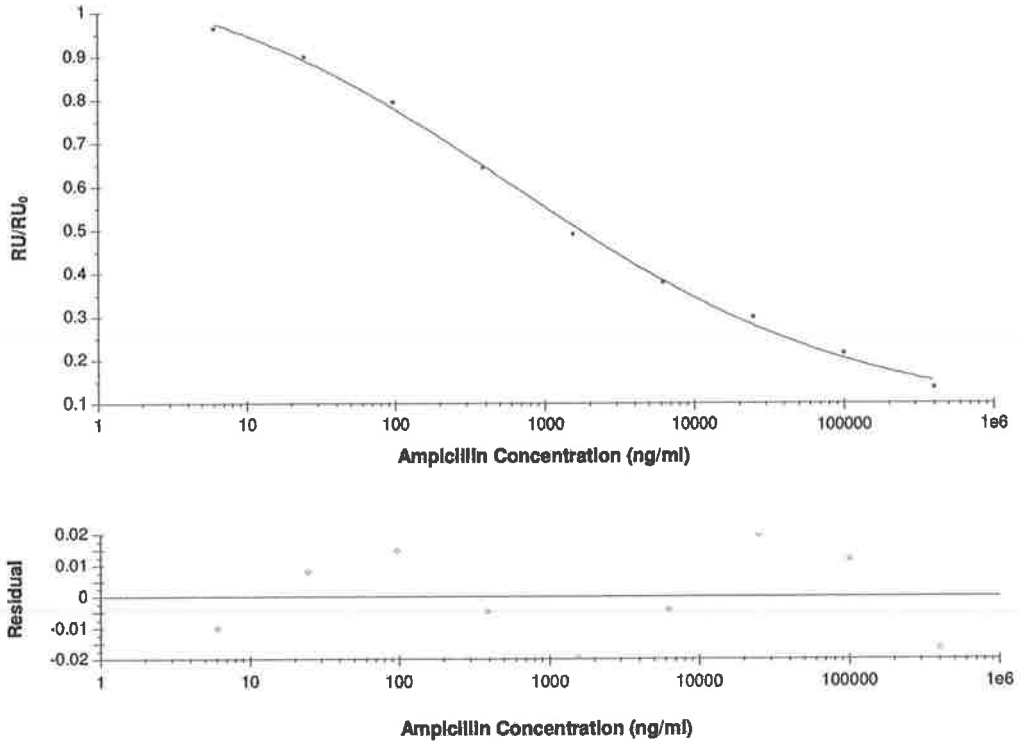


Figure 5.13. *Inter-day calibration curve for the inhibition Biacore assay for the detection of ampicillin in PBS using the anti-ampicillin polyclonal antibody. Three replicates of each standard were analysed on three separate days. Ampicillin was immobilised directly onto the sensor chip surface. The range of detection was found to be 24.4 to 400,000ng/ml. The binding response at each ampicillin concentration (RU) was divided by the antibody response in the absence of free ampicillin (RU_0) to give a normalised binding response (RU/RU_0), which was plotted against the logarithm of ampicillin concentration. The inter-assay limit of ampicillin detection (LOD) was found to be 11.5ng/ml.*

Table 5.3. *Inter-day Biacore inhibition assay coefficient of variation (CVs) for the detection of free ampicillin in HBS. Each standard was assayed in triplicate over three days and the CVs were calculated as the standard deviation (SD) expressed as a percentage of the mean value for each standard. Measured (i.e. back-calculated concentrations) were calculated from the four-parameter model produced using BIAevaluation 4.0.1 software and the percentage recovery expressed as a ratio between measured and added concentrations.*

Concentration (ng/ml)	**$RU/RU_0 \pm SD$**	**CV (%)**	**Back calculated concentration (ng/ml)**	**Recovery (%)**
400,000	0.138 ± 0.004	2.90	N/D	-
100,000	0.217 ± 0.009	4.15	147,041.54	147
25,000	0.300 ± 0.012	4.00	18,858.50	75
6,250	0.379 ± 0.020	5.28	5,953.48	95
1,562.50	0.491 ± 0.039	7.94	1,735.15	111
390.63	0.644 ± 0.054	8.39	405.67	104
97.66	0.796 ± 0.079	9.92	89.45	91
24.41	0.899 ± 0.079	8.79	23.34	96
6.10	0.965 ± 0.054	5.60	6.78	111

* *N/D = not determinable. Values were too high to be calculated from the four-parameter model produced using BIAevaluation 4.0.1 software.*

5.2.5.4 Determination of the range of detection of ampicillin in processed milk using a Biacore inhibition assay format

5.2.5.4.1 Non-specific binding studies

The feasibility of detecting ampicillin in a food matrix such as milk and its possible matrix effects on the optical biosensor were first investigated. Milk composes of water (87%), proteins (3.3%), lipids (3.7%), carbohydrates (4.7%) and enzymes, vitamins and mineral salts with a pH~6.7 (Aerts *et al.*, 1995). Pasteurised and homogenised milk, with a 3.5% fat content (Avonmore) was purchased locally and used in all development. To minimise non-specific binding (NSB) effects, all milk was fractionated into a lipid and aqueous phase by means of centrifugation and the lipid layer removed, as described in *Section 2.5.5.2.3.* This method, referred to as "defatting", has commonly been used (Sternesjö *et al.*, 1995; Strasser *et al.*, 2003) and has shown to have no effect on the resulting antibiotic concentration (Zhi *et al.*, 2001). Both neat and diluted (2-fold in HBS) de-fatted milk samples were injected over both the ampicillin immobilised and control flow cell to evaluate the degree of NSB. Samples were injected for 2 minutes at a flowrate of 10µl/min and the surface fully regenerated with a 30 second injection of 5mM NaOH. The NSB for both preparations was the same, indicating that dilution of the de-fatted milk had minimal effect with a normalised response of between 80 to 90RU obtained for both diluted and neat samples, as illustrated in *Figure 5.14.*

The NSB response observed for a number of defatted milk samples (n=20) was found to be relatively constant in terms of response units (RU) with an average value of approximately 86±5RU. Throughout all further analysis the response obtained from defatted milk (containing no free ampicillin or antibody) was subtracted from all other response values.

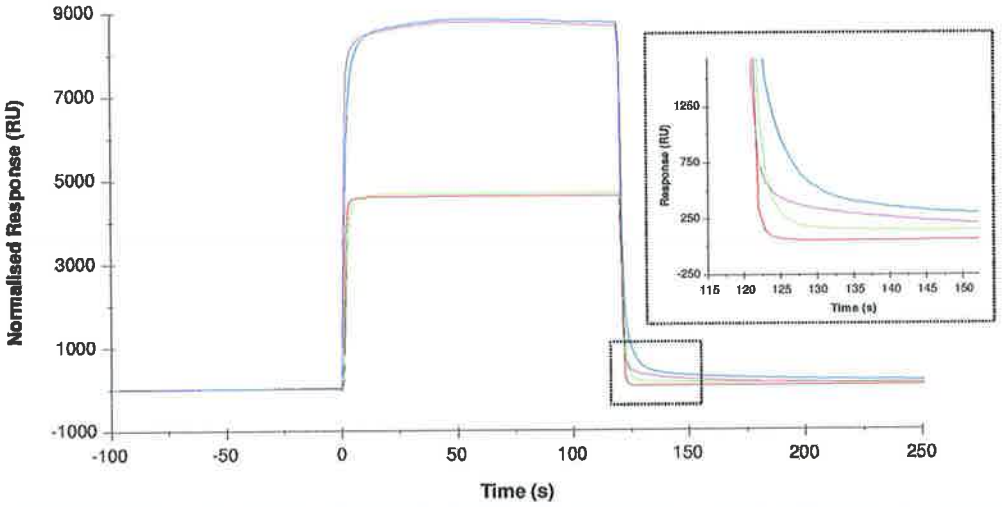


Figure 5.14. *Overlay plot of sensograms of the prepared defatted milk samples containing no antibody injected over the directly immobilised ampicillin surface and control surface. When the diluted (2-fold in HBS) defatted milk was passed over both the ampicillin and control surfaces a response of 135RU and 44RU was obtained, respectively. A higher response of approximately 310 and 219 units was observed when the neat defatted milk was passed over the ampicillin and control surface, respectively. However normalised responses were 91RU and 90RU for the diluted and neat milk sample, respectively.*

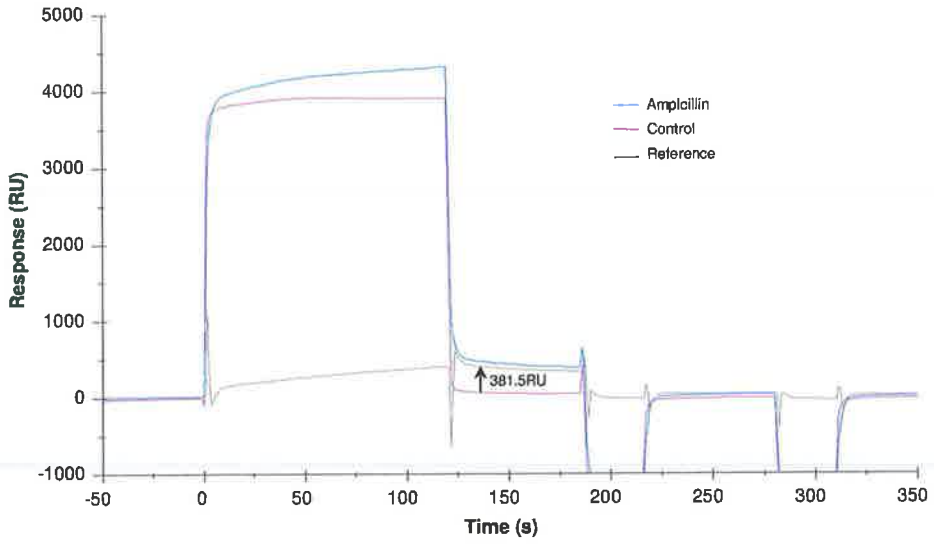


Figure 5.15. *Overlay sensogram plots of the polyclonal anti-ampicillin antibody binding to the directly immobilised ampicillin surface and the capped CM dextran surface. The anti-ampicillin polyclonal antibody, prepared in defatted milk at a concentration of 44µg/ml, was passed over each surface simultaneously at a flow arte of 10µl/min for 2 minutes, the response recorded and the surface fully regenerated following two 30 second injections of 100mM NaOH. Approximately 455RU of antibody bound to the directly immobilised ampicillin surface. However, a response of 72RU was observed when the antibody prepared in milk was passed over the control surface, indicating a small degree of NSB to the dextran surface. Therefore, throughout analysis, the final report response was corrected for the response of the control flow cell. The resulting 'real-time' reference subtraction sensogram is shown above.*

5.2.5.4.2 Evaluation of directly immobilised ampicillin surface stability

For a Biacore-based assay to be cost effective, each immobilised flow cell should be capable of analysing a large number of samples. Hence for multiple analyses, a stable surface and an efficient regeneration process is imperative. To assess the stability of the ampicillin immobilised surface, multiple (*i.e.* 50) binding-regeneration cycles on were performed.

Polyclonal antibody, at a concentration of 44µg/ml in processed defatted milk, was injected over the sensor surface at a flowrate of 10µl/min for 2 minutes and specific bound antibodies removed with two 30 second pulses of 100mM NaOH. There was no decrease in binding response recorded over the fifty binding-regeneration cycles, indicating the surface was stable and could be successfully regenerated without any loss of binding activity (*Figure 5.16*). However, a slight variation (<2%) in binding response was observed but thought to be due to the difference in composition of each milk preparation.

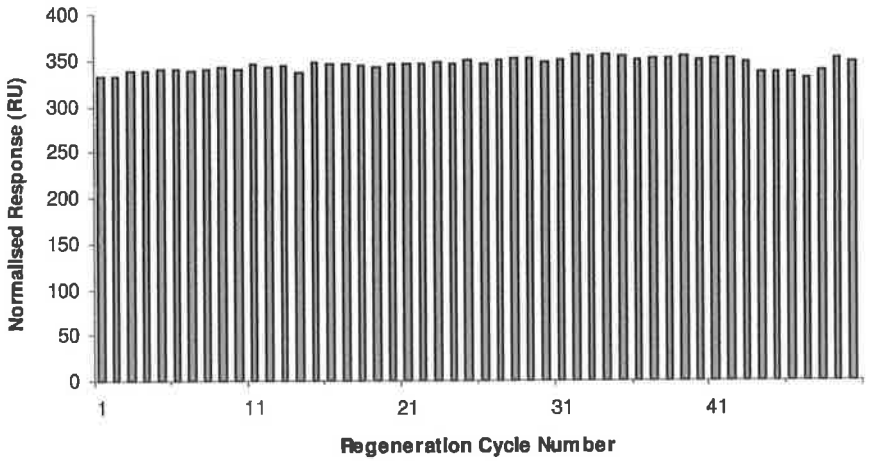


Figure 5.16. *Typical regeneration profile for fifty consecutive binding-regeneration cycles of a 2 minute pulse of purified polyclonal antibody in processed milk to the surface of a chip immobilised with ampicillin. Antibody, at a concentration of 44µg/ml, was used and the surface regenerated with two 30 second pulses of 100mM NaOH. There was negligible decrease in binding activity with only a 1.86% variation within binding responses observed over the fifty consecutive cycles.*

5.2.5.4.3 Development of a Biacore inhibition assay for the detection of ampicillin in processed milk

Processed defatted milk was spiked with known concentrations of ampicillin, ranging from 1.5 to 400,000ng/ml, as described in *Section 2.5.5.2.2,* and mixed with an equal volume of antibody at a concentration of 44μg/ml. Samples were incubated for 30 minutes at room temperature to allow equilibration before they were each injected simultaneously over the ampicillin immobilised and control surface. Each standard was injected in triplicate for 2 minutes at a flowrate or 10μl/minute, followed by two 30 second pulses of 100mM NaOH. The overlaid sensograms, shown in *Figure 5.17 (A)* and *(B)*, illustrate the decreasing response with increasing concentrations of ampicillin.

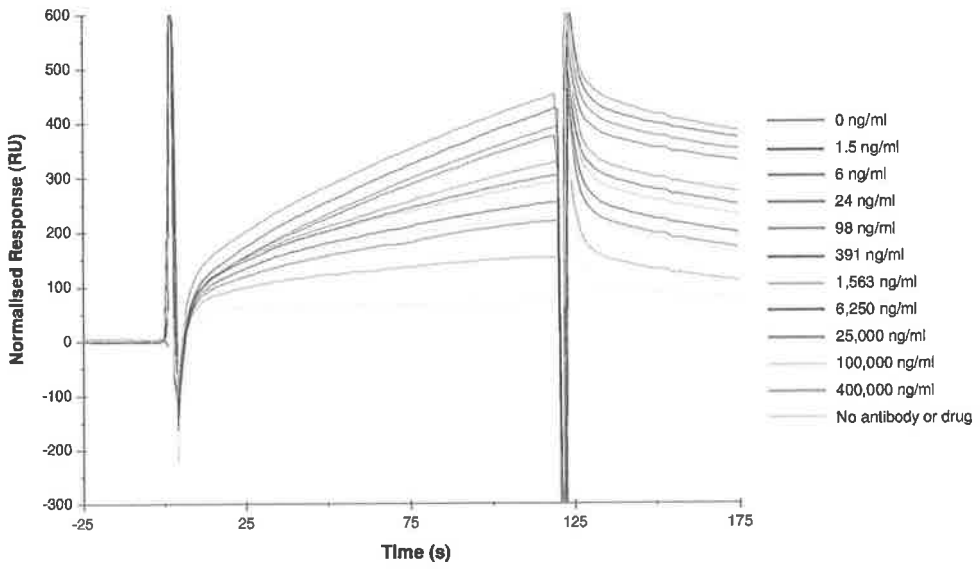


Figure 5.17. *(A) Overlay plot showing the decreasing response units (RU) with each increase in ampicillin concentration. Ampicillin standards, prepared in defatted milk were mixed with an equal volume of the anti-ampicillin polyclonal antibody and injected over the directly immobilised ampicillin and control flow cells.*

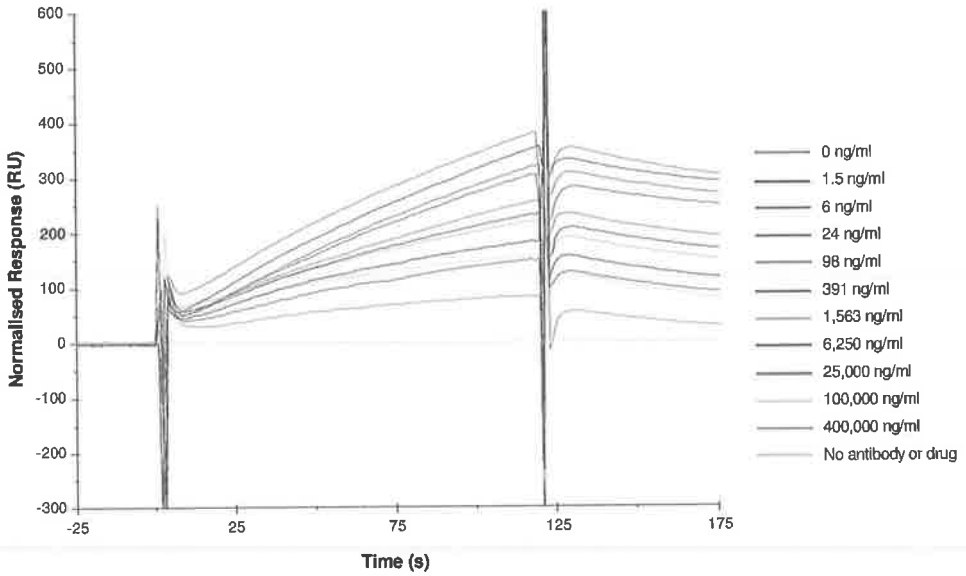


Figure 5.17. (B) *Overlay plot showing the decreasing response units (RU) with each increase in ampicillin concentration following subtraction of the non-specific response from defatted milk.*

To demonstrate the reproducibility of the assay both intra- and inter-variability studies were performed. Intra- and inter-day assay CV's ranged from 0.14% to 8.22% and 2.05% to 17.30%, respectively, indicating adequate reproducibility (*i.e.* less than 20%). The CV's were found to be higher than that calculated when the assay was developed in HBS. This was thought to be as a result of the variation between each treated milk sample and the complexity of the matrix. Percentage recovery values indicated adequate accuracy, with majority of values below 20%. CV's and percentage recovery values are shown in *Table 5.4*. Normalised absorbance values (RU/RU_0) were plotted against the log of ampicllin concentration to generate a calibration curve using BIAevaluation 4.0.1 software. *Figure 5.18* shows the inter-day calibration curve using the polyclonal antibody, where the range of detection was found to be between 6 and 100,000ng/ml. The limit of detection (LOD) was determined as before, by selecting the mean normalised absorbance minus three standard deviations for the negative standard (no drug). This value was found to be

3.6ng/ml, which is below the required sensitivity set out by the EU MRL of 4ng/ml (EEC regulation no. 2377/90).

The overlaid Biacore inhibition assay calibration curves for the detection of ampicillin in HBS and defatted processed milk is shown in *Figure 5.19*. Response values were normalised by the transformation to $\%B/B_0$ according to:

$$\%B/B_0 = \frac{RU - RU_{excess}}{RU_0 - RU_{excess}}$$

where RU is the response, RU_0 is the response at zero dose of analyte and RU_{excess} is the response at excess of analyte. The assays employing the polyclonal antibody in HBS and milk displayed similar sensitivities with an IC_{50} of 1.38μg/ml and 1.68μg/ml for ampicillin, respectively.

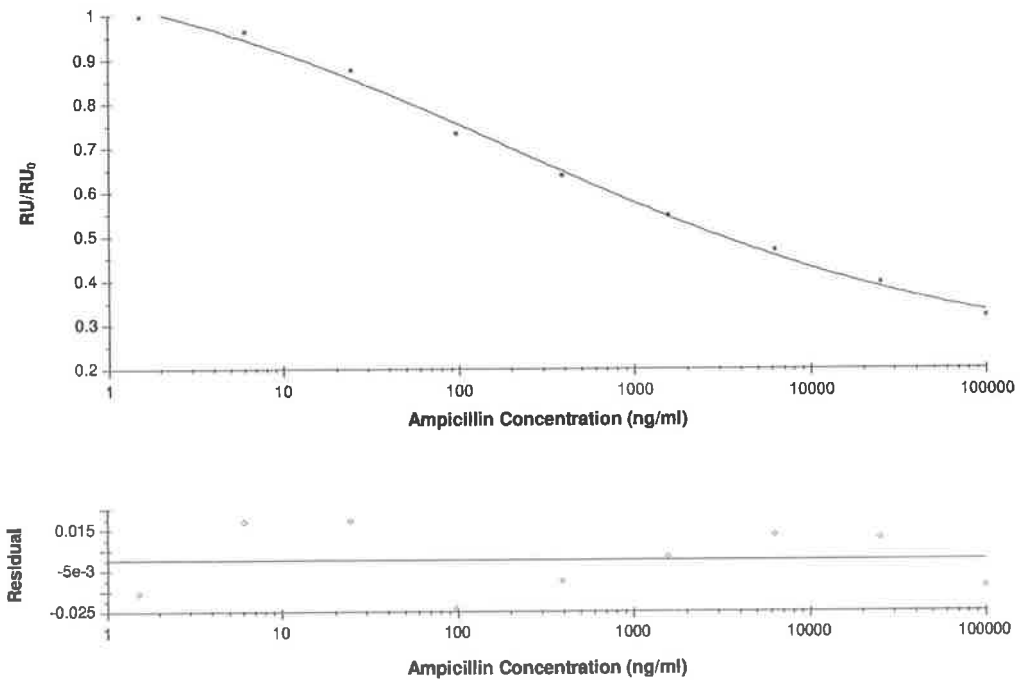


Figure 5.18. *Inter-day calibration curve for the inhibition Biacore assay for the detection of ampicillin in processed milk using the anti-ampicillin polyclonal antibody. A calibration curve was constructing by plotting the mean (n=3) normalised response for each ampicillin concentration. Three replicates of each standard were analysed on three separate days. Ampicillin was immobilised directly onto the sensor chip surface. The assay was found to have a range of detection of 6 to 100,000ng/ml and a limit of detection of 3.6ng/ml.*

Table 5.4. *Inter-day assay coefficient of variation (CVs) for the detection of free ampicillin processed milk using an SPR biosensor. Each standard was assayed over three days and the CVs were calculated as the standard deviation (SD) expressed as a percentage of the mean value for each standard. Measured concentrations (i.e. back-calculated concentrations) were calculated from the four-parameter model fitted to the assay data using BIA evaluation 4.01 software and percentage recoveries expressed a ratio between measured and added concentrations.*

Concentration (ng/ml)	**$RU/RU_0 \pm SD$**	**CV (%)**	**Back calculated concentration (ng/ml)**	**Recovery (%)**
100,000	0.318 ± 0.055	17.30	133,884.10	134
25,000	0.395 ± 0.031	7.85	20,515.68	82
6,250	0.468 ± 0.024	5.13	5,407.41	87
1,562.50	0.548 ± 0.051	9.31	1,561.78	100
390.63	0.645 ± 0.021	3.26	412.84	106
97.66	0.732 ± 0.020	2.73	132.51	136
24.41	0.876 ± 0.018	2.05	18.93	78
6.10	0.963 ± 0.056	5.82	4.93	81

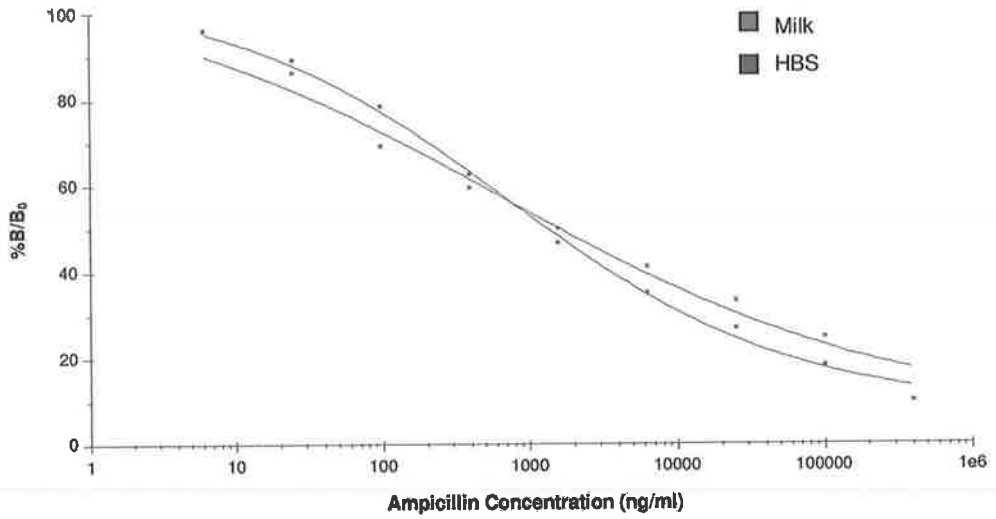


Figure 5.19. *Inhibition Biacore assay for the detection of ampicillin in HBS buffer and defatted processed milk. Normalised response values (expressed as %B/B0) were plotted against the logarithm of ampicillin concentration using Biaevaluation 4.0.1 software and a four-parameter equation fitted to the data. The* IC_{50} *values for the assays employing the polyclonal antibody were found to be 1.38µg/ml and 1.68µg/ml for ampicillin in HBS and milk, respectively.*

5.2.5.4.4 Cross-Reactivity Studies

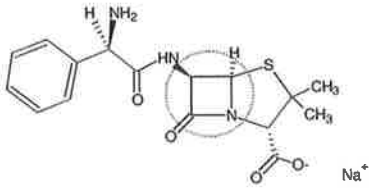
To determine the degree of cross-reactivity of the polyclonal antibody, the Biacore assay was performed, as before, with six structurally related penicillins and two members of cephalosporins, as seen in *Figure 5.20.* Standards of each potential cross-reactant, ranging from 1.5 to 400,000ng/ml, were prepared in milk and mixed with an equal volume of antibody at a concentration of 44μg/ml. Samples were incubated for 30 minutes at room temperature to allow equilibration and injected simultaneously over the ampicillin immobilised and control surface for 2 minutes at a flowrate or 10μl/minute. Normalised responses (RU/RU_0) were plotted against the log of concentration using BIAevaluation software.

Percentage cross-reactivities ($\%CR_{90}$ and $\%CR_{50}$), were estimated by comparing the relevant LDD_{90} and IC_{50} values of the potential cross-reactants to those values of the antigen (*i.e.* ampicillin) (Hennion and Barcelo, 1998), where the IC_{50} value is the analyte concentration that results in 50% inhibition and the LDD_{90} is the least detectable dose that results in 10% inhibition. Results, listed in *Table 5.5*, indicated the polyclonal antibody was specific to ampicillin with minimal cross-reactivity with similar penicillin compounds and no detectable cross-reactivity with cephalosporin compounds.

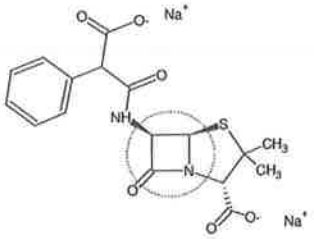
Table 5.5. *Percentage cross-reactivity (CR) values of the polyclonal antibody to various potential cross-reactants.*

Compound	LDD_{90} (ng/ml)	IC_{50} (ng/ml)	CR_{90} (%)	CR_{50} (%)
Ampicillin	10	3,000	100	100
Carbenicillin	45	8,500	22.22	35.29
6-APA	12,500	N/D	0.08	N/D
Cloxacillin	3,000	450,000	0.33	0.67
Amoxicillin	5,500	600,000	0.18	0.50
Oxacillin	4,500	500,000	0.22	0.60
Dicloxacillin	3,000	650,000	0.33	0.46
7-ADAC	N/D	N/D	N/D	N/D
Cephalexin	150,000	N/D	<0.01	N/D

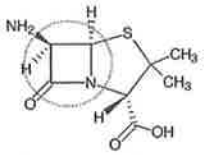
** N/D = not determinable; the ampicillin concentration required to reach the IC_{50} value was greater than 1mg/ml. LDD_{90} = least detectable dose that results in 10% inhibition.*



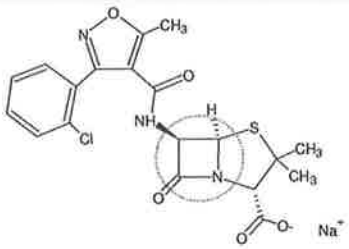
Ampicillin sodium salt



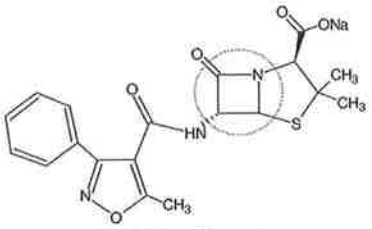
Carbenicillin sodium salt



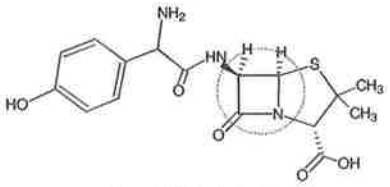
(+)-6-Aminopenicillanic acid



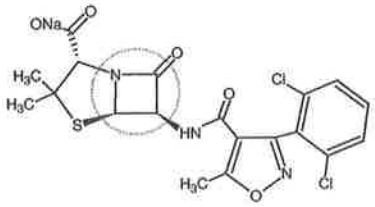
Cloxacillin sodium salt



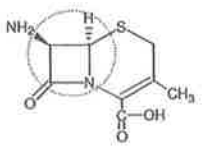
Oxacillin sodium salt



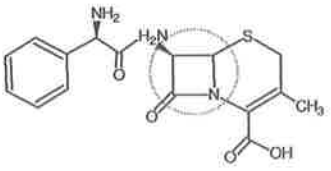
Amoxicillin trihydrate



Dicloxacillin sodium salt



7-Aminodesacetoxycephalosporanic acid



Cephalexin hydrate

Figure 5.20. *Chemical structures of the potential cross-reactants analysed. The common β-lactam ring core structure is highlighted with variability in the structure, caused by two side chain groups.*

5.2.6 Development of a biochip-based assay using the polyclonal antibody for the detection of ampicillin

Although there are a number of assays described in the literature for the detection of antibiotic residues in milk, there is still a definite need for a portable system that can easily be used in non-lab environments but which can still provide the necessary sensitivity and speed of analysis. Thus, a novel, disposable, antibody-based bio-chip sensor was developed by a research group led by Prof. MacCraith of the National Centre for Sensor Research (NCSR), DCU.

The disposable bio-chip detection system is based on fluorescence so the first step was to develop a working assay in plate format with fluorescently labelled reagents. For this purpose, ampicillin was biotinylated, as described in *Section 2.5.5.3.1*, for use in a competitive assay, whereby unlabelled ampicillin competes for antibody binding sites with a known quantity of biotinylated ampicillin. EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce) was linked to the primary amine group of ampicillin, as outlined in *Figure 5.21* and the biotinylated antibiotic could then be traced using a commercially available Streptavidin-Cy5 (Amersham Biosciences). In this manner, the detected fluorescence was reduced in direct proportion to the antibiotic concentration. Unreacted biotin removed by addition of Affigel 102 (Bio-Rad) in excess and BIO-AMP recovered by centrifugation.

5.2.6.1 Fluorescence immunoassay (FIA) development

The concentration of the reagents and all aspects of the assay were optimised initially on a plate format before being transferred to the bio-chip system.

5.2.6.1.1 Checkerboard FIA for the determination of optimal antibody and reagent concentrations

A checkerboard ELISA was performed, as described in *Section 2.5.5.3.2* to determine those concentrations that would give the greatest assay sensitivity. An immunoplate was coated with varying concentrations of the ampicillin-specific polyclonal antibody and blocked with PBS containing 2% (w/v) BSA. Varying concentrations of the biotinylated ampicillin (BIO-AMP) were then added to the plate and the amount of bound BIO-AMP detected with the addition of streptavidin-Cy5. The resulting fluorescence response was measured using a Tecan Safire^2 plate reader and the results

were plotted, as illustrated in *Figure 5.22*. An optimum conjugate coating concentration of 4.38μg/ml of polyclonal antibody was determined as that which gave the highest fluorescence, using the most economical concentration. The optimal BIO-AMP dilution that gave the greatest change in absorbance per change in antigen dilution was chosen to be 1/120.

A checkerboard was also performed in the same manner with varying dilution of BIO-AMP and Streptavidin-Cy5 (Strep-Cy5) against a plate coated with the optimal dilution of antibody (4.38μg/ml) to determine the optimal dilution of the fluorescently labelled tracer, as described in *Section 2.5.5.3.3*. The optimal BIO-AMP dilution was again found to be approximately 1/120 using Strep-Cy5 at a dilution of 1/800 (*i.e.* the dilution which gave the highest fluorescence while being the most economically viable), as illustrated in *Figure 5.23*.

However, a high degree of NSB was observed between the BIO-AMP and blocking solution (BSA) as demonstrated in *Figure 5.22*. When no antibody was coated on the surface a high signal was still observed following the addition of varying dilutions of BIO-AMP. Therefore it was necessary to optimise both blocking and diluent reagents to establish the optimum reagent that would give minimal NSB.

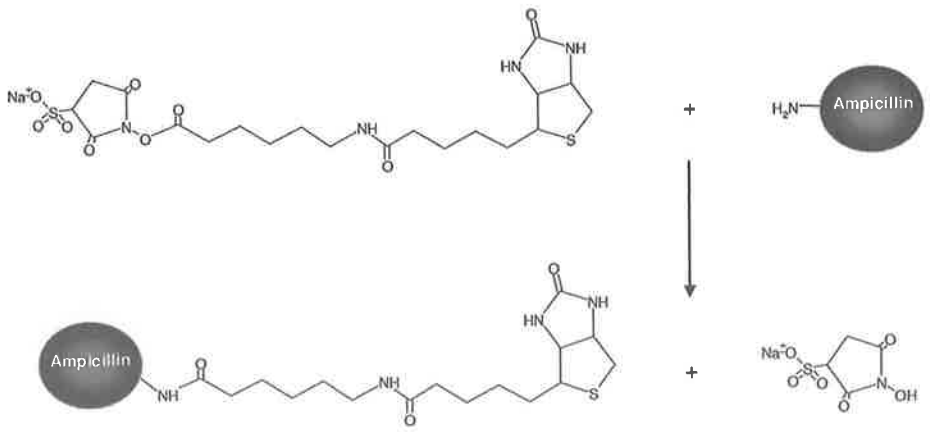


Figure 5.21. *Biotinylation of ampicillin using EZ-Link™ Sulfo-NHS-LC-Biotin reagent (Pierce). The N-hydroxysulfosuccinimide (Sulfo-NHS) esters of biotin react with the primary amine group (-NH2) in pH 7-9 buffers to form stable amide bonds. The biotin derivative has a molecular weight of 556.59Da, a spacer arm length of 22.4Å and is water-soluble.*

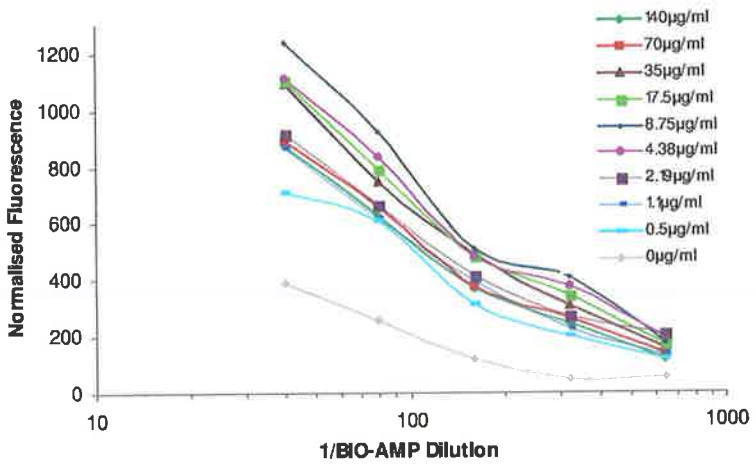


Figure 5.22. *Checkerboard FIA for the determination of optimal ampicillin-specific polyclonal antibody coating concentration and BIO-AMP dilution for use in a competitive FIA. Varying antibody coating concentrations ranging from 0 - 140µg/ml and BIO-AMP dilutions from 1/40 - 1/5,120 were assayed. A 4.38µg/ml antibody concentration and BIO-AMP dilution of 1/120 were chosen for use in a competitive FIA.*

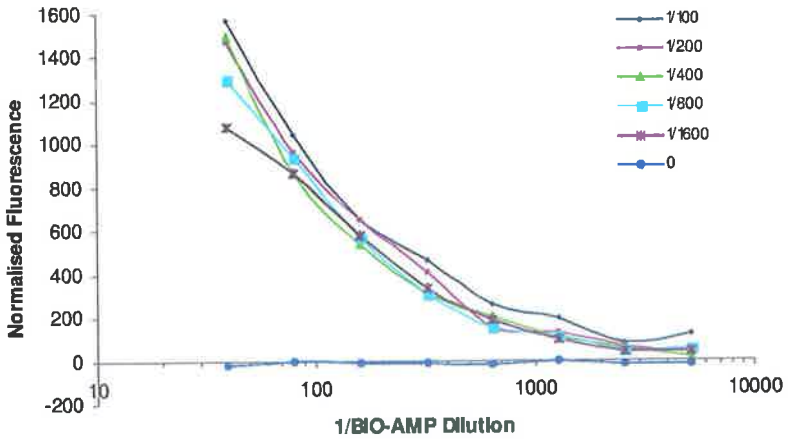


Figure 5.23. *Checkerboard FIA for the determination of optimal BIO-AMP dilution and Strep-Cy5 dilution for use in a competitive FIA. Varying BIO-AMP dilutions ranging from 1/40 – 1/5,120 and Strep-Cy5 dilutions from 1/100 - 1/1,600 were assayed. A 1/120 dilution of BIO-AMP and a Strep-Cy5 dilution of 1/800 were chosen for use in a competitive FIA.*

5.2.6.1.2 *Optimisation of blocking reagents and diluents*

BSA, OVA, casein and the non-ionic detergent Tween 20 were all investigated as potential blocking reagents and diluents. Plates were coated with antibody at the optimal concentration and blocked with PBS containing 2% (w/v) BSA, 2% (w/v) OVA, 2% (w/v) casein or 0.1 - 1 (v/v) Tween 20 surfactant. BIO-AMP, diluted 120-fold was then prepared in various diluents, added to the plate and the respective fluorescence measured following addition of Strep-Cy5 at a dilution of 1/800. Diluents included PBS, PBST (PBS containing 0.05% (v/v) Tween 20), or PBS containing various concentrations of the respective blocking reagent. Raising the ionic strength of the dilution buffers by the addition of various concentrations (0.2 to 0.5M) of sodium chloride was also evaluated as a means to reduce the NSB. Tween 20 was found to be the most suitable blocking reagent as the solution significantly reduced the NSB of the BIO-AMP to the immunoplate, as represented by the bar chart in *Figure 5.24*.

A titre of BIO-AMP against the optimal coating concentration of polyclonal antibody was performed using Tween 20 as a blocking agent. From *Figure 5.25* it can be seen that the use of Tween 20 resulted in only specific interactions between the ampicillin specific antibody and the BIO-AMP. The use of Tween 20 as a blocking agent rather than proteins has previously been shown to be equivalent or even sometimes superior to other methods (Batteiger *et al.*, 1982). However, it should be noted that the non-ionic detergent should not be used as a diluent with coating proteins or protein blocking reagents as it can prevent attachment of proteins to polystyrene or nitrocellulose membranes (Hoffman *et al.*, 1986; Gardas *et al.*, 1988). For subsequent assay development, plates were blocked with a concentration of PBS containing 0.4 (v/v) Tween 20 and BIO-AMP was diluted in PBS.

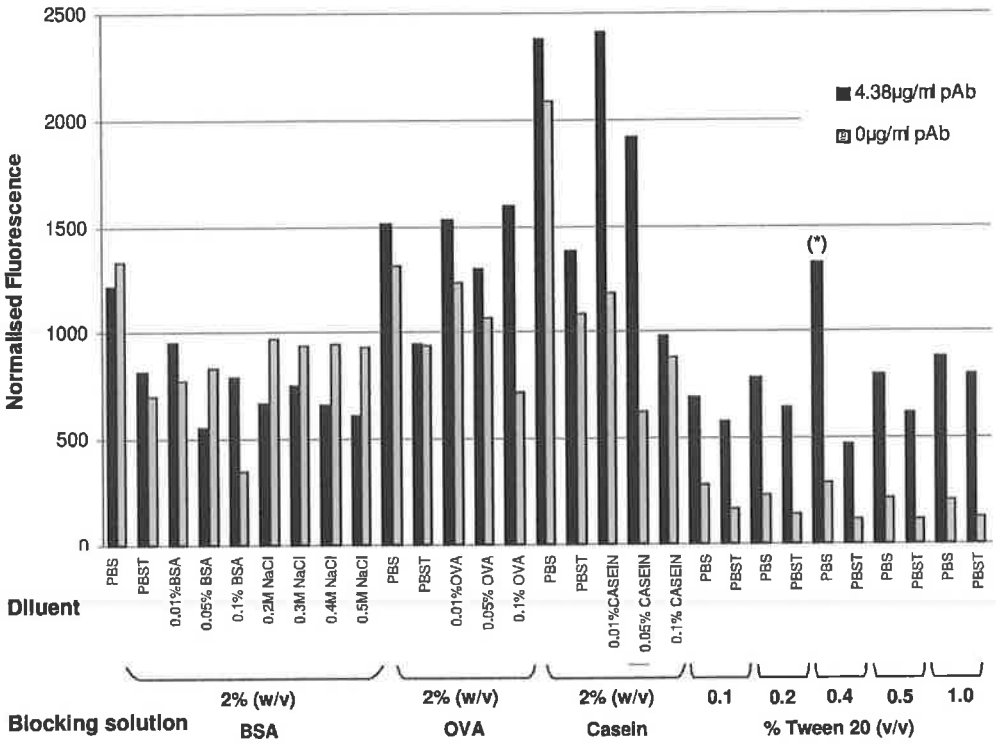


Figure 5.24. *Optimisation of blocking reagent and BIO-AMP diluent. Plates were coated with the optimal concentration of antibody (i.e. 4.38µg/ml) and with PBS containing no antibody (control wells). Each plate was then blocked with a different blocking solution, i.e. PBS containing 2% (w/v) BSA, 2% (w/v) OVA, 2% (w/v) casein or 0.1 - 1 (v/v) Tween 20 surfactant. BIO-AMP, diluted 120-fold, was prepared in various diluent, i.e. PBS, PBST, PBS containing 0.01-0.1% BSA, PBS containing additional sodium chloride (0.2M NaCl-0.5M NaCl), PBS containing 0.01-0.1% OVA and PBS containing 0.01-0.1% casein. Each preparation of BIO-AMP in various diluents was added in duplicate to the plate and the respective fluorescence measured following addition of Strep-Cy5 at a dilution of 1/800. to determine the optimal reagent that would display negligible NSB. Plates blocked with 0.4% (v/v) Tween 20 and the use of PBS as the diluent resulted in minimal non-specific binding (NSB) between the blocking solution and the BIO-AMP preparation (*).*

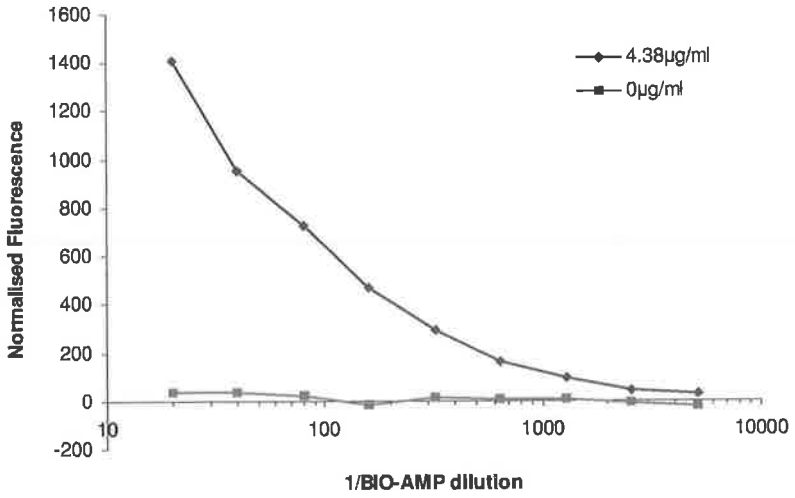


Figure 5.25. *Titre of BIO-AMP against plates coated with ampicillin specific polyclonal antibody and blocked with PBS containing 0.4% (v/v) Tween 20. Only specific binding interactions were observed between the antibody and BIO-AMP.*

5.2.6.1.3 Competitive FIA for the detection of ampicillin in PBS

Using the optimised dilutions determined, a model competitive assay, where free ampicillin in solution and biotinylated ampicillin compete with one another to bind to antibody immobilised on the surface of the plate, was developed. Standards of ampicillin were prepared in PBS ranging from 1.5 to 400,000ng/ml and mixed with an equal volume of BIO-AMP to give a final dilution of 1/120.

Intra-assay and inter-assay variability studies were performed to demonstrate the reproducibility of the competitive FIA. Intra- and inter-day assay CV's ranged from 0.61% to 14.43% and 2.69% to 26.51%, respectively (*Table 5.6*). The assay was found to relatively accurate with back-calculated values found to be within ±15% of the measured concentrations.

Results were normalised by dividing the mean fluorescence obtained at each ampicillin concentration (F) by the fluorescence values determined in the presence of

zero drug (F_0). These values (F/F_0) were plotted against the logarithm of ampicillin concentration using BIAevaluation 4.0.1 software and a four-parameter equation was fitted to the data. *Figure 5.26* shows the FIA inter-day calibration curve using the polyclonal antibody, where the range of detection was found to be between 1,563 and 100,000ng/ml. The assay was found to be far less sensitive (> 200-fold) than the Biacore-based inhibition assay developed for the detection of ampicillin in milk using the same antibody.

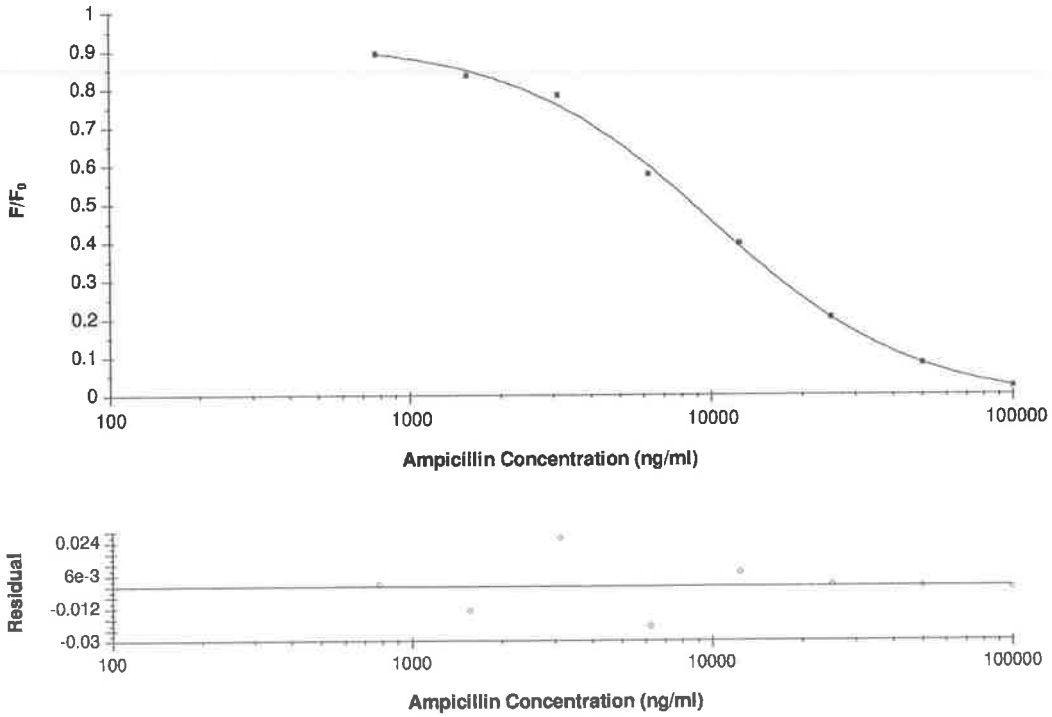


Figure 5.26. *Inter-day calibration curve for competitive FIA for the detection of free ampicillin in PBS using the anti-ampicillin polyclonal antibody. Ampicillin specific antibody was coated on the surface on an immunoplate at a concentration of 4.38µg/ml and a final dilution of 1/120 BIO-AMP was used. Bound BIO-AMP was detected using Strep-Cy5 and the normalised fluorescence calculated. Three replicates of each standard were analysed on three separate days.*

Table 5.6. *Inter-day FIA coefficient of variation (CVs) for the detection of free ampicillin using the ampicllin specific polyclonal antibody. Three sets of each standard were assayed over three days and the CVs calculated. Back-calculated concentrations were determined using BIAevaluation 4.0.1 software and expressed as a percentage of the theoretical concentrations (% recovery).*

Concentration (ng/ml)	**$F/F_0 \pm SD$**	**CV (%)**	**Back calculated concentration (ng/ml)**	**Recovery (%)**
100,000	0.022 ± 0.004	18.18	102,101.25	102
50,000	0.083 ± 0.022	26.51	50,092.41	100
25,000	0.203 ± 0.04	19.70	24,887.65	99
12,500	0.397 ± 0.019	4.79	12,183.62	97
6,250	0.576 ± 0.049	8.51	6,750.69	108
3,125	0.785 ± 0.048	6.11	2,662.67	85
1,562.50	0.835 ± 0.044	5.27	1,794.58	115
781.25	0.893 ± 0.024	2.69	758.00	97

5.2.6.2 Biochip application

The customised biochip system, illustrated in *Figure 5.29*, was developed by the research group led by Prof. Brian MacCraith of the National Centre for Sensor Research (NCSR), DCU (Blue *et al.*, 2005; Stranik *et al.*, 2005). The sensor platform consists of a chip incorporating patented cone structures for enhanced fluorescence detection. On conventional planar biochips the detection system (*e.g.* CMOS camera) is placed either directly above or below the fluorescently-labelled reagent. In these positions, a significant portion of the fluorescence is not captured by the detection system but instead propagates down the length of the planar chip. However the cone structures reflect a much larger fraction of the fluorescence down onto the detector positioned beneath the biochip, as illustrated in *Figure 5.27*. This sensor platform, designed by Prof. Brian MacCraith and colleagues (Patent no. WO02059583), has shown to produce a 25-fold enhancement of luminescence detected (Blue *et al.*, 2005) and has facilitated a 22-fold enhancement of fluorescence detected (personal correspondence with Dr. Helen McEvoy).

The chip, as seen in *Figure 5.28 (A)*, is produced by micro-injection moulding and is housed within a flow cell, enabling sample to be pumped over the sensor surface (*Figure 5.28 (B)*). The excitation and detection set-up employs a laser diode as the light source, as seen in *Figure 5.29*. This causes the fluorescent-labelled antigen bound to the antibodies on the sensor platform to fluoresce and the resultant signal is recorded with a capillary metal oxide semiconductor (CMOS) camera.

In order to demonstrate the capability of the novel platform to function as a highly efficient biochip, its use for the detection of antibiotic residues was investigated. The principle behind the system involves two flow cells, a test and a reference flow cell. A solution containing a known concentration of fluorescently labelled antibiotic is applied to the reference flow cell, while the milk sample mixed with a solution containing a known concentration of fluorescently labelled antibiotic is applied to the test flow cell. In the reference flow cell, the labelled antibiotic binds directly to its corresponding antibody and a reference fluorescent signal is recorded. This fluorescence is directly related to the concentration of labelled antibiotic that has bound to the antibody. While in the test flow cell, the labelled antibiotic and the unlabelled antibiotic in the milk compete with each other to bind to the antibodies on

the sensor platform. By using this approach, the fluorescent signal from both flow cells can be compared and the concentration of antibiotic present in the milk can therefore be determined.

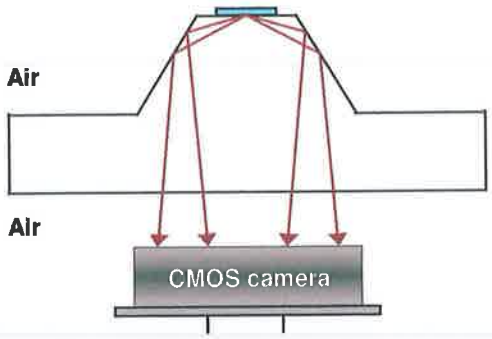


Figure 5.27. *Sensor platform incorporating cone structure for enhancing the capture of fluorescently labelled reagents. The structure designed for this project was that of a frustrated cone, the angle of which was determined by the refractive indices of the cone material, fluorescent layer and the environment. The angle of the cone structure allows total internal reflection of the emitted fluorescence and directs it towards the CMOS camera, which was placed directly beneath it.*

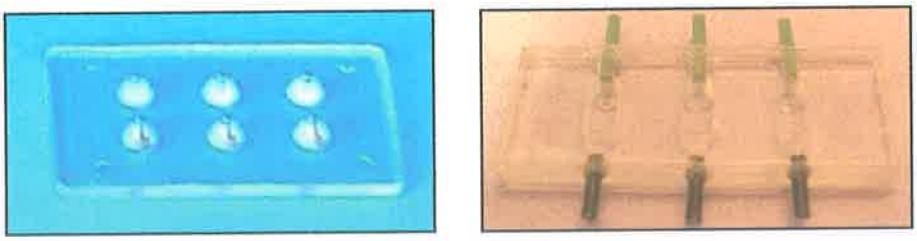


Figure 5.28. *Images of* ***(A)*** *the sensor chip platform incorporating enhanced fluorescence capture structures and* ***(B)*** *housed within a flow cell, enabling sample to be pumped over the sensor surface.*

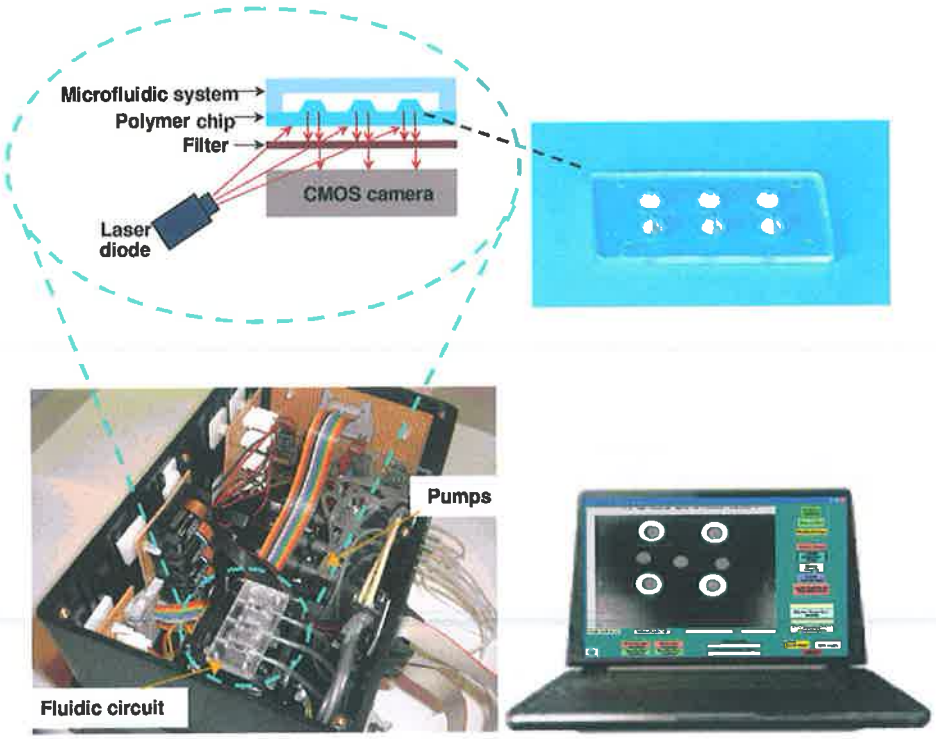


Figure 5.29. *The prototype portable biochip system containing pumps, fluidic circuit, interrogation optics and imaging system. Test samples are mixed with a known quantity of fluorescently labelled analyte and pumped over the antibody immobilised biochip sensor platform. A laser light is used to excite any bound fluorescently labelled analyte and background fluorescence is reduced by an optical filter. The resulting fluorescent signal is captured using a complementary metal oxide semiconductor (CMOS) camera and the signal monitored on a laptop with imaging software.*

Antibodies, at a concentration of 14ng/µl, were immobilised on the surface of the polystyrene cone structures by passive adsorption and any remaining binding sites on the biochip blocked, as described in *Section 2.5.5.4*. Following washing, the chambers were filled with a solution of BIO-AMP, the chip incubated and bound BIO-AMP detected following the addition of Strep-Cy5. The fluorescence signal was captured with a CMOS camera and the resulting image is shown in *Figure 5.30 (C)*.

Mean fluorescent intensity values (n=3) of 151,528 ± 11,913 and 41,910 ± 9,979 were recorded when 14ng and 0ng of antibody were immobilised on the surface of the cones, respectively (*Figure 5.30 (C) (i) and (ii)*). CVs were calculated to determine the reproducibility of spot intensities and were found to be 7.86% and 23.81%, for the antibody immobilised and control cones, respectively. The fluorescent signal from a blank chip was also recorded to estimate the degree of background fluorescence (*Figure 5.30 (B)*). Normalised values (minus background fluorescence) were therefore found to be 121,111 and 11,493 for the antibody immobilised and control sensor surfaces (*Figure 5.30 (C) (i) and (ii)*).

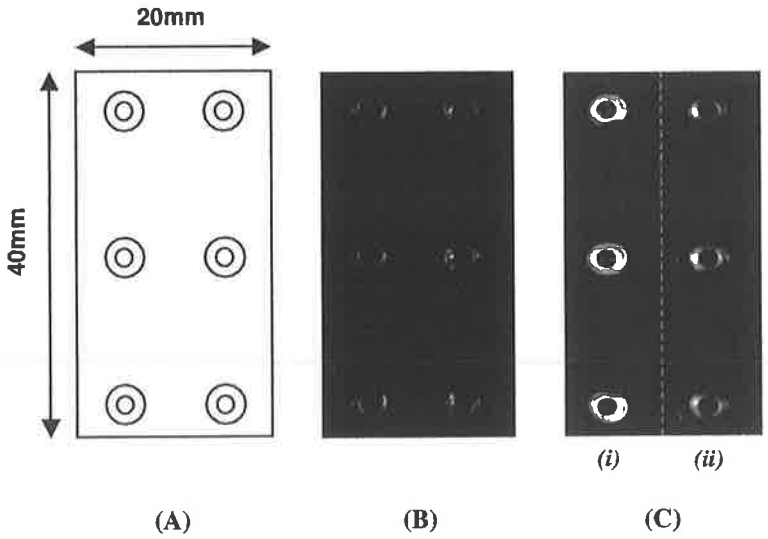


Figure 5.30. *(**A**) Schematic of the patented polystyrene biochip incorporating enhanced fluorescence capture structures. (**B**) Captured image of blank control chip (mean fluorescent intensity (n=6) was found to be 30,417 ± 4,532). (**C**) Captured image of test chip. BIO-AMP was added and captured by ampicillin specific antibodies immobilised on the surface of the chip. Following the addition of Strep-Cy5, the fluorescence was captured with a CMOS camera. (**i**) Antibody immobilised cones (n=3) with a mean fluorescent intensity of 151,528 ± 11,913 and (**ii**) no antibody immobilised (n=3) with a mean fluorescent intensity of 41,910 ± 9,979.*

5.2.7. Selection of recombinant antibodies to ampicillin

An immune phage display antibody library was constructed using a system developed by Krebber *et al.* (1997) for the isolation of a single chain variable fragment (scFv) specific for ampicillin. A naïve antibody library, donated by Cambridge Antibody Technology (CAT) was also screened for the selection of an anti-ampicillin scFv.

5.2.7.1 Production of an immune murine antibody library against ampicillin

An immune library was created for the isolation of an scFv with the highest affinity to ampicillin possible. The immune system of a mouse was primed by sub-cutaneous immunisation with an AMP-BSA conjugate over a twelve-week period, as described in *Section 2.5.2.1*. Tail bleeds were performed seven days post-immunisation, the blood collected and the recovered serum screened against an AMP-THY conjugate for specific antibodies, as described in *Section 2.5.2.2*. When a sufficient titre was attained (>1/100,000), the mouse RNA was isolated from the spleen of a mouse using a standard Trizol method (*Section 2.5.6.1*) and the RNA concentration was estimated. First strand cDNA was synthesised from mRNA using an AMV reverse-transcription polymerase chain reaction (RT-PCR) kit (Promega), as described in *Section 2.5.6.2*. This cDNA, shown in *Figure 5.31*, was then used as a template for the subsequent amplification of heavy and light chain antibody genes using the primers as listed in *Section 2.5.6.3*.

For successful gene amplification all components must be optimal. A commercial PCR optimiser kit was used to determine the optimal magnesium and pH conditions. Both of these factors can have an effect on primer-template specificity, enzyme activity and denaturation temperature. The buffers, listed in *Figure 5.32*, have varying magnesium concentrations (ranging from 1.5mM to 3.5mM Mg^{2+}) with varying pH (pH 8.5 to 10). In this case buffer D (3.5mM Mg^{2+}, pH 8.5) proved optimal for the amplification of heavy chain DNA (*Figure 5.32*), while buffer B (2.0mM Mg^{2+}, pH 8.5) contained the optimal pH and magnesium concentrations for amplification of light chain DNA (*Figure 5.33*).

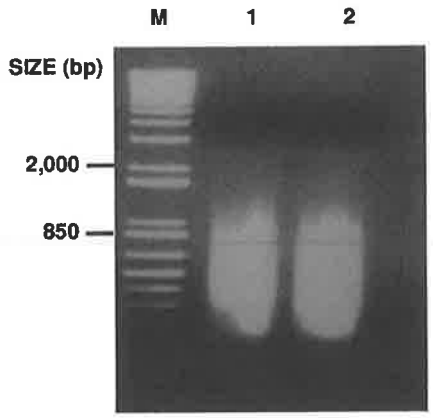


Figure 5.31. *Complementary DNA (cDNA) was synthesised by reverse transcription PCR of mRNA extracted from the spleen of a mouse immunised with AMP-BSA. Lanes:* ***(M)*** *1Kb plus DNA ladder; (1-2) cDNA.*

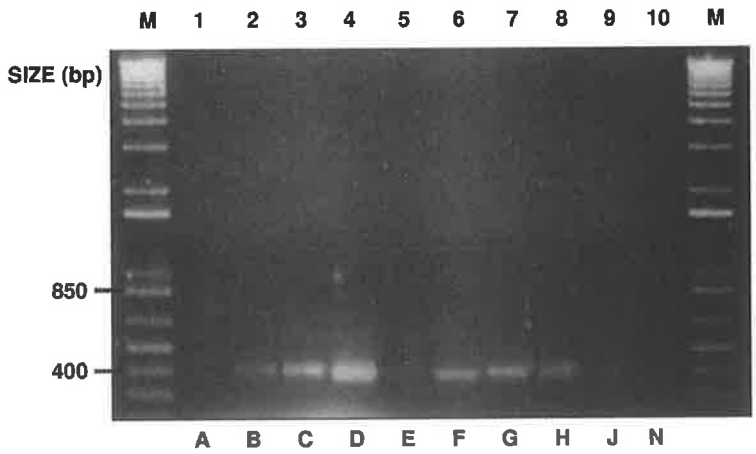


Figure 5.32. *Determination of optimal magnesium and pH conditions for amplification of variable heavy (V_H) chain genes. In this case buffer D proved optimal. A specific 400bp band can be seen for the V_H fragments. Lanes:* ***(M)*** *1Kb plus DNA ladder;* ***(1-10)*** *Heavy chain DNA using various buffers.*

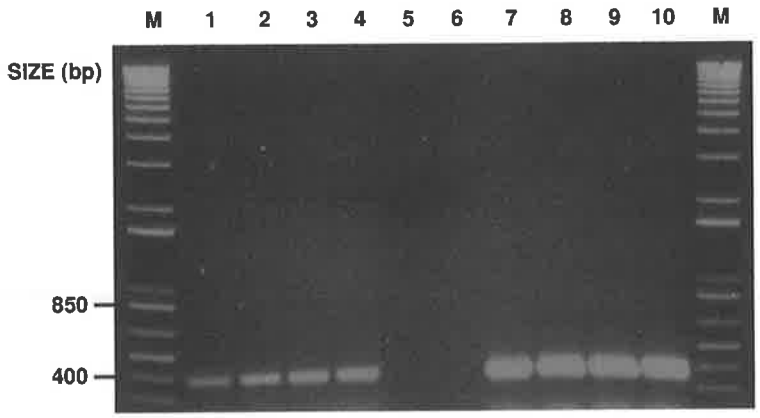


Figure 5.33. *Amplification of variable heavy (V_H) and light (V_L) chain genes. A specific band can be seen at 400bp for both the V_H and V_L fragments. Lanes:* ***(M)*** *1Kb plus DNA ladder;* ***(1-4)*** *Amplified heavy chain DNA;* ***(7-10)*** *Amplified light chain DNA.*

Following amplification of heavy and light chain genes, the fragments were excised from the agarose gel, purified using a commercial DNA purification kit (Eppendorf Perfectprep® Gel Cleanup Kit) and quantified (*Figure 5.34*). Purified variable heavy and light DNA fragments were then assembled by splice by overlap extension (SOE) PCR to form a single chain variable fragment (scFv) of approximately 800bp (*Figure 5.35*). This scFv fragment was digested with the unique restriction enzyme *Sfi*I prior to insertion into a phage display vector pAK100. The restricted scFv fragment, seen in *Figure 5.36*, was purified, quantified and ligated into a previously digested pAK100 vector (*Figure 5.37-5.38*). The vector containing the insert was then transformed into a suitable expression host, in this case competent *E. coli* XL-1 Blue cells. However, initial transformation into the competent XL-1 Blue cells yielded a library of only 1 x 10^2 cfu/ml. The transformation was repeated using electrocompetent *E. coli* XL-1 Blue cells, resulting in an increased library size of 7.4 x 10^3 cfu/ml. The recombinant library was subjected to a number of rounds of biopanning against a number of drug conjugates for the selection of an scFv to ampicillin. However, an scFv specific for ampicillin was never isolated.

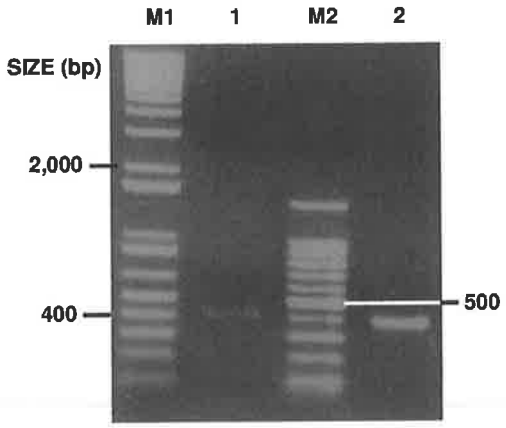


Figure 5.34. *Quantification of purified heavy and light chain DNA. Lanes:* ***(M1)*** *1Kb plus DNA ladder;* ***(1)*** *Purified heavy chain DNA;* ***(M2)*** *Quantification marker (100bp to 1Kb, the 500bp band contains 150ng of DNA, while the other 9 bands contain 5ng when 5µl of the marker is loaded on a gel, allowing quantification of purified fragments);* ***(2)*** *Purified light chain DNA. From the gel picture it was estimated that purified heavy chains and light chains contained approximately 25ng/5µl and 100ng/5µl of DNA, respectively.*

Figure 5.35. *Determination of optimal magnesium and pH conditions for SOE PCR amplification. For this reaction buffer C proved optimal. A specific band at 800bp was seen when the PCR product was run on an agarose gel. Lane:* ***(M)*** *1Kb plus DNA ladder;* ***(1-8)*** *assembled scFv.*

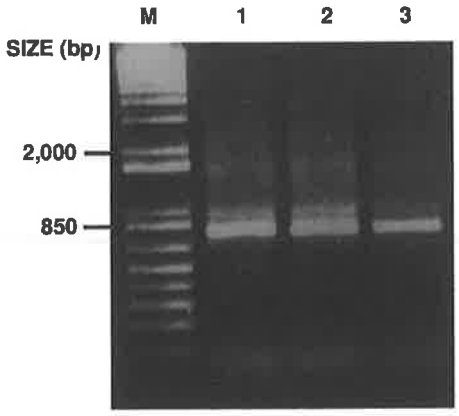


Figure 5.36. *SfiI digestion of scFv fragment. Lanes:* ***(M)*** *1Kb plus DNA ladder;* ***(1-2)*** *SfiI digested SOE product;* ***(3)*** *undigested SOE product.*

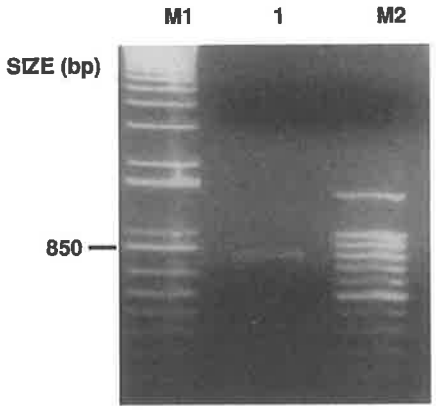


Figure 5.37. *Quantification of purified SfiI digested SOE. Lanes:* ***(M1)*** *1Kb plus DNA ladder;* ***(1)*** *purified digested SOE product;* ***(M2)*** *Quantification marker (100bp – 1Kb). The 500bp band contains 150ng of DNA, while the other 9 bands contain 50ng when 5µl of the marker is loaded on a gel, allowing quantification of purified fragments. From the gel picture it was estimated that the purified SfiI digested SOE product contained approximately 15ng/5µl of DNA.*

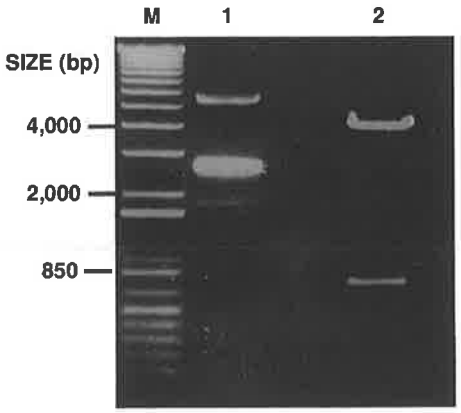


Figure 5.38. *Sfi 1 digestion of pAK 100 plasmid. Lanes:* ***(M)*** *1Kb plus DNA ladder;* ***(1)*** *undigested plasmid;* ***(2)*** *SfiI digested plasmid with an 800bp scFv fragment removed.*

5.2.7.2 Selection of scFvs specific for ampicllin from a naïve human antibody library

A naïve human recombinant library, donated by Cambridge Antibody Technology Ltd. (CAT, Cambridge, U.K.) was screened by bio-panning for the isolation of an scFv specific for ampicillin. This extremely diverse library consists of approximately 1.4 x 10^{10} phage-displaying scFv clones (Vaughan *et al.*, 1996). A number of rounds of biopanning were performed against a number of different ampicillin conjugates coated on Nunc Maisorb^{TM} immunotubes. In addition, a number of different phage elution methods were used, including both non-specific elution (100mM glycine-HCl or 100mM triethylamine) and specific elution (free ampicillin). Following screening by phage ELISA, a number of positive binders were identified. However, when each clone was retested, it was found that they recognised the protein carrier and not the ampicillin.

5.3 Discussion

This chapter described the production and characterisation of polyclonal antibodies to ampicillin and their subsequent use in the development of immunoassays for the detection of the antibiotic in milk. A number of ampicillin-protein immunogen conjugates were successfully produced and characterised indirectly by immunisation of Balb/c mice followed by the determination of the specific antibody produced in serum. An AMP-BSA conjugate induced a high antibody response to ampicillin in a mouse and further investigation revealed the resulting antiserum was specific to the intact β-lactam ring structure. The conjugate was used for immunisation for production of polyclonal antibodies and the anti-ampicillin antibodies were purified from the sera by Protein G and subtractive immunoaffinity chromatography. The purification process was assessed by SDS-PAGE and the presence of rabbit IgG molecules was confirmed by Western blotting.

The polyclonal antibodies were initially used in an ELISA format for the detection of ampicillin in PBS. The resulting ELISA proved reproducible, with inter-assay CVs less than 11%. However, the assay was not as sensitive as desired, with a limit of detection of approximately 73ng/ml, significantly higher than the current EU maximum residue limit (MRL) of 4ng/ml for ampicillin in milk.

In an attempt to improve assay sensitivity, a Biacore inhibition assay employing the polyclonal antibodies and a directly immobilised ampicillin CM5 chip, was developed. Gaudin *et al.* (2001) suggested that an antibody that recognises the closed β-lactam ring would not recognise the ampicillin surface because the β-lactam ring would be quickly hydrolysed during immobilisation or by the continuous flow of HBS buffer. However, this was not found in this case as the anti-ampicillin antibodies were shown to specifically bind the ampicillin-immobilised surface and binding was also inhibited with the addition of free ampicillin with intact ring structure.

An optimal concentration of 44μg/ml of polyclonal antibody was used in the development of the preliminary assay in HBS. Higher amounts of antibody are often required in biosensor assays due to the continuous flow over the sensor surface. Cliquet *et al.* (2005) describe the use of penicillin-specific polyclonal antibody, at a concentration of 50μg/ml, for the detection of penicillins in a Biacore assay. It was

reported by Haasnoot *et al.* (2003) that ten times more antibodies were required in a biosensor assay compared to an ELISA. However the Biacore inhibition assay demonstrated improved reproducibility (in comparison to the ELISA) with intra- and inter-assay CVs below 1.5% and 9.92%, respectively. Percentage recovery values indicated reasonable accuracy with values ranging between 91 and 111% at lower ampicillin concentrations. The assay, with a range of detection between 24.4 and 400,000ng/ml was found to have a LOD of 11.5ng/ml, representing a 6-fold increase in sensitivity compared to the ELISA.

The ability of the biosensor assay employing the anti-ampicillin antibodies to detect the antibiotic in a real sample matrix was then investigated. Pasteurised and homogenised milk was defatted to minimise any matrix effects on the optical biosensor. Regeneration studies were performed to assess the stability of the ampicillin immobilised CM5 sensor surface to determine its suitability for analysing a large number of samples. Various regeneration solutions were evaluated, including glycine (pH 3) and sodium hydroxide containing 20% (v/v) acetonitrile but sodium hydroxide (100mM) proved optimal for complete regeneration of the sensor surface. Although this solution is quite stringent, it did not have an adverse affect on the stability of the ampicillin-immobilised chip surface. No loss of binding activity was observed following fifty binding-regeneration cycles. Over the period of assay development in HBS the ampicillin sensor surface went through over 250 binding-regeneration cycles over a two-week period without a significant (<14%) decrease in binding response.

A Biacore inhibition assay employing defatted milk spiked with ampicillin was successfully developed and displayed increased assay sensitivity. Intra- and inter-variability studies demonstrated adequate reproducibility within the 20% acceptance limit for assay precision (Findlay, 2000). Intra- and inter-day assay CVs were below 8.22% and 17.30%, respectively. The increase in variation compared to the assay developed in HBS was thought to be due to the complexity of the matrix and the variation within each milk sample. The range of detection of the assay for milk spiked with ampicillin was found to be between 6 and 100,000ng/ml. The LOD was found to be 3.6ng/ml, which is below the required sensitivity set out by the EU MRL of 4ng/ml (EEC regulation no. 2377/90). The increase in sensitivity compared to the assay

developed in buffer was due to the more reproducible results obtained in the milk assay, as the limit of detection was by selecting the mean response minus three standard deviations for the negative standard (no ampicillin). Although spiked samples do not mimic real unknown milk samples, the assay could potentially be a valuable tool for the detection of ampicillin in raw milk.

The degree of cross-reactivity of the polyclonal antibody with six structurally related penicillins and two members of cephalosporins was also determined in a Biacore inhibition assay format. Percentage cross-reactivities ($\%CR_{90}$ and $\%CR_{50}$), were estimated by comparing the relevant LDD_{90} and IC_{50} values of the potential cross-reactants to those values of ampicillin. The polyclonal antibody displayed the highest levels of cross-reactivity with carbenicillin (22-35%). Carbenicillin, a more stable analogue of ampicillin, is identical in structure to ampicillin with the exception of a carboxyl group substituted for the primary amine. Variation (in the case of cloxacillin, oxacillin, amoxicillin and dicloxacillin) or removal (in the case 6-APA) of the side chain at the R_1 position led to a significant decrease in cross-reactivity. The polyclonal antibody demonstrated negligible cross-reactivity with the cephalosporin compounds.

The applicability of a disposable, antibody-based biochip sensor for the detection of ampicillin was investigated. Initially reagents were optimised and tested in a fluorescent immunoassay (FIA) plate format as the proposed biochip detection system was based on fluorescence. For this purpose, ampicillin was biotinylated for use in a competitive assay, whereby unlabelled ampicillin competes for antibody binding sites with a known quantity of biotinylated ampicillin and the biotinylated antibiotic could then be traced using a commercially available Strep-Cy5. Unreacted biotin was removed by the addition of Affi-gel 102 (Bio-Rad), an amino-terminal cross-linked agarose gel, and BIO-AMP recovered by centrifugation. However, initial experiments revealed a high degree of NSB between the BIO-AMP and blocking solution (BSA). Therefore, both blocking and diluent solutions were optimised to establish the optimum reagent that would give minimal NSB. The use of Tween 20 as a blocking reagent was found to be the most suitable as the solution significantly reduced the NSB of the BIO-AMP to the immunoplate.

The optimal reagents and dilutions were then used in the development of a model competitive FIA in PBS. The assay proved less reproducible than both the ELISA and Biacore assay with intra- and inter-day assay CV's as high as 14.43% and 26.51%, respectively. The assay was found to relatively accurate with percentage recovery values ranging from between 85 and 115%. However, the assay displayed poor sensitivity with a range of detection between 1,563 and 100,000ng/ml. This represents a dramatic decrease in sensitivity (>200-fold) compared to that of the Biacore-based inhibition assay developed for the detection of ampicillin in milk. The poor sensitivity was thought to be due to the crudely purified biotinylated ampicillin used in assay development. Therefore, the actual concentration of free ampicillin in solution was thought to be far greater than the theoretical concentration. An amino-terminal cross-linked gel, *i.e.* Affi-gel 102 (Bio-Rad), was used to remove any remaining free actiavetd biotin However, biotinylated ampicillin was not purified from non-biotinylated ampicillin resulting in a heterogeneous solution. Various attempts were made to purify the solution including the use of a monomeric avidin resin (Pierce) and a Float-A-Lyzer® dialysis kit (500Da cut-off)(Spectrum® Laboratories, Inc.) but none proved to be successful. Purifying ampicllin via a monomeric avidin column exposed the drug to harsh regeneration solutions during the method, increasing the possibility of ring hydrolysis. The synthesis and purification of fluorescent derivatives is often difficult and expensive due to the instability of the β-lactam ring. However, a novel fluorescent penicillin analogue has been generated for use in an automated fluorescent immunoassay for the analysis of β-lactam antibiotics (Benito-Peña *et al.*, 2005).

Although future work on improving the sensitivity of the FIA is required, the applicability of the biochip system for the detection of antibiotic residues was still investigated. The ampicillin-specific antibodies were immobilised on the surface of the polystyrene chip cone structures and following the addition of BIO-AMP and Strep-Cy5 a fluorescence signal was captured with a CMOS camera. CVs were calculated to determine the reproducibility of spot intensities and were found to be 7.86% and 23.81%, for the antibody immobilised and control cones, respectively. The fluorescent signal from the antibody-immobilised cones was found to be ten times higher than that from the control cones. The antibody consumption was low with only 14ng per cone required to obtain a signal (50 times less antibody than required to

perform a single analysis using the Biacore-based assay). These results indicated that the system could detect fluorescently labelled ampicillin captured by specific antibodies immobilised on the surface of the biochip sensor and could be potentially useful for the detection of antibiotic residues in milk in a laboratory, farm or factory environments. The miniaturised size of the detection system makes it highly suitable for field-testing purposes in comparison to larger and more complicated immunosensors. The cheap, mass-producible chip platforms enabling enhanced fluorescence could also be applied to other food contaminants simply by changing the antibody immobilised on the surface and are promising alternative to the more conventional ELISA method.

A murine recombinant antibody library was also generated for the isolation of an scFv specific to ampicillin, which might display greater sensitivity than the polyclonal antibody in an immunoassay. Recombinant antibody technology allows an increase in the speed of production of antibodies and the possibility of altering affinity and specificity. Also with the development of new expression vectors, it is possible to engineer tags into the antibody sequence to facilitate site-directed orientation on a sensor surface (*e.g. in vivo* biotinylation technique as described in *Chapter 4*). The mRNA isolated from the spleen of a mouse immunised with an AMP-BSA conjugate was used to generate the phage display library against ampicillin using the system developed by Krebber *et al.* (1997). Electrocompetent *E. coli* XL-1 Blue cells with high efficiency were used for library transformation resulting in $7.4x10^3$ transformants. The size of the library generated was very small with a library size of between 10^6-10^8 transformants expected. The library was screened against a number of different ampicillin conjugates for the selection of an scFv specific for the drug. This screening process involves multiple rounds of phage binding to the immobilised drug conjugate, subsequent washing to remove any non-specific phage and finally elution to retrieve specific binding phage. A number of scFvs were isolated against the protein moiety of the conjugate; however, an scFv specific for ampicillin was not isolated. A naïve human recombinant library (CAT Ltd., UK) with greater diversity, *i.e.* approximately 1.4×10^{10} phage-displaying scFv, was screened by bio-panning for the isolation of an scFv specific for ampicillin. A number of rounds of biopanning were performed against a number of different ampicillin conjugates coated on Nunc Maisorb™ immunotubes. In addition, a number of different phage elution methods

were used, including both non-specific elution (with acidic or basic solutions) and specific competitive elution (with excess free ampicillin). Following screening by phage ELISA, a number of positive binders were identified. However, when each clone was retested, it was found that they recognised the protein carrier and not the ampicillin. These results highlight the importance of both library diversity and efficient selection procedures for the isolation of specific antibodies from phage display libraries. Both factors are of critical importance for isolation of specific antibodies and need to be further optimised for future selection of an scFv specific for ampicillin.

In summary, polyclonal antibodies raised against an ampicllin-protein conjugate were evaluated in terms of their potential application for detection of ampicillin in milk. Preliminary studies using an ELISA format demonstrated poor sensitivity, with a limit of detection of 73ng/ml for ampicillin in buffer. However, a Biacore-based assay facilitated a 6-fold increase in sensitivity for the detection of ampicillin in processed milk. The potential applicability of a disposable, antibody-based biochip sensor for the detection of ampicillin was also investigated. Initially a fluorescence-based assay was developed on a plate format prior to biochip assay development and all reagents were optimised. However, the resulting assay displayed poor sensitivity due to the crude preparation of biotinylated ampicillin used in assay development. Although future work on improving the sensitivity of the FIA is required, the applicability of the biochip system for the detection of antibiotic residues was investigated. The ampicillin-specific antibodies were successfully immobilised on the surface of the polystyrene chip cone structures and following the addition of BIO-AMP and a Strep-Cy5 conjugate, a fluorescence signal was captured with a CMOS camera. In an attempt to generate antibodies with increased affinity for ampicillin, a murine recombinant antibody library was also generated. However, despite the use of various screening and elution procedures, an scFv specific to ampicillin was never isolated.

Chapter 6

Overall conclusions

6.1 Overall summary and conclusions

The overall aim of this research was to develop novel immunoassays for the detection of the food contaminants, aflatoxin B_1 and ampicillin, and for the diagnosis of *Corynebacterium pseudotuberculosis* infection in sheep.

In Chapter 3, studies focused on the development of sensitive and specific antibody-based assays for the detection of antibodies to *C. pseudotuberculosis*, the causative agent of caseous lymphadenitis (CLA). The previously cloned phospholipase D (PLD) gene from *C. pseudotuberculosis* (Songer *et al.*, 1990) was successfully produced from the high-level expression vector pTrcHisB in *E.coli* XL10-Gold cells and several parameters affecting recombinant protein expression were optimised. Following IMAC purification of the histidine-tagged PLD protein, milligram quantities (~30mg/L culture) of functional protein were obtained from standard laboratory shake-flask cultures. The ability of the recombinant PLD exotoxin to discriminate between sera from CLA-infected sheep from those with no prior history of infection was confirmed by Western blot analysis and the protein was applied for the development of an indirect ELISA and Biacore-based assay for the diagnosis of CLA in sheep. Assays were validated using a method described for the validation of serological assays for the diagnosis of infectious diseases (Jacobson, 1998) and initial feasibility studies were carried out on nine serum controls selected from sheep experimentally infected with *C. pseudotuberculosis* and sheep free of infection. Optimised assay formats were subsequently applied to a range (n=92) of clinical samples from Irish herds and initial evaluation of the assays yielded promising results. Diagnostic sensitivity (DSn) and specificity (DSp), calculated as a comparison to those results obtained in the ELISA developed in Lelystad, averaged from 76-85% for both assays. The Biacore assay, in particular, proved to be a valuable tool for high-throughput, automated screening of serum samples for *C. pseudotuberculosis* infection. The assay also proved to be highly sensitive and results reflected those obtained using the indirect PLD ELISA with 91.3% of serum samples giving the same result.

Chapter 4 focused on recombinant antibody library construction and selection of phage displaying-Fab antibodies derived from a parent anti-AFB_1 scFv. Two Fab clones, one with identical variable regions comparative to the parent scFv (D11) and

one with a single point mutation in the CDRH3 (G6), were isolated from the recombinant antibody library. The potential benefits of converting an scFv to a Fab format, including expression, stability, sensitivity and specificity, and the affect of the amino acid substitution in the mutant G6 Fab, were addressed. In terms of expression, the yields of histidine-purified scFv and Fab fragments were found to be equivalent and milligram quantities of functional antibodies were produced in standard lab shake-flask cultures. The Fab fragments displayed increased stability in comparison to their scFv counterparts, which was thought to be due to the influence of the additional constant regions and the presence of the interchain disulphide bond. When the three anti-AFB_1 antibody fragments were applied to the development of ELISA and Biacore-based assays, the D11 Fab and parent scFv displayed similar sensitivity, taking into account experimental error. However, the mutant clone (G6) demonstrated increased assay sensitivity and specificity comparative to the parent scFv and D11 Fab fragments. The ELISA-based assay, employing the G6 Fab, was estimated to have a limit of detection of 1.3ng/ml for AFB_1, which is below the required European Union maximum levels, currently set at 2-8ng/g for AFB_1 in various food types. Cross-reactivity studies carried out on the G6 Fab fragment revealed the antibody also displayed greater specificity towards B_1, in comparison to the D11 Fab and scFv antibody fragments. The advantages of the SPR-based biosensor, Biacore™, over conventional ELISA assays were also highlighted. Biacore-based assays employing the anti-AFB_1 antibody fragments enabled a significant increase (up to 27-fold) in sensitivity over the ELISA and displayed excellent precision. The limit of detection of 117pg/ml for AFB_1 using the G6 Fab-based assay compares favourably with other methods of detection in the published literature.

Finally, the advantages of an *in vivo* biotinylation strategy were highlighted. The three antibody fragments were subcloned into the pAK400Bio vector for subsequent expression of *in vivo* biotinylated antibodies in *E. coli* AVB100 cells. The scFv was successfully biotinylated *in vivo* during expression and demonstrated no decrease in sensitivity following biotinylation when applied to an ELISA format. The Fab fragments were unable to be detected with an extravidin conjugate suggesting poor expression yields of biotinylated fragment or an inaccessible biotin tag. However, it is though that these problems could be overcome in the future with further optimisation

of expression conditions or the incorporation of a flexible linker to overcome conformational constraints.

In Chapter 5, polyclonal antibodies raised against an ampicllin-protein conjugate were evaluated in terms of their potential application for detection of ampicillin in milk. Although preliminary studies using an ELISA format demonstrated poor sensitivity, a Biacore-based assay facilitated greater sensitivity for the label-free detection of ampicillin in processed milk. The ampicillin-specific polyclonal antibodies displayed a high avidity for a directly immobilised ampicillin surface and high concentrations of free ampicillin were required to totally inhibit binding to the analyte surface. However, despite some non-specific interactions encountered, the antibodies were found to be capable of detecting ampicillin at an estimated concentration of 3.6ng/ml in spiked defatted milk (below the required European Union MRL set at 4ng/ml). The potential applicability of a disposable, antibody-based biochip sensor for the detection of ampicillin was also investigated. Initially a fluorescence-based assay was developed on a plate format prior to biochip assay development and all reagents were optimised. However, the resulting assay displayed poor sensitivity compared to that of the Biacore-based inhibition assay due to the crude preparation of biotinylated ampicillin used in assay development. Although future work on improving the sensitivity of the FIA is required, the applicability of the biochip system for the detection of antibiotic residues was still investigated. The ampicillin-specific antibodies were successfully immobilised on the surface of the polystyrene chip cone structures and following the addition of BIO-AMP and a Strep-Cy5 conjugate, a fluorescence signal was captured with a CMOS camera. The system enabled rapid detection of biotinylated ampicillin with a significant decrease in reagent volumes required. In an attempt to generate antibodies with increased affinity for ampicillin, a murine recombinant antibody library was also generated. However, despite the use of various screening and elution procedures, an scFv specific to ampicillin was not isolated. Both library construction and selection procedures need to be further optimised for future selection of an scFv specific for ampicillin.

In summary, this work highlights the potential use of immunoassays for the detection of food contaminants and the diagnosis of infectious diseases. It demonstrates the use of an incremental process for the development and validation of sensitive and specific

antibody-based assays for the detection of caseous lymphadenitis. The research also emphasised the use of recombinant antibody technology and demonstrated how genetically-derived antibody fragments can be further engineered to increase sensitivity and incorporate novel peptide tags. Finally, the use of biosensors for rapid, automated detection of antibiotic residue contamination in milk was explored. Although spiked samples do not mimic real unknown samples, the assay could be potentially useful for the detection of ampicillin in raw milk.

Future work arising from the body of this research could entail further validation of the PLD Biacore-based assay using a larger number of serum samples and subsequent application to determine the prevalence of CLA disease in Ireland. Biacore is presently assessing the use of the AFB_1-specific G6 Fab in future commercial applications. Mutagenesis techniques are also currently being employed to further modify the antibody, which could potentially lead to increased sensitivity or broader specificity. Finally, the use of the *in vivo* biotinylation technique for orientated immobilisation of antibody fragments onto the biochip platforms described, would facilitate the generation of highly sensitive, cheap, mass-producible arrays applicable to laboratory, farm or industrial environments.

Chapter 7

References

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Appendix

LELYSTAD ELISA-POSITIVE RESULTS											
		PLD		NO PLD		NORMALISED		RESULTS			
	ID	OD 1	OD 2	OD 1	OD 2	OD 1	OD 2	MEAN	STDEV	CV	Mean/Pos
1	3	0.804	0.552	0.157	0.089	0.647	0.463	0.555	0.130	23.44	35.90%
2	4	2.345	1.991	0.175	0.085	2.17	1.906	2.038	0.187	9.16	132.00%
3	9	1.858	1.867	0.072	0.117	1.786	1.75	1.768	0.025	1.44	114.50%
4	10	2.102	2.009	0.193	0.139	1.909	1.87	1.890	0.028	1.46	122.40%
5	12	0.691	0.664	0.101	0.083	0.59	0.581	0.586	0.006	1.09	37.90%
6	19	0.328	0.34	0.111	0.094	0.217	0.246	0.232	0.021	8.86	15.00%
7	20	0.339	0.332	0.108	0.095	0.231	0.237	0.234	0.004	1.81	15.20%
8	23	1.083	0.843	0.208	0.269	0.875	0.574	0.725	0.213	29.38	46.90%
9	28	0.471	0.453	0.159	0.159	0.312	0.294	0.303	0.013	4.20	19.60%
10	32	0.549	0.552	0.101	0.106	0.448	0.446	0.447	0.001	0.32	29.00%
11	35	0.312	0.413	0.135	0.168	0.177	0.245	0.211	0.048	22.79	13.70%
12	36	1.198	1.149	0.139	0.104	1.059	1.045	1.052	0.010	0.94	68.10%
13	40	0.679	0.58	0.088	0.094	0.591	0.486	0.539	0.074	13.79	34.90%
14	43	0.657	0.674	0.08	0.085	0.577	0.589	0.583	0.008	1.46	37.80%
15	50	0.576	0.561	0.113	0.121	0.463	0.44	0.452	0.016	3.60	29.20%
16	55	0.449	0.517	0.285	0.324	0.164	0.193	0.179	0.021	11.49	11.60%
17	63	0.211	0.203	0.083	0.093	0.128	0.11	0.119	0.013	10.70	7.70%
18	104	0.76	0.982	0.123	0.15	0.637	0.832	0.735	0.138	18.77	47.60%
19	108	0.457	0.873	0.085	0.092	0.372	0.781	0.577	0.289	50.17	37.30%
20	114	1.122	1.114	0.214	0.025	0.908	1.089	0.999	0.128	12.82	64.70%
21	115	1.07	0.559	0.147	0.115	0.923	0.444	0.684	0.339	49.55	48.50%
22	123	0.491	0.487	0.087	0.151	0.404	0.336	0.370	0.048	13.00	26.30%
23	124	1.13	1.178	0.103	0.119	1.027	1.059	1.043	0.023	2.17	74.10%
24	125	0.592	0.705	0.085	0.08	0.507	0.625	0.566	0.083	14.74	40.20%
25	132	1.18	1.316	0.138	0.13	1.042	1.186	1.114	0.102	9.14	79.10%
26	137	0.55	0.709	0.076	0.188	0.474	0.521	0.498	0.033	6.68	35.30%
27	140	0.428	0.479	0.108	0.113	0.32	0.366	0.343	0.033	9.48	24.40%
28	142	2.066	1.504	0.248	0.294	1.818	1.21	1.514	0.430	28.40	107.50%
29	143	1.706	1.651	0.169	0.191	1.537	1.46	1.499	0.054	3.63	106.40%
30	144	0.369	0.351	0.095	0.087	0.274	0.264	0.269	0.007	2.63	19.10%
31	147	0.84	1.929	0.194	0.153	0.646	1.776	1.211	0.799	65.98	86.00%
32	150	0.991	1.084	0.164	0.188	0.827	0.896	0.862	0.049	5.66	61.20%
33	151	0.875	0.433	0.082	0.094	0.793	0.339	0.566	0.321	56.72	40.20%
34	165	0.355	0.396	0.089	0.087	0.266	0.309	0.288	0.030	10.58	20.40%
35	173	0.273	0.293	0.079	0.076	0.194	0.217	0.206	0.016	7.91	14.60%
36	175	1.009	0.948	0.183	0.211	0.826	0.737	0.782	0.063	8.05	55.50%
37	176	0.679	0.612	0.086	0.098	0.593	0.514	0.554	0.056	10.09	39.30%
38	177	0.5	0.45	0.203	0.127	0.297	0.323	0.310	0.018	5.93	22.00%
39	178	0.92	1.15	0.303	0.455	0.617	0.695	0.656	0.055	8.41	46.60%
40	185	2.016	1.698	0.194	0.189	1.822	1.509	1.666	0.221	13.29	158.00%
41	188	1.344	1.284	0.143	0.15	1.201	1.134	1.168	0.047	4.06	110.80%
42	191	1.285	1.309	0.19	0.23	1.095	1.079	1.087	0.011	1.04	103.10%
43	194	0.542	0.557	0.21	0.16	0.332	0.397	0.365	0.046	12.61	34.60%
44	195	1.506	1.492	0.184	0.162	1.322	1.33	1.326	0.006	0.43	125.80%

DCU ELISA RESULTS - SCREEN 1													
	PLD			NO PLD			NORMALISED			RESULTS			
ID	OD 1	OD 2	OD 3	OD 1	OD 2	OD 3	OD 1	OD 2	OD 3	MEAN	STDEV	CV	MEAN/POS
3	0.325	0.319	0.325	0.147	0.148	0.156	0.178	0.171	0.169	0.17	0.00	2.74	14.08%
4	1.202	1.046	1.129	0.264	0.266	0.262	0.938	0.78	0.867	0.86	0.08	9.18	70.24%
9	1.152	1.128	1.187	0.137	0.137	0.125	1.015	0.991	1.062	1.02	0.04	3.53	83.37%
10	1.26	1.102	1.223	0.314	0.343	0.318	0.946	0.759	0.905	0.87	0.10	11.30	70.92%
12	0.994	0.884	0.865	0.2	0.208	0.194	0.794	0.676	0.671	0.71	0.07	9.75	58.18%
19	0.523	0.518	0.489	0.181	0.183	0.178	0.342	0.335	0.311	0.33	0.02	4.94	26.39%
20	0.624	0.604	0.562	0.202	0.207	0.199	0.422	0.397	0.363	0.39	0.03	7.52	32.12%
23	0.879	0.836	0.783	0.227	0.236	0.232	0.652	0.6	0.551	0.60	0.05	8.40	43.48%
28	0.766	0.722	0.747	0.229	0.24	0.221	0.537	0.482	0.526	0.52	0.03	5.65	37.26%
32	0.937	0.892	0.928	0.234	0.235	0.224	0.703	0.657	0.704	0.69	0.03	3.90	49.77%
35	0.904	0.822	0.839	0.283	0.276	0.245	0.621	0.546	0.594	0.59	0.04	6.47	42.46%
36	1.395	1.374	1.308	0.192	0.17	0.172	1.203	1.204	1.136	1.18	0.04	3.30	85.44%
40	0.868	0.907	0.826	0.153	0.155	0.156	0.715	0.752	0.67	0.71	0.04	5.76	53.32%
43	1.033	0.952	0.979	0.18	0.184	0.192	0.853	0.768	0.787	0.80	0.04	5.56	60.08%
50	0.751	0.693	0.714	0.24	0.25	0.236	0.511	0.443	0.478	0.48	0.03	7.12	36.72%
55	0.641	0.608	0.601	0.179	0.174	0.178	0.462	0.434	0.423	0.44	0.02	4.57	32.91%
63	0.483	0.462	0.469	0.131	0.134	0.134	0.352	0.328	0.335	0.34	0.01	3.65	25.23%
104	1.18	1.027	1.138	0.173	0.177	0.186	1.007	0.85	0.952	0.94	0.08	8.51	71.13%
108	0.742	0.676	0.702	0.178	0.172	0.188	0.564	0.504	0.514	0.53	0.03	6.10	53.63%
114	0.91	0.86	0.964	0.192	0.198	0.203	0.718	0.662	0.761	0.71	0.05	6.96	72.58%
115	0.584	0.6	0.649	0.197	0.191	0.196	0.387	0.409	0.453	0.42	0.03	8.07	42.34%
123	0.83	0.984	0.894	0.138	0.134	0.139	0.692	0.85	0.755	0.77	0.08	10.39	77.86%
124	0.843	0.989	0.889	0.137	0.143	0.149	0.706	0.846	0.74	0.76	0.07	9.56	77.69%
125	0.85	0.913	0.797	0.19	0.209	0.199	0.66	0.704	0.598	0.65	0.05	8.14	66.51%
132	0.908	0.903	0.883	0.192	0.171	0.174	0.716	0.732	0.709	0.72	0.01	1.64	73.12%
137	1.017	1.123	0.882	0.179	0.209	0.184	0.838	0.914	0.698	0.82	0.11	13.42	83.05%
140	0.411	0.385	0.352	0.131	0.114	0.14	0.28	0.271	0.212	0.25	0.04	14.52	19.12%
142	1.371	1.34	1.412	0.358	0.365	0.383	1.013	0.975	1.029	1.01	0.03	2.76	78.94%
143	1.182	1.136	1.078	0.248	0.248	0.248	0.934	0.888	0.83	0.88	0.05	5.90	69.39%
144	0.39	0.372	0.357	0.121	0.118	0.131	0.269	0.254	0.226	0.25	0.02	8.74	18.77%
147	1.408	1.283	1.352	0.233	0.229	0.244	1.175	1.054	1.108	1.11	0.06	5.45	87.31%
150	1.152	1.2	1.111	0.305	0.279	0.292	0.847	0.921	0.819	0.86	0.05	6.11	67.69%
151	0.521	0.506	0.444	0.265	0.256	0.255	0.256	0.25	0.189	0.23	0.04	16.00	18.18%
165	0.435	0.395	0.431	0.128	0.148	0.144	0.307	0.247	0.287	0.28	0.03	10.90	21.35%
173	0.308	0.306	0.303	0.126	0.13	0.134	0.182	0.176	0.169	0.18	0.01	3.70	13.38%
175	1.101	0.985	1.035	0.191	0.175	0.183	0.91	0.81	0.852	0.86	0.05	5.86	65.28%
176	0.579	0.508	0.529	0.122	0.127	0.13	0.457	0.381	0.399	0.41	0.04	9.63	31.40%
177	0.953	0.894	0.975	0.122	0.124	0.132	0.831	0.77	0.843	0.81	0.04	4.81	62.03%
178	0.744	0.764	0.722	0.155	0.157	0.163	0.589	0.607	0.559	0.59	0.02	4.15	44.54%
185	0.881	0.871	0.77	0.159	0.164	0.175	0.722	0.707	0.595	0.67	0.07	10.29	52.52%
188	0.743	0.745	0.693	0.122	0.128	0.133	0.621	0.617	0.56	0.60	0.03	5.69	47.55%
191	0.72	0.697	0.68	0.2	0.212	0.18	0.52	0.485	0.5	0.50	0.02	3.50	40.69%
194	0.392	0.334	0.31	0.283	0.253	0.193	0.109	0.081	0.117	0.10	0.02	18.47	8.30%
195	0.912	0.825	0.689	0.237	0.232	0.23	0.675	0.593	0.459	0.58	0.11	18.94	57.76%

DCU ELISA RESULTS - SCREEN 2													
	PLD			NO PLD			NORMALISED			RESULTS			
ID	OD 1	OD 2	OD 3	OD 1	OD 2	OD 3	OD 1	OD 2	OD 3	MEAN	STDEV	CV	Mean/Pos
3	0.461	0.412	0.429	0.3	0.147	0.255	0.161	0.265	0.174	0.20	0.06	28.33	14.14%
4	1.565	1.592	1.572	0.338	0.309	0.326	1.227	1.283	1.246	1.25	0.03	2.27	88.50%
9	1.519	1.515	1.523	0.175	0.18	0.149	1.344	1.335	1.374	1.35	0.02	1.51	95.50%
10	1.545	1.529	1.52	0.362	0.345	0.32	1.183	1.184	1.2	1.19	0.01	0.80	84.05%
12	1.175	1.191	1.21	0.212	0.237	0.219	0.963	0.954	0.991	0.97	0.02	1.99	68.52%
19	0.568	0.509	0.542	0.182	0.176	0.178	0.386	0.333	0.364	0.36	0.03	7.38	25.52%
20	0.634	0.614	0.606	0.184	0.192	0.196	0.45	0.422	0.41	0.43	0.02	4.80	30.21%
23	1.082	1.012	1.019	0.314	0.324	0.334	0.768	0.688	0.685	0.71	0.05	6.60	52.84%
28	0.682	0.653	0.695	0.249	0.25	0.253	0.433	0.403	0.442	0.43	0.02	4.79	31.54%
32	1.19	1.139	1.106	0.294	0.299	0.298	0.896	0.84	0.808	0.85	0.04	5.25	62.78%
35	1.054	1.063	1.02	0.346	0.349	0.387	0.708	0.714	0.633	0.69	0.05	6.59	50.72%
36	1.465	1.387	1.333	0.208	0.209	0.207	1.257	1.178	1.126	1.19	0.07	5.56	87.88%
40	1.153	1.157	1.122	0.218	0.213	0.223	0.935	0.944	0.899	0.93	0.02	2.57	68.66%
43	1.283	1.25	1.289	0.227	0.234	0.236	1.056	1.016	1.053	1.04	0.02	2.14	77.24%
50	0.519	0.484	0.432	0.199	0.179	0.176	0.32	0.305	0.256	0.29	0.03	11.40	21.77%
55	0.76	0.71	0.649	0.234	0.228	0.216	0.526	0.482	0.433	0.48	0.05	9.69	35.62%
63	0.436	0.385	0.354	0.159	0.153	0.153	0.277	0.232	0.201	0.24	0.04	16.15	18.36%
104	0.997	0.978	0.941	0.173	0.177	0.186	0.824	0.801	0.755	0.79	0.04	4.43	70.92%
108	0.716	0.67	0.669	0.148	0.142	0.159	0.568	0.528	0.51	0.54	0.03	5.55	50.11%
114	0.905	0.889	0.914	0.168	0.167	0.175	0.737	0.722	0.739	0.73	0.01	1.27	68.58%
115	0.56	0.535	0.581	0.207	0.211	0.217	0.353	0.324	0.364	0.35	0.02	5.96	32.48%
123	0.893	0.858	0.916	0.122	0.115	0.117	0.771	0.743	0.799	0.77	0.03	3.63	72.17%
124	0.923	0.883	0.923	0.116	0.122	0.124	0.807	0.761	0.799	0.79	0.02	3.11	73.85%
125	0.742	0.737	0.753	0.146	0.149	0.155	0.596	0.588	0.598	0.59	0.01	0.89	55.60%
132	1.084	1.025	0.913	0.124	0.124	0.121	0.96	0.901	0.792	0.88	0.09	9.64	66.47%
137	1.046	0.99	0.981	0.132	0.14	0.144	0.914	0.85	0.837	0.87	0.04	4.75	81.15%
140	0.324	0.386	0.305	0.167	0.174	0.156	0.157	0.212	0.149	0.17	0.03	19.86	14.45%
142	1.049	1.065	1.049	0.183	0.215	0.181	0.866	0.85	0.868	0.86	0.01	1.15	72.10%
143	0.781	0.76	0.805	0.146	0.151	0.149	0.635	0.609	0.656	0.63	0.02	3.72	53.01%
144	0.37	0.37	0.354	0.189	0.193	0.186	0.181	0.177	0.168	0.18	0.01	3.80	14.68%
147	1.218	1.189	1.177	0.154	0.156	0.168	1.064	1.033	1.009	1.04	0.03	2.66	86.66%
150	0.951	0.872	0.807	0.237	0.232	0.222	0.714	0.64	0.585	0.65	0.06	10.02	54.10%
151	0.42	0.427	0.366	0.136	0.144	0.135	0.284	0.283	0.231	0.27	0.03	11.40	19.99%
165	0.495	0.462	0.431	0.16	0.155	0.154	0.335	0.307	0.277	0.31	0.03	9.47	22.88%
173	0.301	0.292	0.265	0.115	0.107	0.103	0.186	0.185	0.162	0.18	0.01	7.64	13.27%
175	1.02	1.066	1.021	0.136	0.13	0.139	0.884	0.936	0.882	0.90	0.03	3.40	67.28%
176	0.535	0.465	0.428	0.11	0.105	0.106	0.425	0.36	0.322	0.37	0.05	14.12	27.56%
177	0.937	0.879	0.814	0.1	0.114	0.125	0.837	0.765	0.689	0.76	0.07	9.69	57.05%
178	0.707	0.679	0.631	0.12	0.123	0.133	0.587	0.556	0.498	0.55	0.05	8.26	40.86%
185	0.809	0.635	0.638	0.114	0.111	0.109	0.695	0.524	0.529	0.58	0.10	16.70	51.14%
188	0.713	0.751	0.728	0.162	0.154	0.149	0.551	0.597	0.579	0.58	0.02	4.03	49.10%
191	0.414	0.364	0.41	0.119	0.108	0.118	0.295	0.256	0.292	0.28	0.02	7.72	23.00%
194	0.298	0.28	0.267	0.176	0.17	0.157	0.122	0.11	0.11	0.11	0.01	6.08	8.87%
195	0.863	0.874	0.821	0.106	0.109	0.113	0.757	0.765	0.708	0.74	0.03	4.15	60.85%

DCU BIACORE RESULTS								
	ID	RU 1	RU 2	RU 3	MEAN	STDEV	CV	MEAN/POS
1	**3**	36.1	37.6	38.9	37.53	1.40	3.73	**7.84**
2	**4**	743.4	810.2	877.7	810.43	67.15	8.29	**169.31**
3	**9**	110.3	118.1	116.8	115.07	4.18	3.63	**24.04**
4	**10**	243.7	250.9	278.4	257.67	18.31	7.11	**53.83**
5	**12**	58.7	60.2	63.8	60.90	2.62	4.30	**12.72**
6	**19**	60.4	61.8	63	61.73	1.30	2.11	**12.90**
7	**20**	52.9	53.7	50.4	52.33	1.72	3.29	**10.93**
8	**23**	64.9	69.8	68	67.57	2.48	3.67	**14.12**
9	**28**	36.3	37.1	37.3	36.90	0.53	1.43	**7.71**
10	**32**	38.7	39.9	42.6	40.40	2.00	4.94	**8.44**
11	**35**	39.4	40.3	41.8	40.50	1.21	2.99	**8.46**
12	**36**	64.9	66.7	68.5	66.70	1.80	2.70	**13.93**
13	**40**	54.2	55.9	57.4	55.83	1.60	2.87	**11.66**
14	**43**	63.2	68.7	74.6	68.83	5.70	8.28	**14.38**
15	**50**	58.9	65.4	57.1	60.47	4.37	7.22	**14.29**
16	**55**	61	65	66.4	64.13	2.80	4.37	**15.16**
17	**63**	22.7	24.3	23.9	23.63	0.83	3.52	**5.59**
18	**104**	34.2	33.4	32.6	33.40	0.80	2.40	**8.84**
19	**108**	42	42.5	40.3	41.60	1.15	2.77	**11.02**
20	**114**	49	49.8	48.2	49.00	0.80	1.63	**12.97**
21	**115**	34.5	35.5	30.1	33.37	2.87	8.61	**8.83**
22	**123**	36.9	36.9	37.2	37.00	0.17	0.47	**9.80**
23	**124**	32.1	31.3	30.7	31.37	0.70	2.24	**8.31**
24	**125**	43.8	42	44.4	43.40	1.25	2.88	**11.49**
25	**132**	48.5	48.3	47.9	48.23	0.31	0.63	**12.77**
26	**137**	39.1	40.1	39.2	39.47	0.55	1.40	**10.45**
27	**140**	26.6	27.1	27.1	26.93	0.29	1.07	**7.13**
28	**142**	133.7	134.5	133.8	134.00	0.44	0.33	**35.48**
29	**143**	47.7	46.4	46.7	46.93	0.68	1.45	**12.43**
30	**144**	35	36.9	34.6	35.50	1.23	3.46	**9.40**
31	**147**	61.3	62.3	60.2	61.27	1.05	1.71	**16.22**
32	**150**	30.8	30.8	30.5	30.70	0.17	0.56	**8.13**
33	**151**	12.6	11.4	9.6	11.20	1.51	**13.48**	**2.97**
34	**165**	22.9	22.7	22.3	22.63	0.31	1.35	**7.05**
35	**173**	32	32.5	33.1	32.53	0.55	1.69	**10.14**
36	**175**	82	79.3	72.1	77.80	5.12	6.58	**24.25**
37	**176**	25.6	26.4	27.7	26.57	1.06	3.99	**8.28**
38	**177**	38.8	38.5	35	37.43	2.11	5.64	**11.67**
39	**178**	61.5	65.1	65.2	63.93	2.11	3.30	**19.93**
40	**185**	63	62.9	62.6	62.83	0.21	0.33	**19.58**
41	**188**	26.3	27.3	26.8	26.80	0.50	1.87	**8.35**
42	**191**	51.7	53.4	53.9	53.00	1.15	2.18	**16.52**
43	**194**	18	15.8	19.2	17.67	1.72	9.76	**5.51**
44	**195**	27.7	32.2	30.6	30.17	2.28	7.56	**9.40**

% POSITIVE CONTROL				
	ID	LELYSTAD	ELISA	BIACORE
1	3	35.90	14.11	7.84
2	4	132.00	79.37	169.31
3	9	114.50	89.43	24.04
4	10	122.40	77.49	53.83
5	12	37.90	63.35	12.72
6	19	15.00	25.95	12.90
7	20	15.20	31.17	10.93
8	23	46.90	48.16	14.12
9	28	19.60	34.40	7.71
10	32	29.00	56.28	8.44
11	35	13.70	46.59	8.46
12	36	68.10	86.66	13.93
13	40	34.90	60.99	11.66
14	43	37.80	68.66	14.38
15	50	29.20	29.24	14.29
16	55	11.60	34.26	15.16
17	63	7.70	21.80	5.59
18	104	47.60	71.02	8.84
19	108	37.30	51.87	11.02
20	114	64.70	70.58	12.97
21	115	48.50	37.41	8.83
22	123	26.30	75.02	9.80
23	124	74.10	75.77	8.31
24	125	40.20	61.05	11.49
25	132	79.10	69.80	12.77
26	137	35.30	82.10	10.45
27	140	24.40	16.79	7.13
28	142	107.50	75.52	35.48
29	143	106.40	61.20	12.43
30	144	19.10	16.72	9.40
31	147	86.00	86.99	16.22
32	150	61.20	60.89	8.13
33	151	40.20	19.09	2.97
34	165	20.40	22.11	7.05
35	173	14.60	13.33	10.14
36	175	55.50	66.28	24.25
37	176	39.30	29.48	8.28
38	177	22.00	59.54	11.67
39	178	46.60	42.70	19.93
40	185	158.00	51.83	19.58
41	188	110.80	48.33	8.35
42	191	103.10	31.84	16.52
43	194	34.60	8.59	5.51
44	195	125.80	59.30	9.40