Production of antibodies for use in a biosensor-based assay for *Listeria monocytogenes*

A thesis submitted for the degree of Ph.D.

By

Paul Leonard B.Sc. (Hons),

August 2003.

Based on research carried out at

School of Biotechnology,

Dublin City University,

Dublin 9,

Ireland,

Under the supervision of Professor Richard O'Kennedy.

This thesis is dedicated to my parents for all their encouragement and support over the last number of years.

"I am not discouraged, because every wrong attempt discarded is another step forward"

-Thomas Edison.

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 the work of others, save and to the extent that such work is cited and acknowledged within the text.

Signed Paul Leonard

Date: Dec. 2003

Acknowledgements

Sincere thanks to Prof. Richard O'Kennedy for his constant support and guidance over the past few years, in particular for sharing his wealth of experience and knowledge throughout my studies. Thanks to all the lab group, past and present and all my friends at DCU for their companionship and some unforgettable (and more often than not better forgotten) nights out! Special thanks goes to Steve, a great scientist and friend, for his support, knowledge and overall enthusiasm over the last few years. To Monty, Macker, Ryaner and the rest of the "Finian's lads" for their continual 'good humoured harassment' and alcohol-based support! Cheers! I would like to thank all my family for their unequivocal support from start to finish, I owe you so much! Finally, I would like to reserve a very special thanks to Nerea, my source of inspiration, for her patience, companionship and constant love and support.

Table of contents:

Abbreviations

Ab	antibody
A/Abs	absorbance
ABS	antigen binding site
ActA	Actin A (Actin Assembly protein)
Ag	antigen
AOAC	Association of Analytical Communities
Arp	actin related protein
BHI	brain heart infusion media
BIA	biomolecular interaction analysis
BNL	Brookhaven National Laboratories
bp	base pairs
BSA	bovine serum albumin
Cam	chloramphenicol
CAT	Cambridge Antibody Technology
cDNA	complementary DNA
CDC	Center for Disease Control and Prevention
CDR	complementarity determining region
cfu	colony forming units
C_H	heavy chain constant domain
C_L	light chain constant domain
CM	carboxymethylated
conc (c)	concentration
CV	coefficient of variation
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidyl triphosphates
dTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
dTT	Dithiothreitol
EB	enrichment broth
E. coli	*Escherichia coli*
EDC	N-ethyl-N'-(dimethylamiopropyl) carbodiimide
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	antigen binding region of an antibody above the hinge region
Fc	constant region of an antibody molecule
FCA	Freunds's Complete Adjuvant

FDA	Food and Drug Administration
FIA	Flow injection analysis
FITC	fluorescein isothiocyanate
^{10}Fn3	tenth fibronectin type III domain
FSAI	Food Safety Authority of Ireland
Fv	variable binding region of an antibody
GW	glycine-tryptophan
g3p	gene-3-protein
HACCP	Hazard Analysis of Critical Control Points
HAT	Hypoxanthine aminopterin thymidine
HGF-R	hepatocytes growth factor receptor
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HT	heat-treated
HBS	Hepes buffered saline
HRP	Horse radish peroxidase
IDF	International Dairy Federation
IFR	Institute for Food Research, Norwich
Ig	immunoglobulin
IgG	immunoglobulin class G
IMAC	immobilised metal affinity chromatography
Inl	Internalin (series of *L. monocytogenes* surface proteins)
InlA	Internalin A
InlB	Internalin B
InlC	Internalin C
IPTG	isopropyl-β-D-galactopyranoside
ISO	International Organisation for Standardisation
Kan	Kanamycin
LB	Luria Bertani media
LE	low expression
LLO	listeriolysin O
Log	logarithmic
LPS	lipopolysaccharides
LRR	leucine rich repeat
mAb	monoclonal antibody
Mpl	metalloprotease
mRNA	messenger RNA
MW	molecular weight

NCTC	UK national collection of type cultures
NE	non-expression
NHS	N-hydroxysuccinimide
NTA	nitrilotriacetic acid
OD	optical density
OPD	o-phenylenediamine dihydrochloride
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PBST	PBS-Tween
PCR	polymerase chain reaction
PDB	Protein Data Bank
PEG	polyethylene glycol
PLC	phospholipase C
RCSB	Research Collaboratory for Structural Bioinformatics
RI	refractive index
rInlB	recombinant InlB
RIDS	ribosomal-interaction display systems
RPM	revolutions per minute
RT	room temperature
RTA	ricin A-subunit
RU	response units
scFv	single chain Fv antibody derivative
SDS	sodium dodecyl sulphate
SIP	selectively infective phage
SOC	super optimal catabolites
SPR	surface plasmon resonance
TBS	Tris buffer saline
Tet	tetracycline
TIR	total internal reflection
TK	thymidine kinase
TY	yeast-tryptone
UV	ultraviolet
VASP	vasodilator-stimulated phosphoprotein
V_H	variable region of heavy chain
V_L	variable region of light chain
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Units

µg	microgram
(k)Da	(kilo) Daltons
µl	microlitre
µM	micromoles
°C	degrees Celcius
cm	centimetres
g	grams
h	hours
kg	kilogram
l	litre
m	metre
M	molar
mg	milligram
min	minute
ml	millilitre
mm	millimetres
nM	nanomolar
mol	molar
pg	picograms
rpm	revolutions per minute
RU	response units
sec, s	seconds
v/v	volume per unit volume
w/v	weight per unit volume

Publications

Leonard, P., Hearty, S., Quinn, J. and O'Kennedy, R. (2003). Development of a surface plasmon resonance-based immunoassay for the direct detection of *Listeria monocytogenes.* Submitted to *Applied and Environmental Microbiology.*

Leonard, P., Hearty, S., Quinn, J. and O'Kennedy, R. (2003). A generic approach for the detection of whole *Listeria monocytogenes* cells in contaminated samples using surface plasmon resonance. *Biosens. Bioelectron.,* accepted 18th November.

Leonard, P., Hearty, S., Brennan, J., Dunne, L., Quinn, J., Chakraborty, T., and O'Kennedy, R. (2003). Advances in biosensors for the detection of pathogens in food and water. *Enzyme Microb. Tech.,* **32**:3-13

Fitzpatrick, J., Fanning, L., Hearty, S., **Leonard, P.,** Manning, B.M., Quinn, J.G., and O'Kennedy, R. (2000). Applications and recent developments in the use of antibodies for analysis. *Anal. Lett.,* **33**(13):2563-2609

Leonard, P., Hearty, S., Quinn, J.G., and O'Kennedy, R. (2001). A pathogenic life cycle of *Listeria monocytogenes*. *INCO COPERINICUS News,* **3**:1-2

Hearty, S., **Leonard, P**., Quinn, J.Q. and O'Kennedy, R. (2002). Background to new rapid technology in detection of pathogens, part II: Book of abstracts, New methods for the detection of *Listeria monocytogenes*. INCO COPERNICUS project ERBIC 15 CT98-0902. May 2002.

Presentations/courses

Leonard, P., Hearty, S. and O'Kennedy, R. (2003). Development of an immunoassay to detect *Listeria monocytogenes* using a scFv antibody selected against purified recombinant Internalin B protein. VIIth International Conference on Agri-Food Antibodies, Uppsala, Sweden, September 10-13.

Completed the EMBO, NUI Maynooth Bioinformatics Summer School, 28th April to 3rd May 2003.

Leonard, P. (2003). Bioinformatics and antibody modelling. Antibody training course. DCU. February 12th.

Leonard, P. (2002). Introduction to Antibodies and the Immune System. CTYI guest lecture. DCU 1st Dec.

Leonard, P. and O'Kennedy, R. (2002). Development of a biosensor based assay to detect *Listeria monocytogenes* in "real time". PhD Transfer meeting. DCU.

Leonard, P. and O'Kennedy, R. (2002). The generation of recombinant antibodies to *Listeria monocytogenes.* The National Centre for Sensor Research cluster seminar. DCU 23rd May 2002.

Leonard, P., Hearty, S., Quinn, J.G., and O'Kennedy, R. (2001). *Listeria monocytogenes:* antibody characterisation, biosensing developments and recombinant studies. *Inco Coperinicus PL 979012 project meeting,* 10th-11th May, Zivkov, Czech Republic.

Hearty, S., **Leonard, P.,** Quinn, J.G., and O'Kennedy, R. (2000). Rapid, specific detection of *Listeria monocytogenes* by antibody techniques and on-line sensor technology; development of improved control of food safety for industry and the consumer. *Inco Coperinicus PL 979012 project meeting,* 22nd-24th Nov., Solvak Technical University, Bratislava, Solvakia.

Leonard, P., Hearty, S., Quinn, J.G., and O'Kennedy, R. (2000). Characterisation of anti-*Listeria monocytogenes* polyclonal antibodies by ELISA and BIAcore. *Inco Coperinicus PL 979012 project meeting,* 25th-26th May, DCU, Dublin.

Summary

The inclusion of *L. monocytogenes* in the list of organisms subject to HACCP has recently driven the search for detection methods suitable for on-line monitoring. The aim of the work presented in this thesis was the development of a biosensor-based immunoassay for the detection of *Listeria monocytogenes* using SPR.

Two polyclonal antibodies were generated from *Listeria monocytogenes* InlB extract and heat-treated *Listeria monocytogenes* cells. Both antibodies were purified and characterised by ELISA, SDS-PAGE and Western blotting. An inhibition ELISA-based immunoassay was developed with each antibody for the detection of *Listeria monocytogenes.* Intra- and interday studies were performed to evaluate the accuracy and intermediate precision of the assays. The feasibility of detecting *Listeria monocytogenes* cells in chocolate milk, a food matrix which has been reported to have been the cause of a well known *Listeria monocytogenes*-associated food poisoning outbreak, was also examined. To determine the potential cross reactivity of each antibody, inhibition ELISAs were performed with a number of bacterial strains. It was concluded that both antibodies can be used as valuable tools for the genus-specific detection of *Listeria* cells, but were severely limited for the species-specific detection of *Listeria monocytogenes* cells.

Expressing *Listeria monocytogenes* invasion-associated proteins in *E. coli* allows the safe and efficient production of high quantities of pure protein for use in the generation of *Listeria monocytogenes* specific antibodies and immunoassay development. Two *Listeria monocytogenes* invasion-associated proteins, Internalin B (InlB) and p60 (also known as iap), were cloned and expressed in *E. coli* XL-10 Gold cells. Expressed protein was purified by immobilised affinity chromatography (IMAC) and used for the selection of specific antibodies (Chapter 5) and for the development a biosensor-based immunoassay for the detection of *Listeria monocytogenes* (Chapter 6).

The emergence of recombinant antibody phage display technology has transformed the way we generate antibodies for the specific detection of a chosen analyte. Chapter 5 describes the development of two combinatorial antibody libraries from mice immunised with *Listeria monocytogenes* cells and InlB protein extract. Phage scFv antibodies were selected from both murine antibody libraries and from a large naïve human antibody library against *Listeria monocytogenes* cells and invasion associated protein. A number of the selected phage-scFv antibodies could not be expressed as soluble scFv and also showed tendencies to cross react with various bacterial strains tested. A soluble scFv

antibody was selected that recognised the *Listeria monocytogenes* invasion-associated protein, Internalin B, but did not recognise *Listeria monocytogenes* cells. An inhibition ELISA was developed with this antibody to indirectly detect *Listeria monocytogenes*.

The two polyclonal antibodies and the selected anti-InlB scFv antibody were used in development of three biosensor-based immunoassays using a BIAcore 3000 instrument (chapter 6). Various assay formats and sensor chip surfaces were evaluated. Intra- and interday assay variability studies performed to determine the precision and reproducibility of each assay. The assay developed with the polyclonal anti-InlB polyclonal proved to be most sensitive and cost effective while the assay developed with the anti-InlB scFv antibody fragment proved most specific for the detection of *Listeria monocytogenes*.

Chapter 1

Introduction

1.1 *Section overview*

The aim of this thesis was to develop a rapid biosensor-based immunoassay for the detection of the food pathogen, *Listeria monocytogenes.* This chapter reviews the important human pathogen, *Listeria monocytogenes* and its pathogenicity life-cycle. The main *Listeria monocytogenes* pathogenicity-related proteins and recent *Listeria monocytogenes* associated food poisoning outbreaks are discussed in detail. The current methods of *Listeria monocytogenes* detection are examined and the use of antibody-based assays for the detection of bacteria discussed. A detailed description of the structure and characteristics of antibodies is given and the rapidly expanding area of antibody engineering introduced and explained. The use of surface plasmon resonance (SPR)-based biosensors for the detection of bacteria is also reviewed. Finally, a thesis outline is presented, highlighting the strategy pursued for the development of a biosensor-based immunoassay for the detection of *Listeria monocytogenes.*

1.2 *Introduction to Listeria monocytogenes*

The bacterial genus *Listeria* consists of a group of facultatively anaerobic, asporogenous Gram-positive rods closely related phylogenetically to *Bacillus, Streptococcus, Enterococcus, Staphylococcus* and *Clostridium* (Vazquez-Boland *et al.,* 2001). Of the six currently recognised species of *Listeria*, (*L. innocua, L. ivanovii, L. monocytogenes, L. grayi, L. seeligeri, and L. welshimeri*), *L. monocytogenes* is the principal human pathogen, with only a few known cases of human listeriosis due to the animal pathogen *L. ivanovii* (Wyatt, 1999). Since its discovery by E.G.D. Murray in 1926 (Murray, *et al.,* 1926), *Listeria monocytogenes* has become regarded as an important food borne pathogen and was the cause of many recent well-publicised food poisoning outbreaks (Schlech, 2000; Donnelly, 2001). Even though *L. monocytogenes* was frequently associated with sepsis and meningoencephalitis in immunocompromised patients in the 1950s and 1960s (Nieman and Lober, 1980), it was not until the 1980s that *L. monocytogenes* was recognised as an important food borne pathogen. Prior to the 1980s, listeriosis was primarily of veterinary concern but as a result of its wide distribution in the environment and its ability to grow at refrigeration temperatures, *L. monocytogenes* can be readily isolated from soil, dust, fertilizer, sewage, water, plants, and even processed foods stored at 4°C (Southwick and Purich, 1996).

L. monocytogenes is a Gram-positive facultatively anaerobic rod-shaped bacterium that grows between 1-45°C. Optimal growth occurs between 30-37°C within the pH range of

pH 5.0 and 9.0, in a NaCl concentration of 10% (Jones and Seeliger, 1992). So far, *Listeria monocytogenes* is the only Gram-positive organism that has been found to contain authentic lipopolysaccharides (LPS). The LPS of *Listeria monocytogenes* and those of all Gram-negative species are also called endotoxins, thereby distinguishing these cell-bound, heat-stable toxins from heat-labile, protein exotoxins secreted into culture media. In addition to causing endotoxic shock, LPS is pyrogenic, can activate macrophages and complement, is mitogenic for B lymphocytes, induces interferon production, causes tissue necrosis and tumour regression, and has adjuvant properties (Peake *et al.,* 1996 and Murray *et al.,* 1998). Most food-borne outbreaks of listeriosis have been caused by *L. monocytogenes* serotype 4b, although no distinct correlation between this serotype and greater virulence has been observed (Schlech, 1997). Pregnant women and newborns, elderly people and people with weakened or suppressed immune systems caused by chemotherapy, AIDS, diabetes and kidney disease are especially at risk to listeriosis. Indeed, immunocompromised patients can be up to 500-1000 times more susceptible to listeriosis than the general population (Jensen *et al.* 1994 and Hof, 2003). In the majority of cases, infection by *L. monocytogenes* results in symptoms such as mild fever, diarrhoea, nausea or vomiting accompanied by a slight headache. However, in more severe cases *Listeria* infection can cause septicaemia, meningitis and meningo-encephalitis. Listeriosis can also cause stillbirths and spontaneous abortions (Doganay, 2003). The number of food poisoning cases due to *L. monocytogenes* may be very few (Table 1) compared to that of other bacteria such as *Salmonella* (1/3 of all cases in the U.S), *Staphylococcus aureus* (25% of all cases in the U.S), *Clostridium perfringens* (17% of all cases) and *Escherichia coli* 0157:H7, (Anonymous, 1997), but the fatality rate is far greater. According to the Center for Disease Control and Prevention (CDC) in the US, approximately 25% of the people (1 in 4 people) who contract listeriosis die as a result of the illness. However, other reports on the disease claim that the fatality rate could be as high as 29% (Fleming *et al.* 1985) or 30% (Cossart *et al.,* 2003).

The outbreaks of listeriosis in late 80s and 90s (discussed later) in both the EU and the United States has lead to the recognition of the hazard caused by this organism and has resulted in tightening of the control of hygiene during the production of food associated with *Listeria* food poisoning, namely soft cheeses and milk. In the United States the Food and Drug Administration has adopted a policy of "zero tolerance" for the presence of *Listeria* in food. Also the food industry, in both the EU and U.S., have introduced a program called Hazard Analysis of Critical Control Point (HACCP) which aims to effectively eliminate *Listeria monocytogenes* from food processing environments and, therefore, processed food products (Schlech, 1997; Anonymous, 1997, and Donnelly,

2001). Due to the increase in the number of human listeriosis cases between 1987 and 1989 in England and Wales, the Dairy Products (Hygiene) Regulations for England and Wales, based on the European Council Directive 92/46/EEC as amended, became law on 9th May 1995. According to these regulations, *L. monocytogenes* should be absent from 1g samples of hard cheese and from 25g samples of certain soft cheeses. Pasteurization still remains the first line of defence against the transmission of foodborne disease through milk and other dairy products contaminated on the farm (Schlech, 1997).

Table 1.1: *A summary of estimated foodborne illnesses, hospitalisations and deaths caused by selected pathogens in the US annually as calculated by the USDA's Economic Research Service.*

Bacteria	***Estimated annual cases***	***Estimated annual hospitalisations***	***Estimated annual deaths***	***Onset***	***Infectious dose (CFUs)***
Salmonella	1,342,532	16,102	556	6 hours to 28 days	10^4 to 10^7
Listeria monocytogenes	2,493	2,298	499	A few days to 3 weeks	400 to 10^3
Campylobacter spp.	1,963,141	10,539	99	2 to 5 days	400 to 10^6
E. coli (0157:H7 and other types)	173,107	2,785	78	12 hours to 3 days	10^1 to 10^2
Clostridium perfringens	248, 520	41	7	18 to 36 hours	$>10^8$
Staphylococcus food poisoning	185,060	1,753	2	1 to 7 hours	$>10^6$

1.3 *A pathogenic life cycle: L. monocytogenes*

To cause infection, many bacteria must first adhere to a mucosal surface such as the alimentary tract mucosa. The alimentary tract mucosa is continually cleansed by the release of mucus from goblet cells and by the peristaltic flow of the gut contents over the epithelium. Similarly, ciliated cells in the respiratory tract sweep mucus and bacteria upward. In addition, the turnover of epithelial cells at these surfaces is fairly rapid. The intestinal epithelial cell monolayer is continually replenished, and the cells are pushed from the crypts to the villar tips in about 48 hours. To establish an infection at such a site, a bacterium must adhere to the epithelium and multiply before the mucus and extruded epithelial cells are swept away. To accomplish this, bacteria have evolved attachment mechanisms, such as pili and colonisation factors that recognise and attach the bacteria to cells (Peake *et al.*, 1996).

Listeria monocytogenes has the amazing capacity to cross three tight barriers in humans, that is the intestinal, the blood-brain and the fetoplacental barriers (Cossart, 2002). During infection, it enters, survives and multiples inside phagocytic and non-phagocytic cells and also spreads directly from cell to cell. These features are considered to be central to the pathophysiology of listeriosis (Vasquez-Boland *et al.,* 2001). *Listeria*-induced internalisation, like all phagocytic processes, is a complex phenomenon that is driven by a finely controlled rearrangement of the actin cytoskeleton coupled to membrane extensions that lead to engulfment of the bacterium (Machesky and May, 2001 and Cossart *et al.,* 2003). Invasive bacteria have been classified into two groups according to the type of morphological change that occurs at the site of entry (Finlay and Cossart, 1996). Bacteria such as *Salmonella* and *Shigella* belong to a group of bacteria that enter cells by a process known as a "trigger mechanism". In the trigger mechanism, a bacterium in contact with a cell delivers virulence factors directly into the host cytoplasm and these virulence factors activate both the cytoskeleton machinery and several signal transduction pathways, resulting in the formation of large membrane ruffles that internalise the bacterium in a type of macropinocytosis (reviewed by Cossart *et al.,* 2003). Alternatively, internalisation can result from the tight apposition of the plasma membrane over the entering bacterium. This process, also called the "zipper mechanism" is used by *Listeria monocytogenes* and appears different from the above mentioned "trigger mechanism" (Cossart, 2002). In the zipper mechanism a bacterial ligand interacts with a surface molecule on the host cell, which is generally a protein involved in cell adhesion and/or activation of the cytoskeleton machinery. Interaction of this receptor with the bacterial ligand induces local rearrangements in the actin cytoskeleton and other signals that culminate in the tight envelopment of the bacterial body by the plasma membrane (reviewed by Cossart *et al.,* 2003).

As discussed, *L. monocytogenes* has been observed to have a very unusual life-cycle with only a few species of bacteria like *Shigella flexneri* (Bernardini *et al.,* 1989) and *Rickettsia* (Heinzen *et al.* 1993) using the same method of intra- and intercellular spread through interaction with f-actin. A family of bacterial cell-wall surface proteins, called internalins (described later), are present at the cell surface of *L. monocytogenes* which promote entry into epithelial cells and hepatocytes (Gaillard *et al.* 1991; Dramsi *et al.* 1995 and Cossart and Bierne, 2001). *L. monocytogenes* also uses the body's own defence systems to gain entry into the cytoplasm of infected cells. Phagocytosis encloses the bacterium in a subcellular organelle called a phagolysosome, which is a toxic and hostile environment for most bacteria. The pH inside the phagolysosome drops which in turn activates listeriolysin O (LLO) and the bacterial metalloprotease (Mpl) resulting in lysis of the organelle within

30 minutes (Cossart, 2002), allowing the bacterium to escape into the cytoplasm. All pathogenic strains of *Listeria* produce listeriolysin O as entry of the bacteria into the cytoplasm of the host cell is required for pathogenesis (Kathariou *et al.*, 1987).

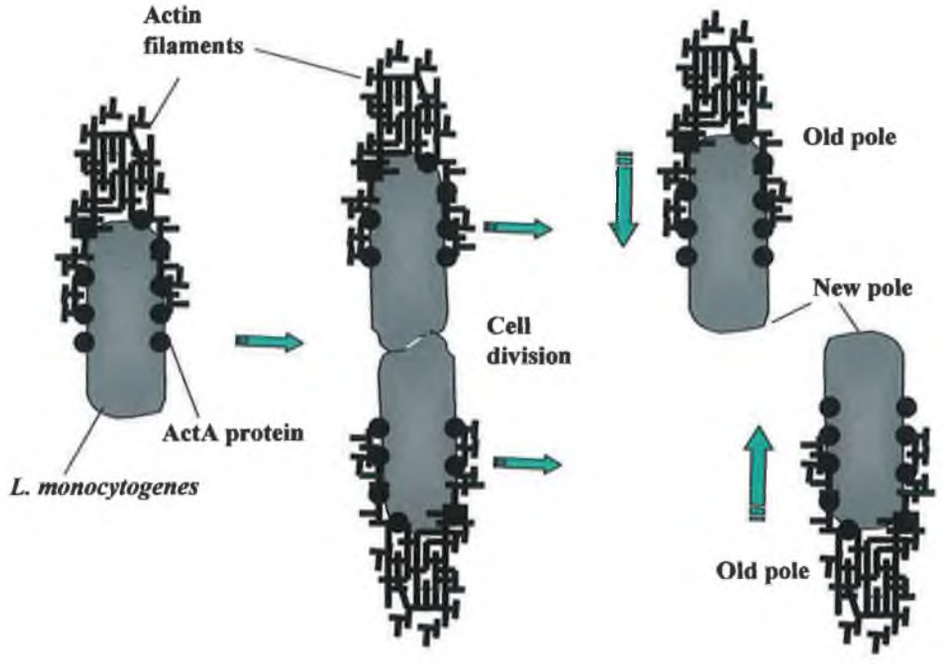


Figure 1.1: *Cell cycle distribution of ActA protein (black dots) and actin filaments (black lines) in L. monocytogenes (adapted from Maddock et al. 1993). When free in the cytosol, Listeria starts to replicate while inducing the recruitment and the polymerisation of cellular actin. ActA, required for the bacterial-induced actin assembly, is distributed around the cell except at the pole following cell division (Knock et al., 1993). The direction of movement of the new cells is therefore in opposite directions as indicated by the arrows.*

Within the cytoplasm, *L. monocytogenes* can proliferate and becomes surrounded by a high density of cytoplasmic actin. Actin is the most abundant protein in the cytoplasm of mammalian cells, accounting for 10 to 20% of the total cytoplasmic protein. It exists either as a globular monomer (G-actin) or a filament (F-actin), the latter formed by head to tail polymerisation of asymmetric monomers (Southwick and Purich, 1996). As the cell divides or proliferates, ActA protein (Figure 1.1) and actin filaments are distributed around the cell with the exclusion of the new pole. As the septation region is devoid of ActA, the movement of the progeny is usually in opposite directions to each other (Maddock *et al.* 1993).

Using this actin-based motility process, the bacterium migrates to the peripherary of the cell where it creates a protrusion or "filopod" into the adjacent cell. This filopod can be

ingested into the adjacent cell forming a now double membraned phagolysosome (Figure 1.2) where the life cycle begins over again (Southwick and Purich, 1996).

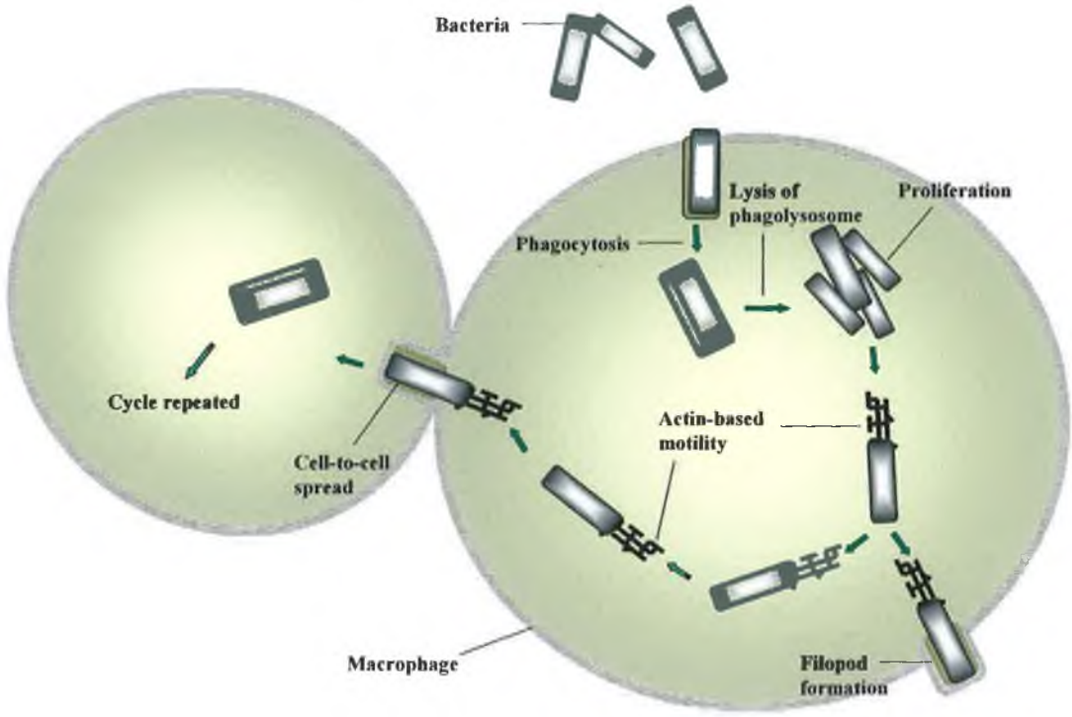


Figure 1.2: *Life cycle of L. monocytogenes in host cells (Adapted from Southwick and Purich, 1996). The Listeria intracellular life cycle involves a number of stages (indicated) starting with the entry of the bacterium into the host cells using the virulence factors discussed below. Phagocytosed Listeria monocytogenes survives within the phagocytic vacuole and within less than 30 minutes disrupts the phagosomal membranes and escapes into the cytosol. In the cytosol, the Listeria monocytogenes cells replicate and utilise actin-based motility to direct cell-to-cell spread.*

1.4 *Virulence factors involved in the entry of Listeria monocytogenes into host cells*

Listeria monocytogenes is able to efficiently invade normally non-phagocytic cells by triggering an induced phagocytosis involving a number of specific proteins (listed in Table 1.2). Actin A (ActA) and p60 (iap gene product) are involved in the invasion of some cell types (discussed later), but clearly the most important listerial factors involved in uptake by host cells are the internalin proteins. These proteins are encoded on a multi-gene family, the first two, Internalin (InlA) and Internalin B (InlB) (discussed below), have been detected in the *inlAB* operon and are the most extensively studied (reviewed by Kreft *et al.*, 2002). The function of other internalins during infection has not yet clearly been elucidated, although a mutant lacking the *inlE, inlG* and *inlH* genes is avirulent in a mouse infection model (Schubert *et al.*, 2001). The products of all these genes (Internalin multi-gene family) are rather larger proteins with a characteristic structure. The primary structure of internalins is characterised by a modular architecture (see Figure 1.3) essentially

comprising two repeat regions separated by an inter-repeat (IR)-region (Schubert *et al.*, 2001). At their N-terminus, a canonical export-mediating sequence, the signal peptide, is found, followed by a stretch of 6-16 leucine-rich repeats (LRRs) (depending on the respective protein and also on the definition of the LRR; Kreft *et al.*, 2002). Leucine-rich repeats (LRRs) are 20-29 residue sequence motifs present in a number of proteins with diverse functions. The primary function of these motifs appears to be to provide a versatile structural framework for the formation of protein-protein interactions (Kobe and Kajava, 2001). In the case of *Listeria,* these are blocks of 22 amino acids with a characteristic signature of leucines; the repeat units together forming a right-handed parallel beta-helix (Kreft *et al.*, 2002).

Table 1.2: *Summary of the main Listeria monocytogenes invasion-associated proteins and their receptors.*

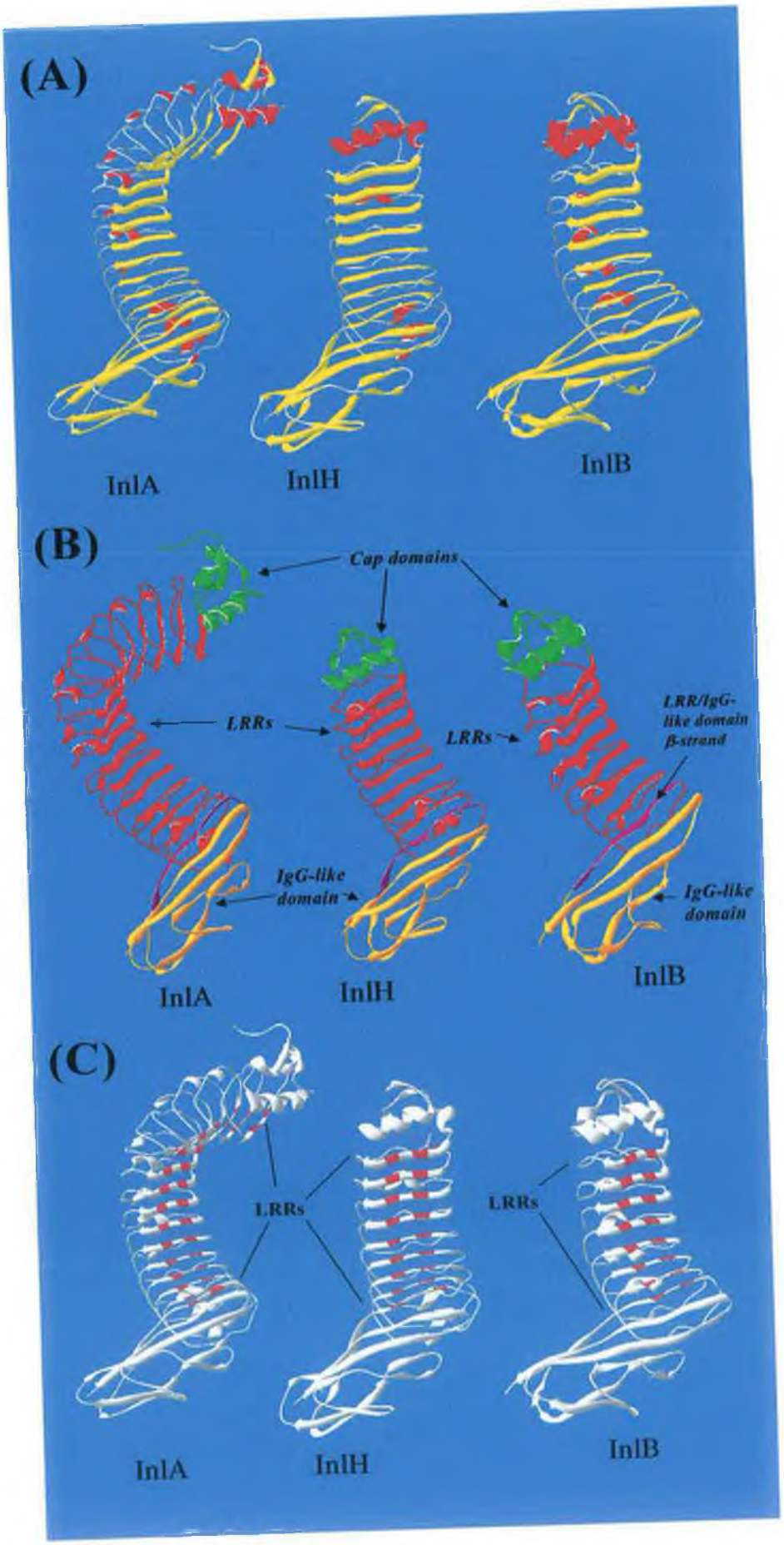
***Listeria monocytogenes* associated protein**	**Protein function**	**Host receptor/ligand**
Listeriolysin O (LLO)	Lysis of phagolysosome in conjunction with the bacterial metalloprotease (Mpl)	Activated by drop in pH
Actin A (ActA)	Intracellular movement by polymerisation of host cellular actin	Actin
Internalin (InlA)	Invasion associated protein promoting phagocytosis in cells expressing its receptor	E-Cadherin
Internalin B (InlB)	Invasion associated protein promoting phagocytosis in a broad spectrum of cells by activation of phosphatidylinositol-3-kinase	Hepatocytes growth factor receptor (HGF-R/Met) and a glycoproteins called gC1q-R
p60 (also known as iap)	Invasion associated secreted protein required for invasion into non-phagocytic host cells	Unknown

The receptor for InlA is the cell adhesion molecule E-cadherin (Mengaud *et al.*, 1996a). E-cadherin is a calcium-dependent cell adhesion molecule composed of five extracellular domains and a cytoplasmic tail (Takeichi, 1990). It plays a key role in embryogenesis by mediating the sorting of cells in tissues (Larue *et al.*, 1994). In adult life, it contributes to cell cohesion and tissue architecture (Hermistion and Gordon, 1995 and Cossart, 2001b). E-cadherin mediates adhesion between epithelial cells through homophilic interactions which require the first extracellular domain (EC1). Both lateral dimerisation of the ectodomain and connection of the cytoplasmic tail of E-cadherin to the actin cytoskeleton via catenins are required for strong homophilic interactions and formation of "adherens junctions" between epithelial cells (Lecuit *et al.*, 1999). InlA is an 80kDa protein that

promotes phagocytosis in cell lines expressing its receptor, E-cadherin (Mengaud *et al.*, 1996a). Expression of InlA in the non-invasive bacterium *L. innocua* renders the organism capable of invading caco-2 cells suggesting that InlA may be sufficient for entry (Gaillard, 1991). Recently, InlA was shown to promote the translocation of *Listeria monocytogenes* through the intestinal barrier (Lecuit *et al.*, 2001). By using anti-E-cadherin antibodies in a microtiter plate binding assay, Mengaud *et al,* (1996b) demonstrated that binding of Caco-2 cells to internalin was strictly E-cadherin-dependent. The nature of the amino acid at position 16 of E-cadherin is crucial for InlA-E-cadherin interaction and is species-specific (Lecuit *et al.*, 1999). The primary structure of internalin can be characterised by two different regions of repeats. The first region is made of fifteen 22 amino acid leucine-rich repeats (LRRs) (Cossart, 2001b). The second region is formed of three consecutive repeats, two of 70 amino acids and one of 49 amino acids (Mengaud *et al.*, 1996a,b). The carboxy-terminal region of InlA contains an **LPXTG** motif (where X can be any amino acid), a signature sequence necessary for sorting and cell wall anchoring of many surface proteins of gram positive bacteria (Lecuit *et al.*, 1997 and Cabanes *et al.*, 2002). LPXTG surface proteins are anchored to the cell wall by transpeptidases named sortases (Navarre and Schneewind, 1999). It has been suggested that internalin is anchored to the cell wall of *Listeria monocytogenes* by covalent linkages via the threonine residue of the conserved motif **LPXTG** (where X is any amino acid), (Fischetti *et al.*, 1990 and Schneewind *et al.*, 1995).

While InlA promotes the infection of human enterocyte-like cell lines, InlB shows a much broader host cell spectrum and promotes invasion by activation of phosphatidylinositol-3-kinase (Ireton and Cossart, 1997 and Schubert *et al.*, 2001). InlB is an agonist of the hepatocyte growth factor receptor (HGF-R/Met), a widely expressed receptor tyrosine kinase involved in complex cellular processes, such as cell proliferation, dissociation, migration and differentiation (Shen *et al.*, 2000). InlB also interacts with gC1q-R, a ubiquitous glycoprotein (Braun *et al.*, 2000), and with proteoglycans (Jonquieres *et al.*, 2001) that might potentiate interactions with Met. InlB is a 630 amino acid protein that is one of the 24 member *L. monocytogenes* internalin-related protein family (Glaser *et al.*, 2001 and Bierne and Cossart, 2002). It has been shown to mediate entry into cultured hepatocytes (Dramsi *et al.* 1995), and some epithelial, endothelial and fibroblast cell lines, including HeLa, HEP-2, CHO, L2, and Vero (Dramsi *et al.* 1997 and Parida *et al.*, 1998). InlB contains leucine rich repeats (LRRs) similar to those in InlA, (Dramsi *et al.* 1997). As shown in Figure 1.3, most Internalin-like proteins have a short N-terminal conserved cap region, followed by several leucine-rich tandem 22-residue repeats (LRR) and an inter repeat (IgG-like) region. In some cases, a second repeat region of up to three repeats of

~70 residues, the B-repeats are present (Bierne and Cossart, 2002). According to Parida *et al.* (1998) and Schubert *et al.* (2001), InlB possess 7 LRRs and one B repeat. However, InlB has also been reported to contain 6 (Cabanes *et al.,* 2002) and 8 (Bierne and Cossart, 2002 and Cossart, 2001b) leucine-rich repeat units (see Figures 1.3 and 1.4). The C-terminal region of InlB contains three tandem ~80-residue repeats, which are highly basic and start with the dipeptide glycine-tryptophan (GW) (Braun *et al.,* 1997). The mechanism of association of InlB with the bacterial surface is different from that of InlA. InlB protein does not contain any known cell-surface anchoring region, such as a hydrophobic C-terminal region or an LPXTG motif. Instead InlB is attached to the bacterial surface via the 232 amino acid GW repeat region beginning with the sequence glycine-tryptophan (GW) that is also present in a newly identified surface-associated bacteriolysin of *L. monocytogenes*, called Ami (Cabanes *et al.,* 2002). These repeats mediate a loose association of the protein with the bacterial surface, mainly through non-covalent interactions with lipoteichoic acid, a membrane-anchored polymer present on the surface of Gram-positive bacteria (Jonquieres *et al.,* 1999).


(A)
InlA
InlH
InlB
(B)
Cap domains
LRRs
LRRs
LRR/IgG-like domain β-strand
IgG-like domain
IgG-like domain
InlA
InlH
InlB
(C)
LRRs
LRRs
InlA
InlH
InlB

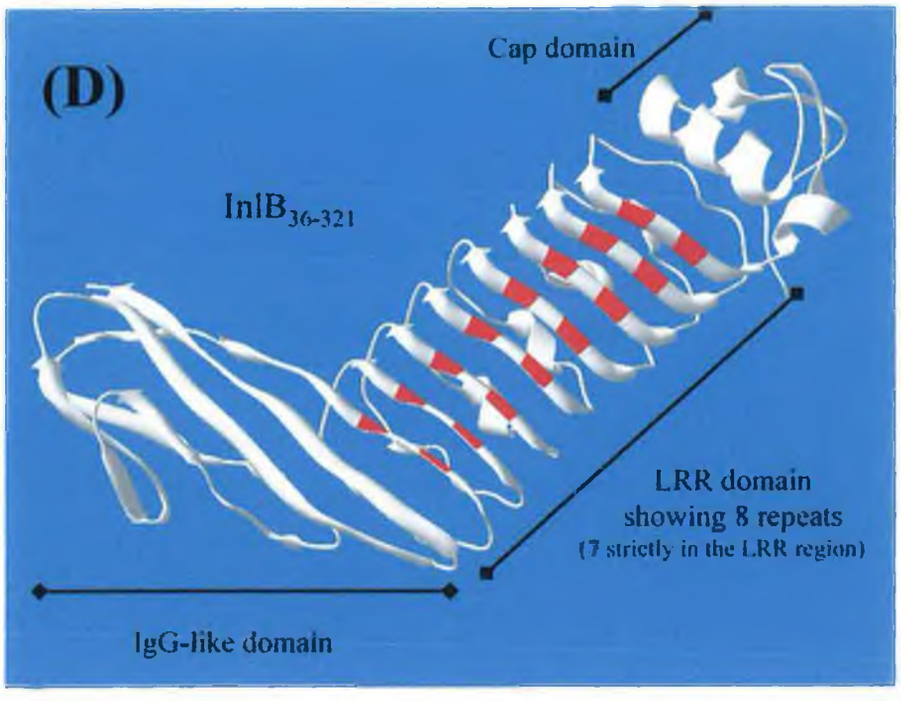


Figure 1.3: *Structures of $InlA_{36-496}$ (PDB code 1O6T), $InlH_{36-343}$ (PDB code 1H6U) and $InlB_{36-321}$ (1H6T) viewed using Swiss-PDB viewer. Panel A shows the 3D structures of InlA, InlH and InlB in a view towards the concave surface of the leucine rich repeat (LRR) region. α-helices are coloured in red, loops in solid grey and β strands as yellow arrows pointing in a N-terminus to C-terminus direction. Panel B shows the structures presented in panel A coloured according to domain structure. The area coloured in green represents the cap domain, the LRR region is coloured in red and the IgG-like domain coloured in orange. The final β strand (coloured purple) of the LRR region is simultaneously the first strand of the IgG-like domain. Panel C shows the structures coloured in grey with the leucine rich repeats coloured in red. Generally LRRs are 20-29 residues long and contain a conserved 11-residue segment with the consensus sequence LxxLxLxxxxL where x can be any amino acid and L positions can also be occupied by valine, isoleucine and phenylalanine (Kobe and Kajava, 2001). In Listeria, the internalin family of LRR proteins have varying numbers of repeats of 22 amino acid residues. In the diagram only the two repeat residues (i.e. LxxLxLxxxxL) present on the β-strands are coloured in red. InlA has 15 tandemly arranged leucine-rich repeats, InlH has 8 repeats and InlB has 7 repeats. Panel D shows an enlarged view of the InlB structure shown in C with the leucine-rich repeat residues coloured in red. As shown in panel B, the last β-strand of the LRR region simultaneously forms the first strand of the IgG-like domain. Depending on the classification of the LRR region (i.e. inclusion of the last β-strand) the InlB structure presented can be classified as having 7 or 8 repeats. In the literature InlB have been reported to have between 6 and 8 repeats (see text).*

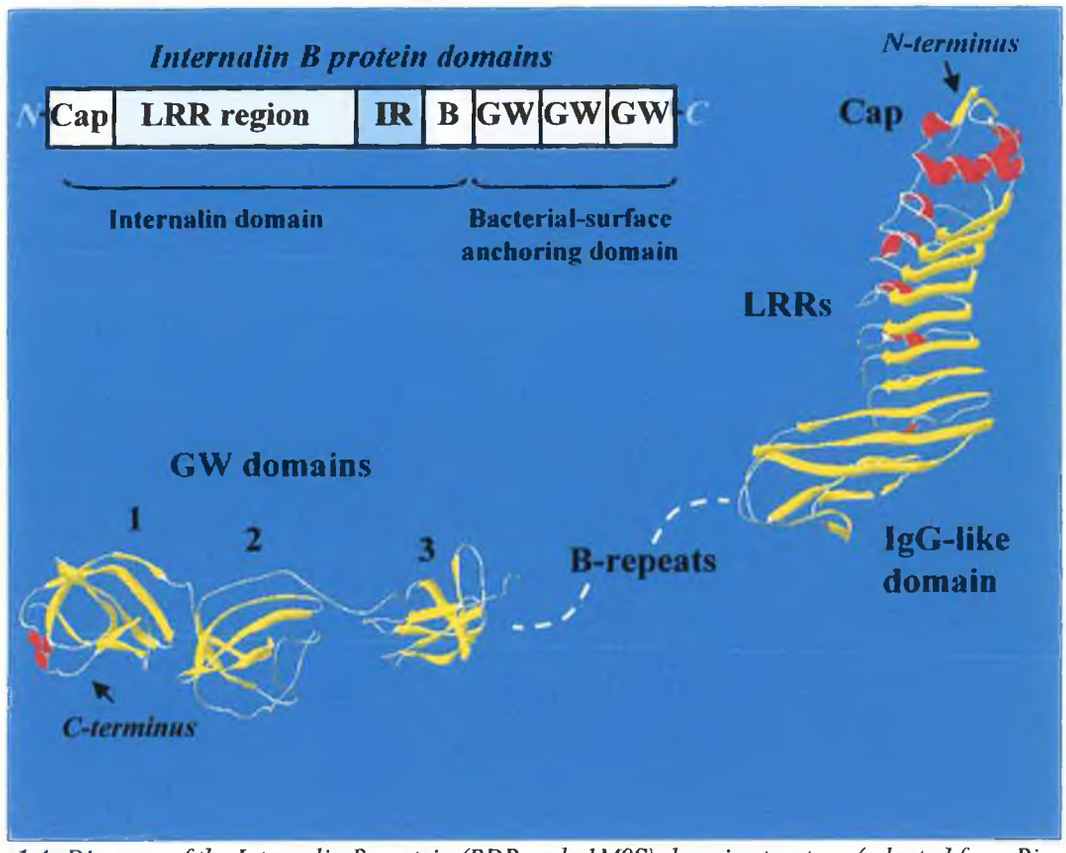


Figure 1.4: *Diagram of the Internalin B protein (PDB code 1M9S) domain structure (adapted from Bierne and Cossart, 2002 and Cossart et al., 2003). The Internalin domain of InlB contains four domains; a cap region, leucine rich repeats (LRRs), an IgG-like domain also known as an inter-repeat (IR) region and a B-repeat region. The amino terminal part encompassing the LRR and IR regions is involved in Met recognition. The protein is anchored to the cell surface via it carboxy-terminal GW repeat domains. The InlB protein tertiary structure shown was taken from the Protein data bank (1M9S) and viewed using the Swiss-PDB viewer program. The white dotted line represents the B-repeat region which could not be modelled.*

L. monocytogenes requires a 60-kDa major extracellular housekeeping protein for normal cell division (Bubert *et al.*, 1994 and Rowan *et al.*, 2000). This major extracellular 60 kDa protein (pI 9.5) was originally reported by Kuhn and Goebel (1989) and termed as p60. This p60 protein is transcribed independently of the central virulence regulator PrfA, and it is also involved in the attachment of *L. monocytogenes* to certain eukaryotic cells (Rowan *et al.*, 2000). The gene coding for p60 is called *iap* (invasion-associated protein) and its expression is controlled at the posttranscriptional level (Bhunia, 1997). P60 is a modular protein containing two LysM domains, a bacterial Src homology 3 (SH3) domain and a carboxy-terminal NLPC/P60 domain (Cabanes *et al.*, 2002). The LysM domain (40 residues long) is found in a variety of enzymes involved in degradation of the bacterial cell wall and is thought to have a general peptidoglycan-binding function. It has been shown that the p60 protein is among the strongest antigens in listeriae for B- and T-cell responses

(Geginat *et al.*, 1999 and Kolb-Maurer *et al.*, 2001). It has also been shown that p60 possesses murein hydrolase activity without which cells tend to form long chains, where septum formation between individual cells was intact but cells do not separate (Kuhn and Goebel, 1989). Further studies revealed that the lack of p60 protein was responsible for the loss of cell invasion ability (Kuhn and Goebel, 1989 and Park *et al.*, 2000b) and only the addition of purified p60 into culture media restored the cell internalisation capacity of these mutant cells. The addition of purified p60 also conferred increased cellular uptake when expressed in *Salmonella typhimurium* (Hess *et al.*, 1995) and, therefore, it was evident that p60 is an essential factor in the invasion into non-phagocytic host cells (Park *et al.*, 2000b).

Intracellular movement of *Listeria monocytogenes* requires the expression of a surface protein called "actin assembly" or ActA (Bhunia, 1997), and the two-membrane vacuole is lysed by Listeriolysin O and a lecithinase (PC-PLC) (Cabanes *et al.*, 2002). Listeriolysin O, a 58 kDa protein encoded by *hly,* is a member of a large family of oxygen-labile, sulphydryl-activated cytolysins present in many Gram-positive bacteria (Golfine and Wadsworth, 2002). When inside the cell, the cell recognizes the endocytosed vacuole as a target for degradation. How this recognition occurs is not fully understood, but the sudden drop in pH activates listeriolysin O (LLO) and metalloprotease (Mpl), the latter cleaving the bacterial-secreted phospholipase C (proPC-PLC) to its active form with vacuolar lysis occuring rapidly (Robbins *et al.* 1999).

1.5 *Intracellular spread of Listeria: An actin-based process*

Once escaped from the phagolysosome, the bacterium resides within the host cytoplasm where it harnesses the host machinery required for actin assembly, generating a comet-like tail (Figure 1.5) which propels the bacterium within the host cell (Sanger *et al.*, 1992 and Theriot *et al.*, 1992). It is this actin-based motility that enables *Listeria* to spread infection from one cell to the next via a membrane-bound protrusion. ActA, a 639 amino acid protein with a C-terminal hydrophobic region (Laurent *et al.* 1999) involved in anchoring to the membrane, is needed for polymerization of host actin and movement (Domann *et al.*, 1992). Genetic studies have shown that ActA possesses two regions important for motility. The NH_2-terminal region (1-262) appears solely responsible for actin nucleation, a low-probability process in which three actin monomers combine simultaneously to form a thermodynamically unstable trimer, the first step in the formation of filaments. The central domain (263-390), which contains four proline rich repeats, controls the rate of movement (Pistor *et al.*, 1995; Lasa *et al.*, 1997 and Goldberg, 2001). The proline-rich repeats of ActA form the binding site for vasodilator-stimulated phosphoprotein (VASP) (Laurent *et*

al., 1999) one of the many cellular proteins including alpha-actinin, profilin, cofilin, vinculin and the Arp2/Arp3 complex, which is involved in actin-based movement (Ireton and Cossart, 1997; Cossart, 2000; Bierne *et al.*, 2001 and Merz and Higgs, 2003).

VASP is associated with microfilaments in focal contacts and lamellae in spreading cells (Goldfine, 2001). This tetramer of identical 40-kDa subunits binds to profilin, a host cell component which binds to actin monomers in a one-to-one complex, alters the confirmation of the actin monomer, and accelerates the exchange of ATP for actin-bound ADP (Goldschmidt-Clermont, 1992) and ActA. ActA has four potential VASP-binding sites and VASP has 16 potential profilin-binding sites. As a result, this two-step binding-mechanism allows one ActA molecule to concentrate up to 64 profilin molecules, strongly amplifying the ability of *Listeria* to promote the assembly of actin filaments (Southwick and Purich, 1996).

ActA protein also contains a positively charged amino acid sequence thought to be involved in promoting actin nucleation by attracting negatively charged actin monomers (Southwick and Purich, 1996). The multiprotein complex isolated from platelet extracts, containing the actin-related proteins Arp2 and Arp3 (Cooper and Schafer, 2000 and Cossart, 2000), is sufficient to initiate actin polymerization at the surface of *Listeria*, most likely by interacting with ActA (Welch *et al.*, 1998). The Arp2/3 complex has been observed to remain bound to the pointed ends of nucleated filaments and is uniformly distributed throughout the actin tail while the bacterium is moving (Welch *et al.*, 1997). Although the direct binding of Arp2/3 to ActA has not been demonstrated, the domain of ActA that was found to synergize with Arp2/3 in nucleating actin assembly in-vitro, maps to the N-terminus of the protein, the same region of ActA that has been shown to be essential for nucleation of actin assembly on the surface of the bacterium within the cytoplasm of the mammalian cell (Beckerle, 1998). Indeed, when ActA and Arp2/3 are mixed together with monomeric actin, the nucleation phase of actin assembly which is normally slow, is essentially instantaneous (Beckerle, 1998 and Welch *et al.*, 1998). It is thought that Arp2/3-dependent capping of actin filaments may also enhance their stability by slowing disassembly from the pointed end of the polymers (Berkerle, 1998).

Other proteins such as capping proteins, ADF/cofilin and alpha-actinin play an important role in enhancing filament turnover, i.e. in increasing the site-directed barbed end-growth (Carlier, 1998). Alpha-actinin, a homodimer with an actin-binding site on each monomer, has the ability to cross-link actin filaments (Ireton and Cossart, 1997). It has been shown that microinjection of infected cells with a 53-kDa proteolytic fragment of this protein,

causes the disappearance of bacteria-containing filopodia and an arrest in movement (Dold *et al.,* 1994), indicating that this fragment acts as a dominant negative protein and that cross-linking of actin filaments is important for growth. Cofilin(ADF), a small G- and F-actin binding protein, increases the rate of actin depolymerization and shortens actin tails in human platelet extracts, while addition to highly diluted platelet extracts increased the rate of bacterial movement (Carlier, 1997). From these results it was suggested that cofilin may affect bacterial motility by stimulating the rate of actin filament turnover.

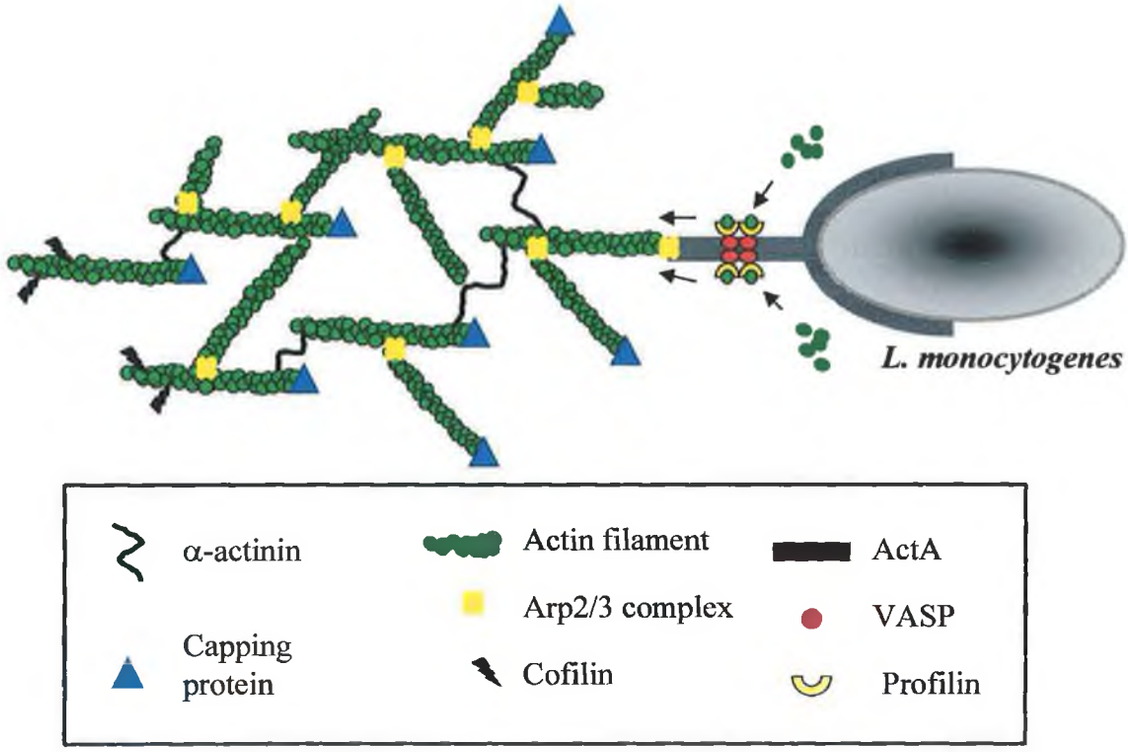


Figure 1.5: *Model of the Listeria monocytogenes actin-based motility process adapted from Cossart and Bierne (2001). The network of elements known as the actin cytoskeleton provides the supportive framework of the three-dimensional structure of eukaryotic cells (Goldberg, 2001). Intracellular microbial pathogens such as Listeria, have evolved mechanisms to utilise pre-existing pathways of actin cytoskeleton rearrangements to generate their own motility within cells. ActA binds the Arp2/3 complex at its Arp2/3 binding domain and an actin monomer at its actin-binding domain. ActA also binds VASP at its proline-rich repeat region with VASP in turn binding profilin. The actin nucleation activity of the Arp2/3 complex is stimulated by its binding to ActA and mediates the addition of actin monomers to the barbed end and caps the pointed end of a new actin filament. As the filament extends, the original Arp2/3 complex is released from ActA and another Arp2/3 complex binds both ActA and the side of the existing actin filament. Repeated rounds of filament branching, filament nucleation, filament extension, and Arp2/3 complex release generate a network of actin filaments linked at 70^{0} angles (Goldberg, 2001). At a distance from the bacterial surface, filament barbed ends are capped, thereby halting the addition of more actin monomers. Also filaments debranch and, with the assistance of the actin severing protein cofilin, depolymerise,*

thereby maintaining a local pool of actin monomers (Goldberg, 2001). The continuous assembly of an actin tail provides sufficient force to propel the organisms through the cytoplasm of the infected cell and into adjacent cells (see Figure 1.2 for a diagram of the intracellular spread of Listeria).

1.6 *Recent outbreaks of listeriosis*

Although usually associated with soft cheeses and unpasteurised milk (Harvey and Gilmour, 1992 and Rea *et al.,* 1992), *L. monocytogenes* can be found in a wide variety of foods. Listeriosis outbreaks in the United States and the UK, have been linked to foods such as hot dogs, pâté, cheese and chocolate milk. A survey of ready-to-eat (RTE) foods in Northern Ireland reported that high numbers of *L. monocytogenes* were also found in foods such as chicken and fish (Wilson, 1995). The isolation of *L. monocytogenes* from a wide variety of foods and recently reported *Listeria*-associated food poisoning outbreaks, have prompted the recognition of *Listeria monocytogenes* as a potentially very dangerous food-borne pathogen.

Cheese, milk, pâté and luncheon meats contaminated with *L. monocytogenes* all have a history of causing listeriosis. In January 2000, the Food Safety Authority of Ireland (FSAI) ordered the withdrawal of two French pâtés by retailers throughout the country. The pâtés were Rillettes (goose, duck or pork) and Langotine (pork tongue in aspic) and were manufactured under the brand name Paul Predault by Société Coudray in France. An advertisement placed in the Irish Times newspaper on Monday 10th January 2000 by the FSAI warned consumers not to consume these pâtés following a number of cases of *Listeria* poisoning in France (de Valk *et al.,* 2001) which have been associated with the pâtés. While there were no reported cases of such poisoning here in Ireland, a 75-year-old man and a baby contaminated in the womb died from *Listeria* suspected to have come from the company's Rillettes. Four other cases of *Listeria* poisoning were also reported in France with one victim in a coma. In a large epidemic in California in 1985, soft Mexican-style cheese was implicated as the vehicle (Schlech, 1997), in which at least 142 cases of listeriosis including 48 deaths, were reported. According to the Institute of Food Science and Technology (UK), an outbreak in Switzerland between 1983 and 1987, involving at least 122 cases (including 34 deaths) was eventually reported to be due to contamination of Vacherin Mont d'Or cheese (Bille, 1990). In 1989 samples of pâté imported into the UK were found to contain high numbers of *L. monocytogenes* and the pâté was withdrawn from sale. *L. monocytogenes* isolated from the pâté and comparison with strains from patients with listeriosis during the outbreak from 1987-1989 provided strong evidence that many cases of listeriosis, in that period, were attributable to the consumption of the pâté (Mclauchlin *et al.,* 1991). Due to the potentially long incubation period of *L.*

monocytogenes and because of the "flu-like" symptoms caused by mild listeriosis, most cases of listeriosis go undiagnosed or the vehicle for transmission goes undiscovered. An outbreak of gastroenteritis and fever among people at a picnic, in Illinois, on July 9th 1994, was later traced to chocolate milk consumed at the picnic (Dalton *et al.,* 1997). A subsequent examination of the production plant showed no defects in the pasteurization process. It later emerged that there was a breach in the lining of a holding tank, which allowed pasteurized milk to leak into the insulation jacket, creating a pool of sequestered milk. As the tank was drained, sequestered milk containing *L. monocytogenes* could re-enter through the breach (Dalton *et al.,* 1997).

L. monocytogenes was the cause of one of the biggest and most recent recalls of food in the United States. On the 22nd December 1998, Bil Mar Foods a meat processing subsidiary of the Sara Lee Corp, announced a 15million lb. recall of hot dogs and deli meats with an estimated cost of $50-70 million (Hollingsworth, 1999). As of February 4th 1999, the outbreak had allegedly caused 16 deaths and three miscarriages, and sickened about 72 people.

In Ireland, food poisoning statistics arise from notifications by the Health Boards under the Infectious Disease Regulations, 1981. Under the regulations statutory notifications are made by the attending clinician who is then obliged to notify the Department of Health and Children. But according to the Food Safety Authority of Ireland, there is a large variation throughout the country in the investigating and reporting procedures used for identifying cases of food poisoning. In 1998 the Food Safety Authority of Ireland conducted a survey in collaboration with the Public Health Infections Working Group with a view to developing a standard system for investigating and reporting cases of food poisoning throughout the country. With a new and more sophisticated system for investigating and reporting cases of food poisoning, food poisoning cases due to *Listeria* and other bacteria will become more apparent (1273 cases notified in '98 compared to 448 cases in '97), increasing the need for stricter production processes and rapid detection systems.

The availability of records and statistical data is very poor in the Republic of Ireland. This contrasts with other nations such as the U.S. and EU member states which keep accurate records of listeriosis and food contamination and such information is made available to the general public. Such records are essential for the implementation of effective preventive measures to reduce incidences of listeriosis. The work undertaken in this project will provide a rapid detection system for the presence of pathogenic *Listeria*. This technology if implemented by the relevant food safety authority and health services would greatly

facilitate the recording of both the prevalence of *Listeria* in food and in the environment, giving realistic measure of the dangers of contracting listeriosis. It is important that this information is conveyed between the Public Health Services, the Food Safety Authority, and the general public. The lack of documented cases in the Republic of Ireland, does not necessarily reflect the true rate of infection. Many cases remain undetected due to the similarity of symptoms with flu and because the general incidence of food poisoning caused by *L. monocytogenes* remain undiagnosed and are recorded as general bacterial food poisoning. The lack of a rapid assay for the detection of *Listeria* spp. may account for the absence of documented cases of human listeriosis in Ireland.

1.7 *Current detection techniques for Listeria*

In recent years, the hazard analysis critical control point (HACCP) concept has been proposed as the best approach to assure food safety (Silva *et al.*, 2003). HACCP is a methodical and systematic application of science and technology to evaluate, plan, control, and document the safe and efficient manufacture of products, from raw materials to end use (Vega-Mercado *et al.*, 2003). The goal of HACCP is to ensure product safety before, during, and after manufacture. The inclusion of *L. monocytogenes* in the list of organisms subject to HACCP has recently driven the search for detection methods suitable for on-line monitoring (Almeida and Almeida, 2000).

Given the severity of *Listeria* infections and its ability to grow at refrigeration temperatures, the US FDA has adopted a 'zero-tolerance' policy for the presence of the bacterium in food and with the use of HACCP strategies aims to effectively eliminate *Listeria monocytogenes* from food processing environments and, therefore, processed food products (Donnelly, 2001). The critical issue facing the implementation of any "zero tolerance" policy relates to the lack of reliable procedures for the detection of low numbers of *Listeria* in foods. The U.S Food and Drug Administration (FDA), the International Organization for Standardization (ISO), and the International Dairy Federation (IDF) have formulated methods for the detection and identification of *L. monocytogenes* in dairy products. The ISO method 11290 and the IDF Standard 143A:1995 method for the detection and enumeration of *Listeria monocytogenes* in foods are used in most countries, including the Republic of Ireland (Scotter et al., 2001a,b). According to these standards, in general, the detection of *Listeria* spp. necessitates at least three successive stages.

Enrichment in selective liquid medium is the first stage of the process. As the numbers of *Listeria monocytogenes* present in food, especially in dairy products, are usually low,

attempts to directly isolate *Listeria monocytogenes* usually fail (Doyle and Schoeni, 1987). Selective medium (enrichment broth (EB)) is usually inoculated with the test portion of the sample and incubated at 30 ± 1°C for 48 ± 2 h (Vlaemynck and Moermans, 1996). The second stage, isolation and presumptive identification, involves the inoculation from the culture, obtained in the enrichment medium, into the isolation medium (Oxford agar). The inoculated medium is incubated at 37 ± 1°C and examined after 48 ± 2 h to check for the presence of colonies which are considered to be presumptive *Listeria* spp. In the third stage of the process colonies of presumptive *Listeria* spp. are subcultured onto a non-selective solid medium for confirmation of identity by means of appropriate morphological, physiological and biochemical tests.

While cultural methods provide the "gold standard" for the detection of *L. monocytogenes* (Golsteyn Thomas, 1991), these are labour intensive and time-consuming, taking up to a week with some methods, and hence present difficulties in the quality control of semi-perishable foods. Detection of *L. monocytogenes* by DNA amplification has been shown to be more suitable (see Table 1.3). The polymerase chain reaction (PCR) can be used to enhance the sensitivity of nucleic acid-based assays. Target nucleic segments of defined length and sequence are amplified by repetitive cycles of strand denaturation, annealing, and extension of oligonucleotide primers by the thermostable DNA polymerase, *Thermus aquaticus* (Taq) DNA polymerase. PCR has provided the basis for many assays developed for the detection of *L. monocytogenes* in recent years with the listeriolysin O (*hlyA*) (Deneer and Boychuk, 1991; Golsteyn Thomas, 1991; Johnson *et al.*, 1992; Bsat *et al.*, 1994 and Norton and Batt, 1999), the p60 (*iap*) (Hein *et al.*, 2001 and Schmid *et al.*, 2003), the Actin A (*ActA)* (Longhi *et al.*, 2003) and the internalin (*InlAB*) (Ingianni *et al.*, 2001; Kaclikova, *et al.*, 2003 and Jung *et al.*, 2003) genes being frequent targets. Also with the advent of microarray technology, microarrays have been coupled with PCR, serving as a set of parallel dot-blots for enhanced product detection and identification of *Listeria* spp. (Volokhov *et al.*, 2002; Borucki *et al.*, 2003 and Call *et al.*, 2003). Use of selective enrichment media overcomes the difficulties that may arise in direct PCR by increasing the amount of target DNA and decreases the amount of inhibitors of PCR that may be present in samples. From an industrial point of view, detection of *L. monocytogenes* using PCR can be a time consuming technique with enrichment broth required to ensure that only DNA from viable cells is detected. Nucleic acid-based amplification systems can still have severe limitations due to the complexity of sample handling and processing, especially when the detection of PCR products are achieved by such methods as Southern blot, gel electrophoresis or dot blot hybridization.

Table 1.3: *List of recent nucleic acid-based detection assays for Listeria monocytogenes highlighting the format used, limit of detection achieved and source of the assay.*

Assay format	Limit of detection	Reference
Direct PCR (InlB)	10^4 cfu/ml	Pangallo et al. (2001)
PCR (InlB)	<10 cfu/25g	Kaclikova et al. (2003)
Competitive PCR (hlyA)	10^3 cfu/0.5ml	Choi and Hong (2003)
Real-time PCR	10^3 cells/ml	Bhagwat (2003)
Real-time PCR (iap)	10^1-10^2 cfu/ml	Hein et al. (2001)
PCR/DNA probe	1-10 cfu/25ml	O'Connor et al. (2000)
PCR/blot	10^3 cfu/ml	Li et al. (2000)
IMS/PCR	25 cells/25g (ham)	Hudson et al. (2001)
IMS/PCR	10^2 cfu/ml	Hsih and Tsen (2001)

Immunological detection with antibodies (see sections 1.8 and 1.9 for a description of antibody structure and diversity) is perhaps the only technology that has been successfully employed for the detection of cells, spores, viruses and toxins alike (Iqbal *et al.*, 2000). Polyclonal antibodies (section 1.10) can be raised quickly and cheaply and do not require the time or expertise associated with the production of monoclonal antibodies (Cahill *et al.*, 1995). However, polyclonal antibodies are limited both in terms of their specificity and abundance. Since the development of hybridoma techniques (Kohler and Milstein, 1975) and the emergence of recombinant antibody phage display technology (section 1.11), developed during the past decade, immunological detection of microbial contamination has become more sensitive, specific, reproducible and reliable with many commercial immunoassays (Table 1.4) available for the detection of a wide variety of microbes and their contaminants. While nucleic acid-based detection may be more specific and sensitive than immunological-based detection, the latter is faster, more robust and has the ability to detect not only contaminating organisms but also their biotoxins that may not be expressed in the organism's genome (Iqbal *et al.*, 2000).

Other methods such as using Phospatidylinositol-specific Phospholipase C activity as a marker to distinguish between pathogenic and non-pathogenic *Listeria* spp. (Notermans *et al.*, 1991) and immunomagnetic capture on beads to recover *Listeria* from environmental samples (Mitchell *et al.*, 1994), have failed to remove the time-consuming culture step, although the latter proved more rapid and more sensitive than a standard culture method for detection of *Listeria* in environmental samples.

Table 1.4: *Table of commercially available antibody-based tests for Listeria and L. monocytogenes. Adapted from the University of Californa Davis, (http://seafood.ucdavis.edu/HACCP/Compendium/Chapt.15.htm)*

Test	Analytical Technique	Approx. Total Test Time[1]	Supplier
Assurance *Listeria* EIA[2] Used to identify *Listeria* spp. including *L. monocytogenes*	Enzyme immunoassay	50 h	BioControl Systems, Inc. www.rapidmethods.com
Transia Plate Used to identify *L. monocytogenes*	Sandwich-type enzyme immunoassay	45 h	Diffchamb www.diffchamb.com
Dynabeads anti-*Listeria* Used to identify *L. monocytogenes*	IMS[3]	48 h	Dynal, Inc. www.dynal.no/
EIA Foss *Listeria*	Combination of ELISA and IMS[3]	48 h	Foss N. America, Inc. www.Fossnorthamerica.com
***Listeria* Rapid Test**[2] Used to identify *Listeria* spp. including *L. monocytogenes*	EIA	42 h	Oxoid, Inc. E-mail: jbell@oxoid.ca
***Listeria*-Tek™**[2]	ELISA	48 h	Organon Teknika Corp. E-mail: casey@orgtek.com
ListerTest ™[2]	IMS[3]	24 h	Vicam, L.P. www.vicam.com
MICRO-ID Listeria[2] Used to identify *Listeria* spp.	Latex agglutination	24 h	Remel, Inc. www.remelinc.com
Reveal® for *Listeria*[2] Used to identify *Listeria* spp	Sandwich ELISA	48 h	Neogen Corporation www.neogen.com
VIDAS LMO Used to identify *L. monocytogenes*	Enzyme linked fluorescent assay	48 h	(Sewell *et al.*, 2003) BioMerieux Inc. www.biomerieux.com
VIP for *Listeria*[2] Used to identify *Listeria* spp. including *L. monocytogenes*	Visual immunoprecipitate	48 h	BioControl System, Inc www.repidmethods.com

[1] Includes enrichment. [2] AOAC Approved (Web: www.aoac.org). [3] Immunomagnetic separation.

1.8 *Antibody Structure*

As presented in Table 1.4, a number of commercially available antibody-based tests for the detection of *Listeria* have been developed. To understand the fundamental principles of antibody-based detection systems a comprehensive knowledge of antibody structure and function is required. This section describes the structure of antibodies and antibody fragments and discusses the antibody components involved in binding to a target analyte.

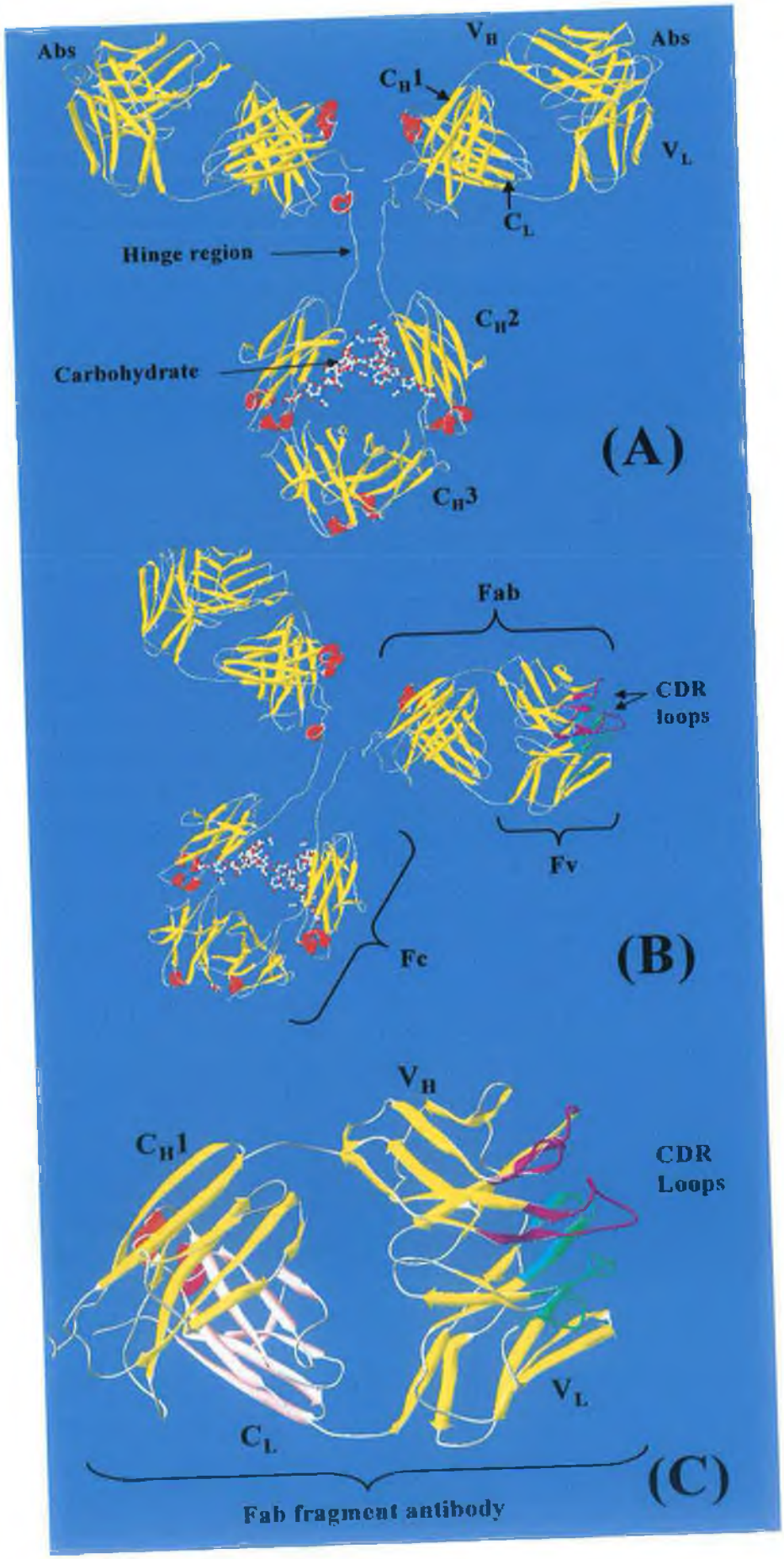
Antibodies are highly soluble serum glycoproteins involved in the defense mechanism of the immune system. The basic structure of all antibody molecules comprises of two identical light chains and two identical heavy chains linked together by disulphide bonds (Figure 1.6). All antibodies are constructed in the same way from paired heavy and light polypeptide chains, and the generic term immunoglobulin (Ig) is used for all such protein molecules. Determination of the amino acid sequences of many immunoglobulin heavy and light chains has revealed, that each chain consists of a series of similar, although not identical amino acid sequences, each about 110 amino acids in length (Branden and Tooze, 1991). The light chain comprises of two such sequences (domains), whereas the heavy chain of IgG contains four. This suggests that the immunoglobulin chains have evolved by repeated duplication of an ancestral gene corresponding to one folded domain of the protein (Janeway *et al.*, 1999). The amino-terminal sequences of both the heavy and light chains vary greatly between different antibodies and are responsible for antigen binding.

Five classes of immunoglobulin IgM, IgD, IgG, IgA and IgE can be distinguished biochemically as well as functionally, whereas more subtle differences confined to the variable region account for the specificity of antigen binding (Janeway *et al.*, 1999). IgG, IgD and IgE are monomeric immunoglobulin molecules built up from two copies each of two different polypeptide chains, heavy (H) and light (L). The light chain folds into two domains, a V_L domain with variable sequence between different immunoglobulin molecules and a C_L domain with constant sequence between different immunoglobulin molecules. The heavy chain folds into four domains, one variable (V_H) and three constant domains (four in the case of IgE), C_H1, C_H2 and C_H3 (C_H4 also present in IgE). An additional polypeptide chain, the J chain, is associated with the immunoglobulins A and M, allowing the linking of two immunoglobulin units to form the dimeric IgA and five immunoglobulin units to form the pentamer, IgM. IgGs are the major components of the five different classes of immunoglobulins with serum concentrations of up to 13.5 mg/ml (Shantha Raju, 2003). There are four isotypes of IgGs, classified as IgG1, IgG2, IgG3 and IgG4, each having different numbers and arrangements of interchain disulphide bonds. In

IgG1 the disulphide bond linking the heavy and light chains goes to the hinge region, whereas in IgG2, IgG3 and IgG4 subclasses the disulphide bond goes to the junction between the variable and constant regions (Shantha Raju, 2003).

The IgG antibody molecule (with a molecular weight of approximately 150 kDa) is composed of two heavy (50 kDa) and two light (25 kDa) polypeptide chains. Proteolytic enzymes (proteases) that cleave polypeptide sequences have been used to dissect the structure of antibody molecules and to determine which parts of the molecule are responsible for its various functions. Figure 1.6, panel B, shows that the IgG molecules comprise of three equally-sized globular portions (Fc and two Fab portions) joined by a polypeptide chain known as the hinge region, forming a crude Y shaped molecule. The hinge region that links the Fc and Fab portions of the antibody molecule is in reality a flexible tether, allowing independent movement of the two Fab arms, rather than a rigid hinge. Genetic engineering techniques (discussed in detail in sections 1.11-1.15) also now permit the construction of a truncated Fab fragment comprising only the V region of the heavy chain linked by a stretch of synthetic peptide to a V region of a light chain (known as a single chain Fv fragment antibody).

The discrete globular domains of immunoglobulin chains fall into two distinct structural categories, corresponding to V and C domains. The similarities and differences between the two domains can be seen in Figures 1.7 and 1.8, which show the characteristic tertiary structure of the immunoglobulin domains. Each immunoglobulin domain is connected in tandem and has a characteristic "immunoglobulin fold" composed of two roughly parallel layers of anti-parallel β-pleated sheets connected with an intrachain disulphide bond. The positions of the two cysteine residues in the β-strands that form the disulphide bond are highly conserved (Mayforth, 1993). Linking the β-strands are loops of amino acids that are more variable in sequence. The amino acids comprising the β-strands are arranged so that the interior of the domain is hydrophobic and exterior is hydrophilic. The C domains have seven β-strands, while the V domains have an extra β-strand pair and loop (the extra loop forming the second hypervariable regions as shown in Figure 1.8). The tertiary structure of the homology domains promotes interaction between the faces of the β-sheets and subsequently allows the correct folding into a functional antibody molecule.


Abs
V_H
Abs
C_H1
V_L
C_L
Hinge region
C_H2
Carbohydrate
(A)
C_H3
Fab
CDR
loops
Fv
Fc
(B)
V_H
C_H1
CDR
Loops
V_L
C_L
(C)
Fab fragment antibody

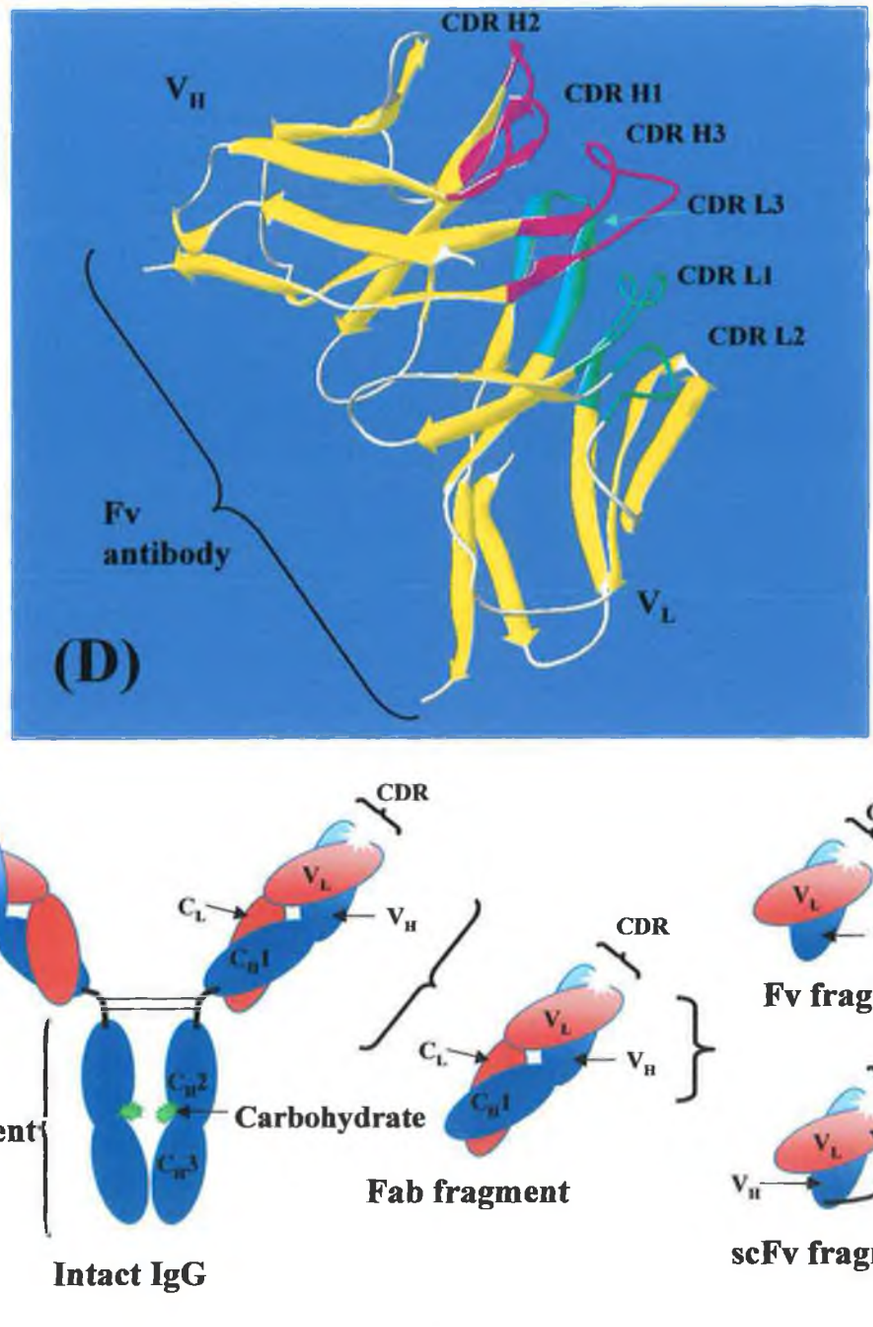


Figure 1.6: *Structure of an antibody and antibody fragments. Immunoglobulin G (IgG) antibodies are made up of four polypeptide chains, comprising of two identical light chains and two identical heavy chains, and can be thought of as forming a flexible Y-shaped structure. Each of the four chains has a variable (V) region at its amino terminus, which contributes to the antigen-binding site (ABS) and a constant (C) region, which in the heavy chain determines the isotype and hence the functional properties of the antibody. The light chains are bonded to the heavy chains by many non-covalent interactions and by disulphide bonds, and the paired V regions of the heavy and light chain domains generated two identical antigen-binding sites (ABS), which lie at the tips of the Y –shaped antibody structure. (A) Ribbon structure of an IgG antibody showing the various polypeptide domains, region of gylcosylation, hinge region*

(flexible region) and the antigen binding sites. β-strands are coloured in yellow, α-helices in red and loops in solid grey. (B) Ribbon structure of an IgG antibody indicating the constant fragments (Fc), antigen binding fragments (Fab), variable fragments (Fv) and the complementarity determining regions (CDR). The heavy chain CDR regions are coloured in purple and the light chain CDR regions in green. (C) Ribbon structure of an enlarged view of the Fab fragment indicated in panel B. A Fab antibody fragment consists of the constant light (C_L) and variable light (V_L) domains associated with variable heavy (V_H) and constant heavy domain 1 (C_H1). The β-strands of the light chain constant domain are coloured in salmon for clarity. (D) Ribbon structure of a Fv fragment antibody with CDR loops indicated. (E) Diagrammatic representation of the antibodies and antibody fragments presented in panels A-D. The fragments presented above may be produced by genetic, enzymatic or chemical manipulation. The whole antibody may be initially broken up into either Fab (antigen-binding fragment) or $F(ab')_2$ (two antigen-binding fragments linked) and Fc (constant domain fragments) regions. Fab fragments may be further broken up into Fv (variable fragments) and scFv (single chain Fv), which are all capable of antigen binding. A scFv antibody contains a synthetic 15-20 amino acid linker peptide (usually a glycine-serine repeat linker) which acts to stabilise the antibody fragment without interfering with domain association. The complementarity-binding regions (CDR) or hypervariable regions located on both the variable heavy and light chains are responsible for antigen binding (Branden and Tooze, 1991).

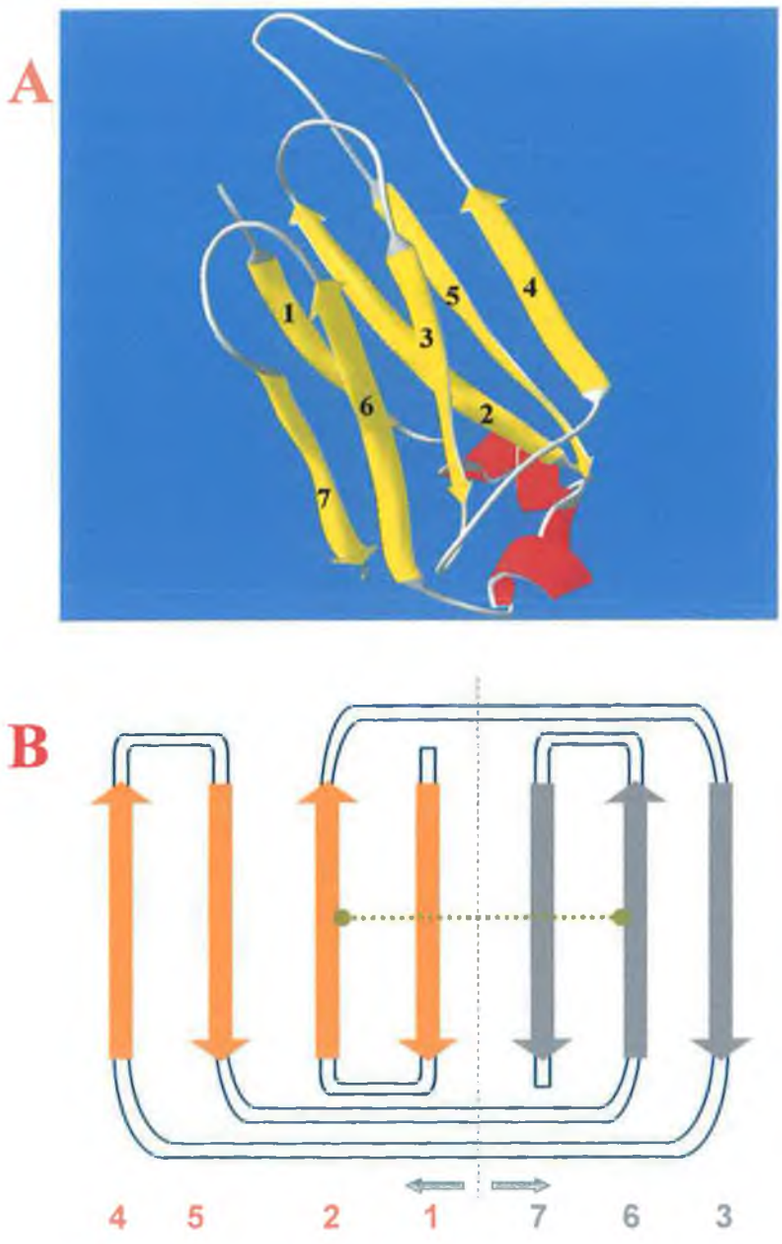


Figure 1.7: *Structure of the immunogolubulin constant domain. All constant domains are built up from seven β strands arranged so that four strands form one β sheet and remaining three strands form a second sheet. The sheets are closely packed against each other and joined together by a disulphide bridge from β strand 6 in the three strand sheet to β strand 2 in the four strand sheet. Most of the invariant residues of the constant domain, including the disufide bridge, are found in the sheets of the framework region. These invariant residues have two important functions. One is to form and stablise the framework by packing the β sheets through hydrophobic interactions to give a hydrophobic core between sheets. Their second function involves interactions between constant domains of different chains to form a complete immunoglobulin molecule. Panel (A) shows a ribbon structure of the constant light domain of an IgG1 antibody (PDB code 1IGY). β strands are coloured in yellow, loops in grey and α-helices in red. The β strand arrows point from*

the N terminus to the C terminus and are numbered in black. From the view presented, the β sheets consisting of strands 3,6 and 7, and strands 1,2,4 and 5 can be clearly differentiated. Panel (B) shows a simple diagramatic representation of the direction and layout of each β strand. The β strands are coloured orange and grey representing the two β sheets consisting of 4 and 3 strands, respectively. The disulphide bond between strands 2 and 6 is indicated by the horizontal broken line. The veritical broken line indicates the axis on which both sheets can be folded together forming a solid 3D antiparalled β barrel consisting of the 3 stranded β sheet packed tightly against the four stranded β sheet (Branden and Tooze, 1991).

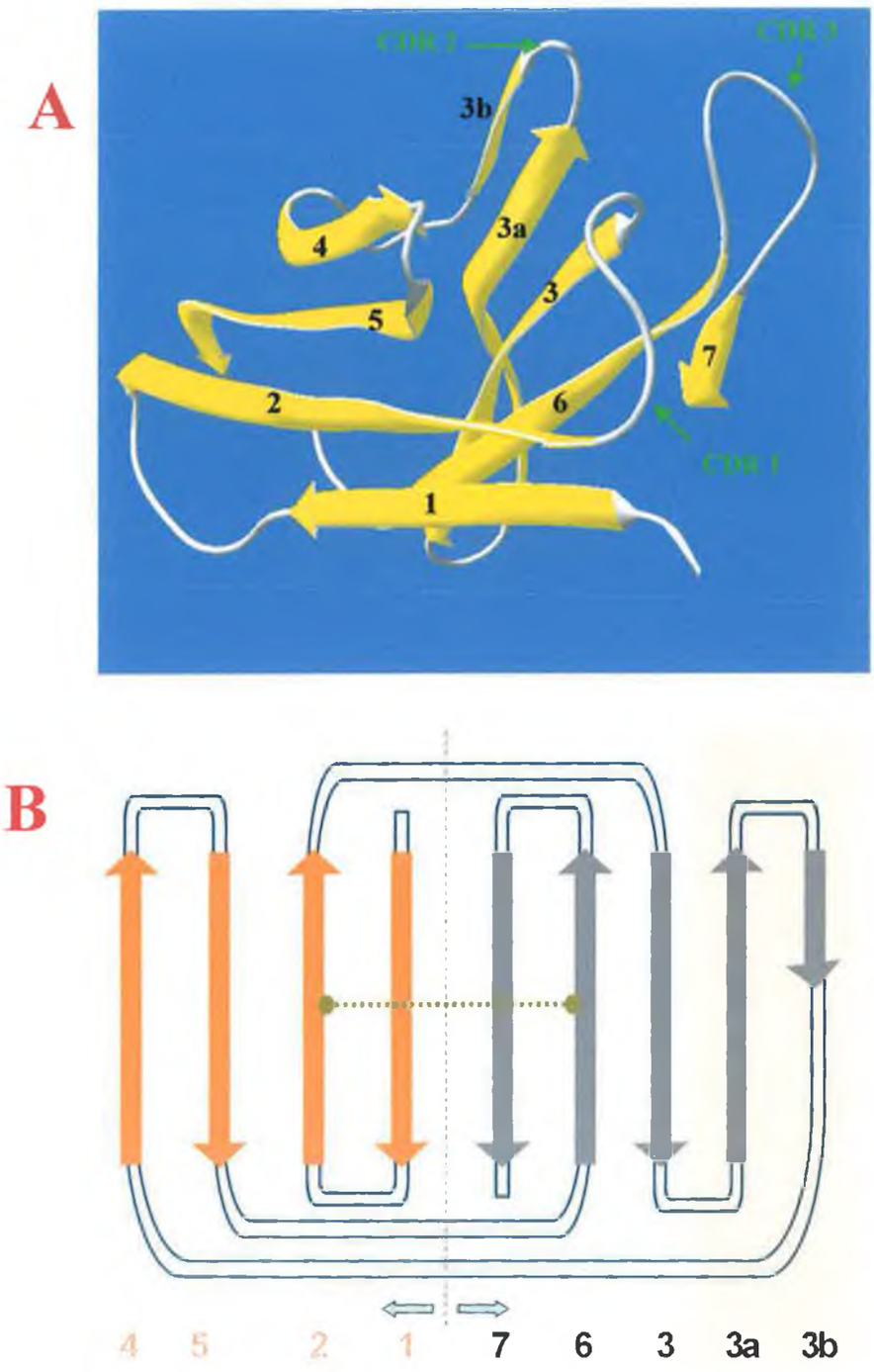


Figure 1.8: *Structure of the immunogolubulin variable domain. The overall structure of the variable domain is very similar to that of the constant domain but there are nine β strands instead of seven. The two additional β strands are inserted into the loop region that connects β strand 3 and 4 (labelleled 3a and 3b). Functionally, this part of the polypeptide chain is important since it contains the hypervariable region CDR 2. The two extra β strands provide the framework that positions CDR 2 close to the other two hypervariable regions in the domain structure. The CDR 1 and 3 loops are found between β strands 2 and 3, and 6 and 7, respectively. Panel (A) shows a*

ribbon structure of the variable light domain of an IgG1 antibody (PDB code 1IGY). β strands are coloured in yellow, loops in grey and the CDR regions indicated by green arrows. The β strand arrows point from the N terminus to the C terminus and are numbered in black. From the view presented, the β sheets consisting of strands 3, 3a, 3b, 6 and 7, and strands 1,2,4 and 5 can be clearly differentiated. Panel (B) shows a simple diagramatic representation of the direction and layout of each β strand. The β strands are coloured orange and grey representing the two β sheets consisting of 4 and 5 strands, respectively. The two additional β strands are numbered 3a and 3b (shown in the shaded area of the diagram). The disulphide bond between strands 2 and 6 is indicated by the horizontal broken line. The veritical broken line indicates the axis on which both sheets can be folded together forming a solid 3D antiparalled β-barrel consisting of the 5 stranded β-sheet packed tightly against the four stranded β sheet.

1.9 *The generation of diversity in the humoral response*

The immune repertoire is remarkably diverse, expressing as many as 10^{11} antibodies with different specificities. It has been estimated that a human body generates 50 million new antibody-producing B cells every day (Branden and Tooze, 1991). The size of the immune repertoire makes it impossible for each antibody to be coded for by its own germline DNA. Rather, unique genetic mechanisms generate the diversity in the variable regions of antibodies.

The germline genes encoding immunoglobulins are located at three different loci on the human chromosomes. The heavy-chain locus is on chromosome 14, the κ light-chain locus is on chromosome 2, and the λ light chain locus is on chromosome 22 (Mayforth, 1993). Each immunoglobulin gene locus contains gene segments (exons) that code for the V and C domains. The germline DNA that codes for the V regions is further divided into two or three groups of gene segments, each group containing anywhere from four to hundreds of gene segments. One gene segment from each group is rearranged or assembled together to form the DNA that codes for the variable region. In the light chain, each V domain is encoded in two separate DNA segments. The first segment encodes the first 95-101 (approximately) amino acids of the light chain and is termed a V gene segment because it makes up most of the V domain. The second segment encodes the remainder of the V domain (up to 13 amino acids) and is termed a joining or J gene segment. The process of rearrangement that leads to the production of an immunoglobulin heavy and light-chain genes is shown in Figure 1.9. The joining of a V and a J gene segment creates a continuous piece of DNA encoding the whole of the light chain V region. The J gene segments are separated from the C region genes by non-coding DNA, and are joined to them by RNA

splicing after transcription, not by DNA recombination (Janeway *et al.*, 1999). The heavy chain V regions are encoded in three gene segments. In addition to the V and J gene segments, there is a third gene segment called the diversity or D gene segment, which lies between the V and J gene segments. The process of recombination that generates a complete heavy chain V segment occurs with the joining of a D gene segment to a J gene segment, followed by a V gene rearrangement to DJ completing the heavy chain V region exon (Figure 1.9).

Further diversity can be generated in the areas where the gene segments join. The positions where the gene segments are fused together are not constant and so imprecise DNA recombination can lead to changes in the amino acids at these sites, called junctional diversity. Bases can be inserted, deleted, copied or altered at the sites of combination and these can be part of the hypervariable regions that produce the antigen-binding site leading to greater diversity in the immunoglobulin specificity (Tonegawa, 1983).

Point mutations occur at a much higher frequency in the V(D)J gene structure than normal. This is referred to as somatic hypermutation and it is antigen-driven mechanism. It allows the secondary and further immune responses to produce antibodies with increased affinity for the antigen of interest. Somatic hypermutation occurs in the germinal centers of lymphoid tissue and is a critically timed process (Mayforth, 1993). In the primary response the antibodies produced are usually of low affinity and are close to germline sequence but as the response matures the amino sequence diverges from the germline. This gives the immune system the ability to provide more active and specific antibodies.

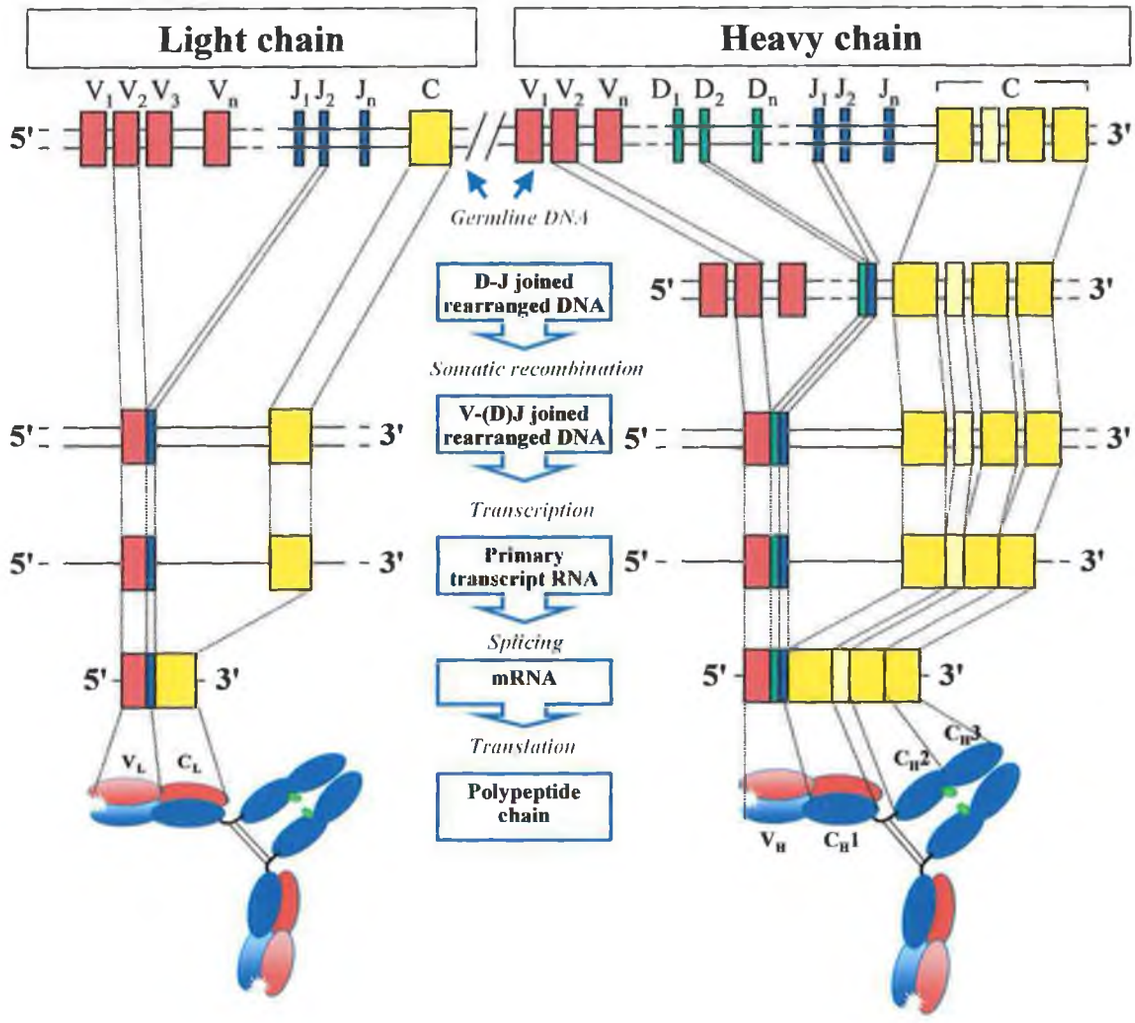


Figure 1.9: *Construction of the variable regions by somatic recombination of separate gene segments. Light chain V-region genes are constructed from two segments as shown on the left hand side of the presented diagram. A variable (V) and joining (J) gene segment in the genomic DNA are joined to form a complete light-chain V-region exon. The C region is encoded in a separated exon and is joined to the variable region exon by splicing of the light-chain RNA to remove the J to C introns. Heavy chain V regions are constructed from three gene segments as shown on the right hand side of the diagram. First the diversity (D) and J gene segments join, then the V gene segment joins to the combined DJ sequence, forming a complete V_H exon. The heavy chain C-region genes (only one shown here) are each encoded by several exons (the exon encoding the hinge region is shaded in a pale yellow). The C-region exons are spliced to the V-domain sequence during processing of the heavy chain RNA transcript. Light chain and heavy chain genes are translated into large polypeptide chains that associat to form the antibody molecule. The diagram present here was adapted from Janeway et al. (1999).*

1.10 *Polyclonal and monoclonal antibodies*

It is possible to obtain antibodies against a very broad range of antigens including conformationally intact or denatured proteins, short peptides, carbohydrates, drugs, hormones and small chemicals (Delves, 1997). An antigen is traditionally defined as any substance that can be bound by the antigen-combining site of an antibody molecule, whereas an immunogen is any antigen capable of eliciting an immune response. Antigens used for immunization may be highly purified preparations containing a single molecular species, a mixture of molecules, or an extremely complex "antigen" such a microorganism or cell. All of the antibodies that a given B-cell clone secretes have exactly the same specificity and avidity for antigen and are called monoclonal antibodies. However, when the immune system is challenged with antigen, many different B cells respond and secrete antibodies. They all recognise the antigen but in slightly different ways and comprise different specificities, avidities, and cross-reactivities. This is called a polyclonal response and antibodies derived from the challenged immune system are known as polyclonal antibodies (Mayforth, 1993).

Monoclonal antibodies (mAb) provide reagents with a single epitope specificity and potentially limitless amounts of identical antibody. On the other hand, polyclonal antibody mixtures usually contain multiple epitope specificities and are limited in quantity to the amount of serum that can be obtained from the immune animal. For commercial purposes larger animals such as sheep, pigs and goats can be used for antibody production. However, for laboratory-scale production of polyclonal antibodies medium-sized species such as rabbits provide perfectly adequate amounts of serum and can yield high-titer antibodies which will last, for most applications, for several years (Delves, 1997).

Paradoxically, a polyclonal antibody antiserum can sometimes provide greater specificity than a mAb raised against the same antigen. This is because undesirable cross-reactions can not be removed from a mAb because all of the molecules have identical specificity. The specificity of a polyclonal antiserum can also be improved by affinity chromatography using purified antigen.

Hybridoma technology was first described by Milstein and Kohler in 1975 and revolutionized the production of antigen specific antibodies. Primed B cells obtained from experimental animals (such as mice and rats) immunized with the desired antigen can be fused to myeloma cells, forming hybridoma cells capable of producing antibody of the desired specificity. The myeloma variants that are chosen as fusion partners for hybridoma

technology, do not secrete any antibodies, fuse well, carry a selectable trait(s) and give rise to stable hybrids (Mayforth, 1993). The hybrid cell (hybridoma) inherits antigen specificity from the normal B cell and immortality from the tumor B cell. Unfused normal B cells are not immortal and die in culture. The myeloma cell lines used are mutants lacking either of the enzymes hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK) and they are unable to use the salvage pathways of nucleic acid synthesis. The mutant myeloma therefore dies if the pathway for purine and pyrimidine synthesis is blocked using the folic acid analog aminopterin since neither pathway for DNA synthesis is then available to it. The hybridoma, however, inherits the salvage pathway genes from the normal B cell and is able to utilize hypoxanthine and thymidine, thus surviving in HAT (hypoxanthine-aminopterin-thymidine) medium (Köhler and Milstein, 1975; Köhler and Milstein, 1976 and Delves, 1993).

The production of mAbs of either murine or rat origin is now a routine procedure easily within the capabilities of most investigators. However, it is a time-consuming and expensive process and serious thought should therefore be given to the necessity of embarking upon a program of mAb production. The emergence of recombinant antibody phage display technology, developed during the past decade, has revolutionized the approaches available to generate specific antibodies. In theory, phage display technology (discussed later) allows the generation of high affinity antibodies and antibody fragments and provides a direct link between the expressed phenotype and encapsulated genotype.

1.11 *An Introduction to Recombinant Antibodies*

As part of our immune system, antibodies identify and attack foreign particles that enter our body. Antibodies and antibody fragments (Figure 1.6) have the ability to detect antigens ranging from illicit drugs and hormones to pathogenic bacteria such as *Listeria monocytogenes*. In recent years, the emergence of recombinant antibody phage display technology, has transformed the way in which we generate antibodies for the specific detection of a chosen analyte. Phage display is a molecular diversity technology that allows the presentation of large peptide and protein libraries on the surface of filamentous phage. Having a direct link between the experimental phenotype and its encapsulated genotype, phage display allows the evolution of selected binders into optimised molecules (Maynard and Georgiou, 2000 and Azzazy and Highsmith, 2002). Moreover, modifications are easily introduced in the primary antibody sequence leading to affinity maturations, often resulting in higher affinity for binding to the antigen, generation of fusion proteins and addition of detection and purification tags. The power of phage selection to choose those ligands having the desired biological properties, permits us to mimic the immune system and synthesise "tailor-made" antibodies for use in diagnosis, immunotherapy or for immunoassay development.

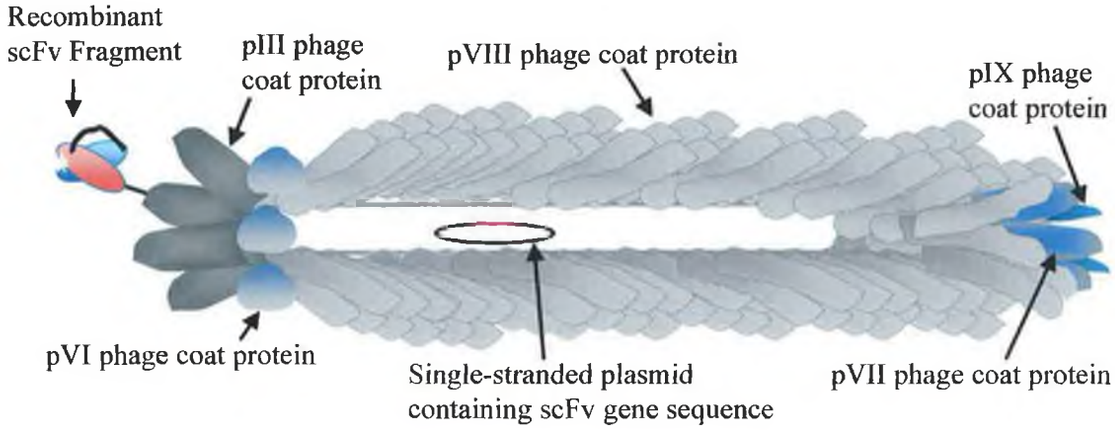


Figure 1.10: *Display of a scFv antibody on a filamentous bacteriophage particle. The location of the capsid proteins and the scFv antibody are clearly indicated by arrows. Filamentous phage particles are covered by approximately 3000 copies of the small major coat protein pVIII. Few copies of the minor coat proteins pIII and pVI are displayed at one extremity of the phage particle, while pVII and pIX proteins are located at the other extremity. The scFv is displayed as a fusion to g3p (pIII) protein at the tip of the phage. The phage particles may incorporate either pIII derived from the helper phage or from the scFv fusion protein and therefore in reality, the scFv is not fused to all pIII protein molecules, allowing the phage to retain its ability to infect bacteria.*

1.12 *Phage display technology*

Phage display was first introduced in 1985 by Smith, who described the use of non-lytic filamentous bacteriophage fd for the display of specific binding peptides on the phage coat. The filamentous bacteriophages (genus *Inovirus*) are a group of viruses containing a circular single-stranded DNA (ssDNA) genome encapsulated in a long flexible protein capsid cylinder. The Ff class of filamentous phages (f1, fd and M13) use the tip of the F conjugative pilus as a receptor and are, therefore, specific for *E. coli* containing the F plasmid. The three Ff class of filamentous phages are 98% homologous with respect to the DNA sequences and consequently, the protein sequences of the gene products are practically the same (Barbas *et al.*, 2001). Filamentous phage does not produce a lytic infection in *E. coli*, but rather induces a state in which the infected bacteria produce and secrete particles without undergoing lysis. The filamentous phage fd can infect strains of *E. coli* containing the F conjugative plasmid by attaching to the tip of the F pilus that is encoded by genes on this plasmid, and translocating the phage genome, a circular single-stranded DNA molecule into the host cytoplasm (Hoogenboom *et al.*, 1998). Filamentous phage (Figure 1.10) are covered by approximately 3000 copies of a small major coat protein (pVIII), with few copies of the minor coat proteins pIII (the product of gene III) and pVI displayed at one extremity of the phage particle, while pVII and pIX are present at the other extremity (reviewed by Nilsson et al., 2000). The principle of phage display involves displaying certain ligands, usually an antibody, fused with the carboxy terminal to a phage coat protein (pIII or pVIII). Initially, complete phage vectors or bacteriophage, which carried all the genetic information required for the phage life cycle were used as the display vector (Barbas *et al.*, 2001). Now small plasmid vector or phagemids, which contain the appropriate packaging signal and cloning sites, have become a more popular type of vector for display (Daly *et al.*, 2001). Phagemids are hybrids of phage and plasmid vectors designed to contain the origins of replication for both the M13 phage and *E. coli* in addition to gene III. They also contain appropriate multiple cloning sites, and an antibiotic-resistance gene, but lack all other structural and non-structural gene products required for generating a complete phage. (reviewed by Azzazy and Highsmith, 2002). Phagemids have high transformation efficiencies, making them ideal for generating large repertoires. Many phagemids also utilize the *lacZ* promoter as a means of controlling expression. For display of scFv-pIII fusion product, the catabolic repressor (glucose) is removed and expression induced using isopropyl-β-D-galactopyranoside (IPTG). The phagemid encoding the scFv-pIII fusion product will be preferentially packaged into phage particles using a helper phage such as M13 KO7 or VCS-M13, which supplies all the structural proteins.

1.13 *The Krebber system for the isolation of specific scFv*

Recombinant antibodies can be generated by PCR from cDNA isolated from hybridoma cells or from the spleens of immunised animals and assembling scFv or Fab fragments by genetic engineering or by a combinatorial approach. This is usually achieved by constructing an antibody gene library by PCR-amplification of the rearranged antibody genes from B-lymphocytes. The combinatorial libraries can be generated from the B cells of immunised animals (immune libraries) or from non-immunised animals (naïve libraries) (Burmester and Plückthun, 2001 and Dübel and Kontermann, 2001). Due to the random light and heavy chain combinations, combinatorial antibody libraries may contain antibody fragments not found in nature, and therefore libraries can be used to isolate antibodies directed against antigens not suited for immunisation, e.g. recognising highly toxic substances or self-antigens.

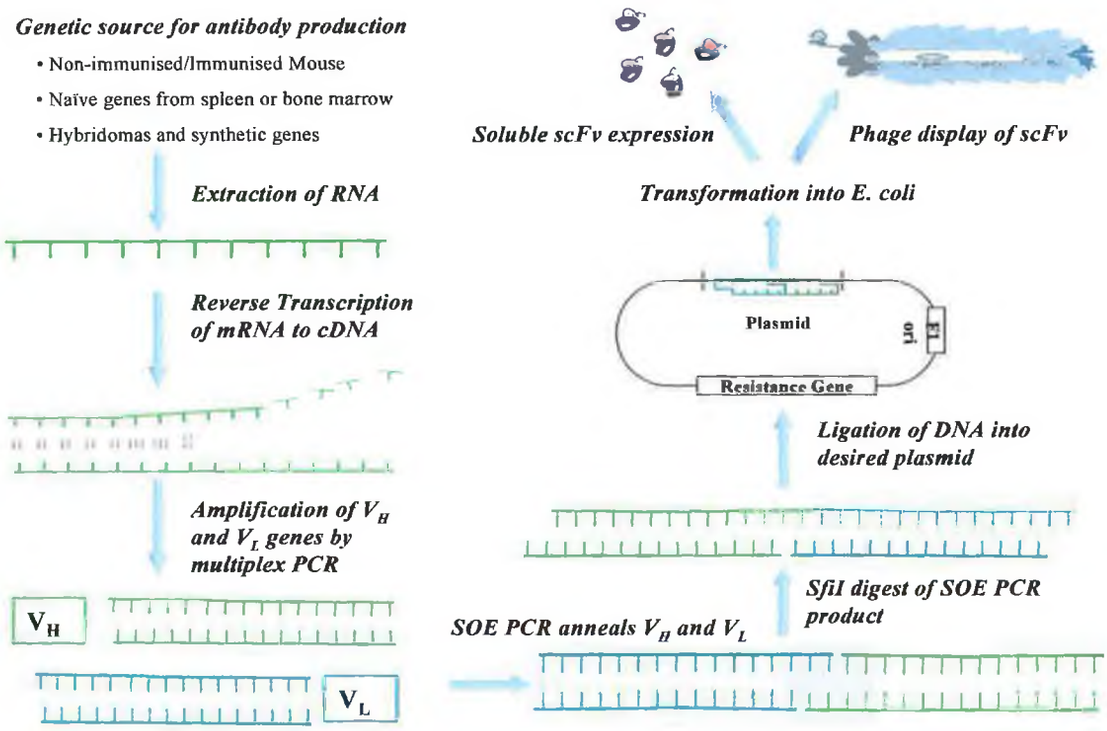


Figure 1.11: *Illustration of the production of antibody fragments using the Krebber system. RNA is extracted from spleen cells or hybridomas and cDNA synthesised with random hexamer primers by reverse transcription PCR. V_L and V_H genes are amplified from the cDNA template using primer mixes designed for the amplification of mouse antibody variable genes. V_L and V_H PCR products are assembled into the scFv format (**S**plice **O**verlap **E**xtension PCR) and purified product digested with the SfiI restriction enzyme. Restricted scFv PCR product is ligated into the pAK100 vector and transformed into E. coli. With the addition of helper phage, the scFv insert is displayed on the tip of the phage whereas the genetic information encoding for the particular scFv fragment is packaged as single stranded DNA in the phage interior. By subcloning the scFv gene into pAK300-600 plasmids the scFv can be expressed in soluble form.*

A prerequisite for the use of recombinant antibody technologies starting from hybridomas or immune repertoires as the source of DNA, is the reliable cloning of functional light and heavy chain genes. For this purpose, Krebber developed a standard phage display system optimized for robustness, vector stability, tight control of scFv-Δgene III expression, primer usage for PCR amplification of variable region genes, scFv assembly strategy and subsequent directional cloning using a single rare cutting restriction enzyme. The Krebber (Krebber *et al.*, 1997a) system allows the rapid harvesting of antigen binding single chain Fv fragment (scFvs) antibodies derived from spleen cell repertoires of mice immunised with antigens such as internalin protein or whole *Listeria monocytogenes* cells. ScFv antibodies (Figure 1.6 panels D and E) generally retain the same affinity as the parent Fv module and have been observed to express to higher levels than Fabs in bacteria (Hudson, 1998). To maximize soluble expression of functional scFvs subsequent to cloning, Krebber and colleagues also constructed a compatible vector series (pAK100-600) (Figure 1.12) to simplify modification, detection, multimerisation and rapid purification of recombinant antibody fragments. An outline of the Krebber system is shown in Figure 1.11. Antibody mRNA (containing the full repertoire of antigen specificities) is isolated from hybridoma or spleen cells and undergoes reverse transcription using random hexamer primers converting mRNA to cDNA. This cDNA is then used as a template for the PCR amplification of heavy and light chain genes encoding a restriction site for cutting with SfiI. The amplified heavy and light genes are then joined together by splice overlap extension (SOE) PCR and this product is purified and digested along with pAK100 vector with the SfiI restriction enzyme. The scFv genes are then ligated into the digested vector and transformed into *E. coli*. Packaging of the scFv into phage (known as Phage rescue) involves the packaging of phagemid DNA as recombinant M13 phage with the aid of helper phage that contains a slightly defective origin of replication (such a M13KO7 or VCSM13) and supplies, *in trans*, all the structural proteins required for generating a complete phage. (reviewed by Azzazy and Highsmith, 2002).

Affinity selection of scFv by panning (Figure 1.13) and subsequent reinfection into *E. coli* greatly enhances the number of specific strong binding scFvs. During panning the phage library is incubated in an immunotube coated with specific antigen, unbound phage particles are washed away and the bound phages are then removed under strict elution conditions. Successive rounds of panning are carried out to enrich antigen binders and ensure antibody fragments with the strongest binding affinities are isolated.

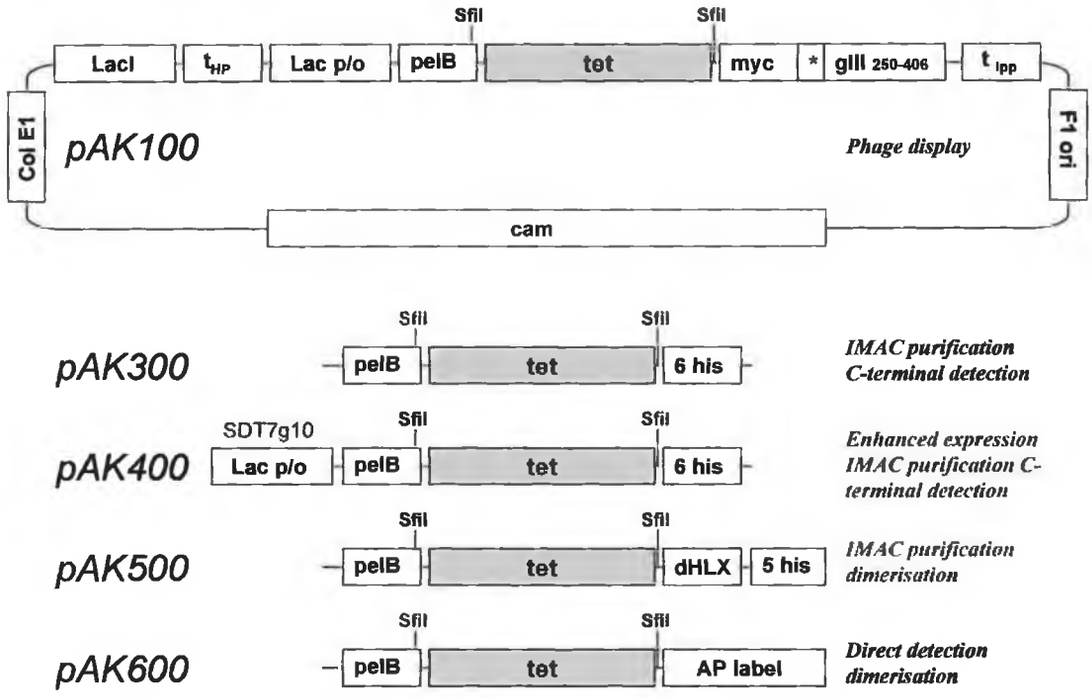


Figure 1.12: *Schematic diagram of the pAK vector series used by Krebber et al. (1997a). The phage display vector pAK100 and related vectors can be used to build modifications into antibody fragments cloned by the strategy outlined in Figure 1.3. Vectors pAK300-600 contain the same elements as in vector pAK100 except for the modifications shown. The pAK100 vector allows fusion of the scFv gene to the g3p protein for antibody production by phage display. The vectors pAK300 and 400 allow the soluble expression of scFv antibody. pAK400 contains a modification (SDT7g10) in the ribosome binding sequence (known as a Shine-Dalgarno sequence named after the workers who first recognised its significance), which results in a significant enhancement of protein expression. PAK500 allows dimerisation of antibody and pAK600 allows both dimerisation of antibody and direct detection through fusion of the scFv with alkaline phosphatase (AP).*

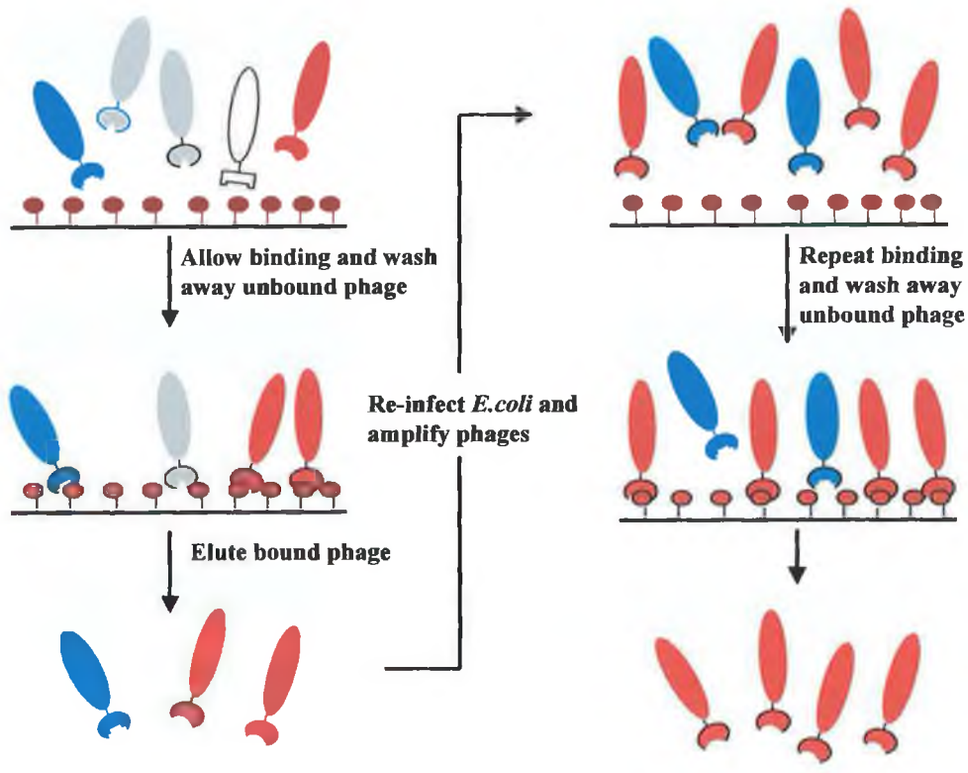


Figure 1.13: *Illustration of the affinity-selection bio-panning process. Phage displaying scFv antibodies are incubated in immunotubes coated with the antigen of interest. Non-binding phage scFv are washed away and antigen-specific phage eluted and subsequently re-infected into E. coli for amplification. The process (bio-panning) is repeated several times, with an enrichment of antigen-specific phage scFv antibodies observed after each round of bio-panning.*

1.14 *The Vaughan naïve human antibody library*

A naïve human antibody library, donated by Cambridge Antibody Technology (CAT) was used to select for scFv antibodies against *Listeria monocytogenes* cells and invasion proteins Internalin B and p60. The antibody library was developed by Vaughan *et al.*(1996), from the functional V gene segments of 43 non-immunised human donors resulting in a repertoire of 1.4 x 10^{10} single-chain Fv (scFv) fragments displayed on the surface of phage. Fragments were cloned in a phagemid vector (pCantab 6), which allows both phage displayed and soluble scFv antibodies to be produced without the need for subcloning. The library was designed similar to the library described by Marks *et al.* (1991), except that a more efficient two fragment PCR assembly process was used (Figure 1.6). Here, the library was made 1000-fold larger by performing several hundred electroporations into *E. coli* TG1 cells. All scFv genes cloned in this vector incorporate a C-terminal stretch of six histidines (HIS) for immobilised metal affinity chromatography

(IMAC) and a sequence derived from c-myc enabling detection with the monoclonal antibody 9E10. An amber codon (A UAG stop codon named "amber", a literal translation of the German word "Bernstein", the name of one of the investigators who discovered it; Dale, 1998) is present between the c-myc tag and gene III sequence, enabling switching between soluble scFv expression induced by the addition of IPTG and phage display using M13KO7 helper phage.

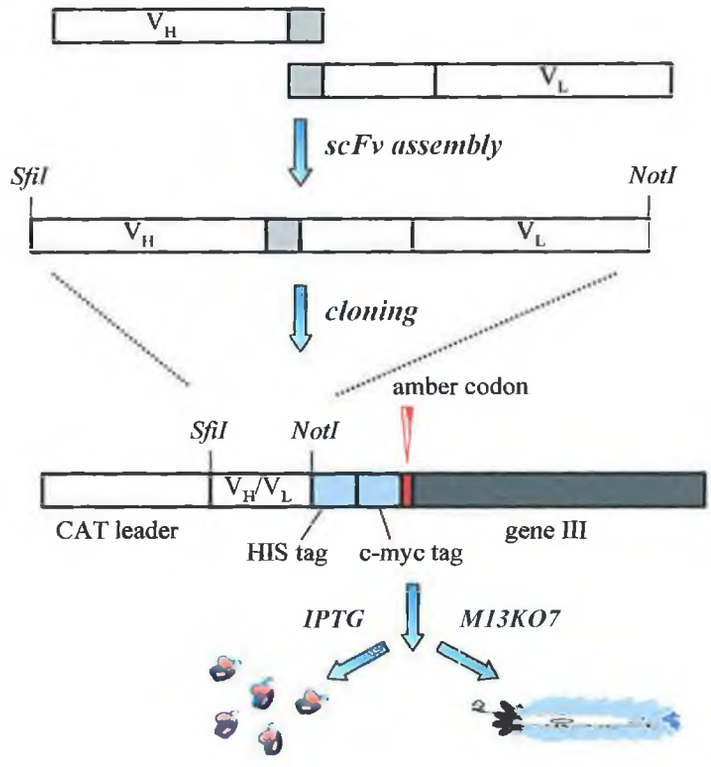


Figure 1.14: *Simplified schematic outline of the cloning scheme used to construct the naïve human scFv repertoire described by Vaughan et al. (1996). The variable heavy and light chain genes were amplified by PCR and assembled by overlap of the glycine linker resulting scFv antibodies of the orientation V_H-linker-V_L which is different from that described by Krebber et al. (1997a). The positions of the Cambridge Antibody Technology (CAT) leader sequence and the His and c-myc tags used for purification and/or detection are clearly shown. The position of the amber codon allows switching from phage displayed scFv antibodies to soluble scFv antibodies without the need for subcloning.*

1.15 *Recent Innovation in phage display technology*

The last decade has seen remarkable developments in the techniques used to generate and isolate antibody fragments of desired specificity. Recombinant DNA techniques now enable us to alter the affinity, valency or size of antibodies and allow us to improve the antibody-antigen interaction introducing novel biological functions, thus extending the antibodies abilities beyond nature.

1.15.1 *Selectively infective phage*

Selectively infective phage (SIP) consist of a filamentous phage particle made non-infective by replacing its gene-3-protein (g3p) N-terminal domains with a ligand-binding protein (e.g. antibody) and an "adapter molecule" in which the ligand is linked to the N-terminal domains of the g3p that are missing from the phage particle (Spada *et al.,* 1997). In SIP, in contrast to phage display, the desired specific protein-ligand interaction is itself directly responsible for restoring infectivity in an otherwise non-infective display phage (Figure 1.15). SIP has the advantage of directly coupling the productive protein-ligand interaction with phage infectivity and amplification, without the need of an elution step from a solid matrix (Jung, *et al.,* 1999). SIP exploits the modular structure of the gene-3-protein, which consists of three domains of 68 (N1), 131 (N2) and 150 (CT), connected by glycine-rich linkers of 18 (G1) and 39 (G2) amino acids, respectively (Spada *et al.,* 1997). The N-terminal N1 domain of g3p is involved in penetration of the bacterial membrane and is absolutely essential for infection into *E. coli* while the N2 domain forms a complex with N1 on the phage and specifically interacts with the *E. coli* F-pilus (Jakes *et al.,* 1988; Stengele *et al.,* 1990 and Krebber *et al.,* 1997b). In the phage particles, the C-terminal domain (CT) is part of the phage coat structure and thereby anchors the N-terminal domains to the phage (Davis *et al.,* 1985). A library displayed in the SIP phage can be constructed by fusing it N-terminally either to CT or to N2-CT. In general, the N2-CT fusions give higher infectivities (Krebber *et al.,* 1997b). Both types can be combined with either an N1-ligand or an N1-N2-ligand, thereby having zero, one or two copies of N2 in each reassembled g3p (Jung, *et al.,* 1999). Phage displaying a protein (e.g. antibody) and the N1 adaptors or N1-N2 adaptors with coupled ligand, can be separately purified and combined in defined amounts *in vitro* to yield infective phages, provided the ligand interacts with the protein. Alternatively, specific protein antigen can be fused to the adapter encoded on an expression plasmid and both phage and adapter produced in the same cell (*in vivo* SIP). *In vivo* SIP, the specific protein does not have to be first expressed and purified as in phage display. Instead, its DNA is all that is needed, and only very small quantities have to be functionally expressed in the selection system (Jung, *et al.,* 1999). Some of the tasks of conventional phage display are to minimize adsorption of non-specific phage to the ligand and to enrich phage displaying high-affinity molecules over abundant low affinity binders. In SIP, phage infectivity is restored only when the non-infective phage displaying protein or peptide, specific for the ligand, comes in contact with adapter molecule. SIP technology is therefore a low background procedure that eliminates the need for inefficient physical separation of specific from non-specific binders.

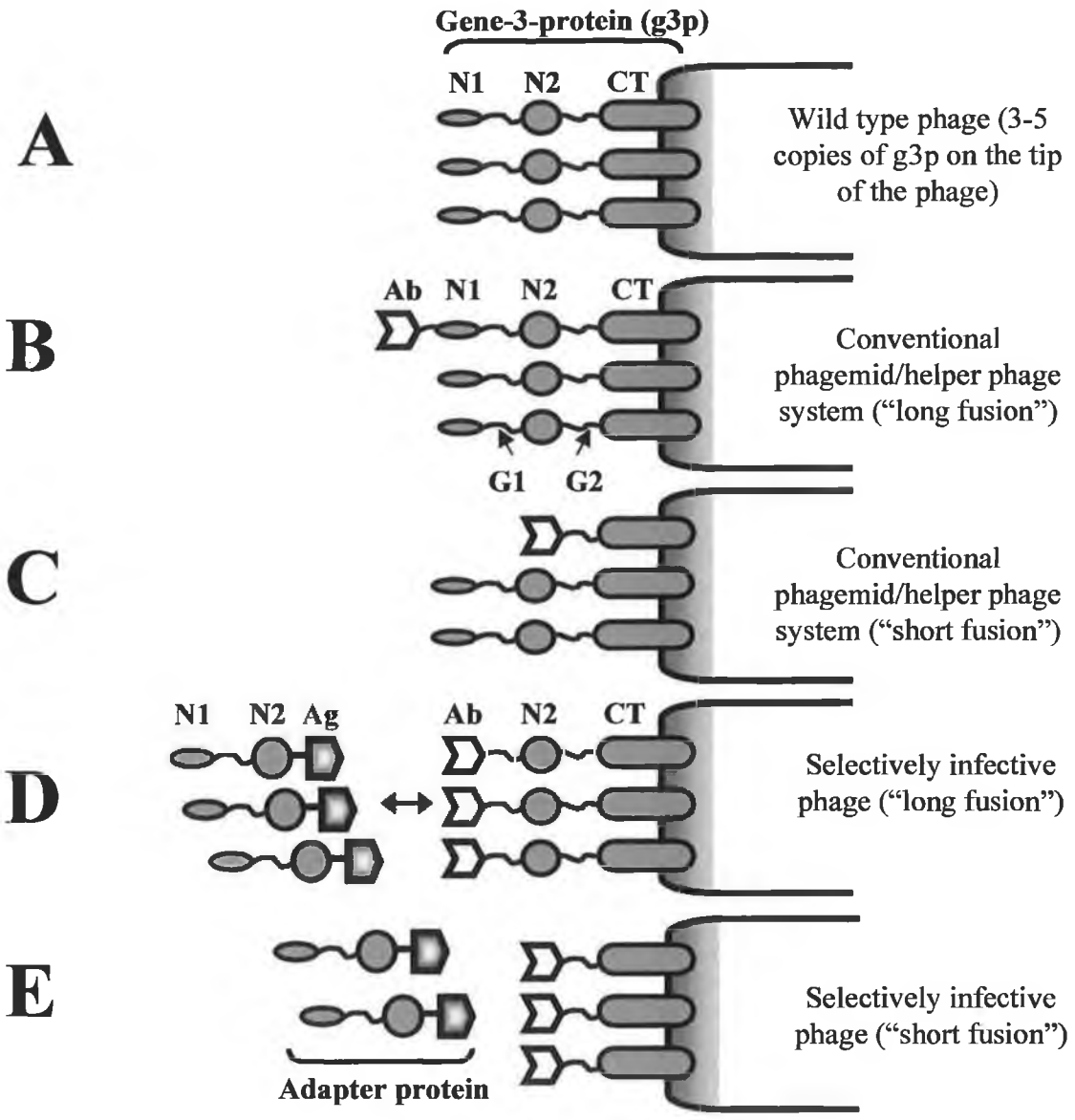


Figure 1.15: *Diagram showing a comparison of the conventional phage display and SIP display systems. (A) 3-5 copies of the minor M13 phage coat protein gene-3-proteins (g3p) are present at the tip of wild type phage. The gene-3-protein consists of three domains, N1, N2 and CT, which are connected by glycine rich linkers (G1 and G2). (B,C) In conventional phagemid/helper phage systems, infectivity is maintained by the presence of wild type (wt) g3p, encoded on the helper phage. Displayed proteins (e.g. antibodies) can be (A) fused to all three g3p domains (long fusion) or (C) fused only to the C-terminal domain of the g3p (short fusion). (D,E) The N-terminal N1 domain of g3p is essential for infection into E. coli and in the selectively infective phage, all copies of g3p lack the N1 domain that is replaced by a specific protein, usually an antibody (Ab) fragment. Proteins can be (D) fused to the N2 domain (long fusion) or (E) fused directly to the CT domain (short fusion). Such phages are totally non-infectious, but infectivity can be restored when the missing domain(s) are supplied in an adapter-linked protein, usually an antigen (Ag). The adapter protein connects the missing domains of g3p to the phage by a non-covalent interaction such as the binding of an antibody (Ab) to a specific antigen (Ag). The diagram presented here was adapted from Spada et al. (1997).*

1.15.2 *Affinity maturation of antibodies*

Recent advances in antibody engineering and phage display technology allow cloned antibody variable domain genes to be further engineered producing antibody variants with lower immunogenicity (Thompson *et al.*, 1998), higher affinity (Schier *et al.*, 1996a,b) altered antigenic specificity (Ohlin *et al.*, 1996), enhanced stability (Wörn and Plückthun, 1999) or increased solubility and expression (Pini *et al.*, 1997). Recombinant antibodies can be easily expressed with a number of fusion proteins and affinity tags for the rapid and efficient detection and purification of the desired antibody. The affinity of recombinant antibody fragments for their antigen can be affinity maturated by processes such chain shuffling, error prone PCR and mutagenesis, resulting in the formation of new antibody fragments with enhanced sensitivity and specificity.

1.15.2.1 *Chain Shuffling*

Chain shuffling relies on the natural mutation of V-genes that occurs *in vivo* in response to antigens that the host has been exposed to for the generation of sequence diversity. For chain shuffling of scFv antibodies, the gene for one of the variable domains (e.g. V_H) is cloned into a repertoire for the second chain (V_L) resulting in a library of scFv antibodies containing the V_H domain gene of the parent antibody and random V_L genes. The new library is then panned against the target antigen to identify clones with improved binding characteristics. The new V_L gene of the scFv antibody with the desired binding characteristics is then cloned into a repertoire of V_H chains and the process of selection repeated (Adams and Schier, 1999 and Nielsen and Marks, 2001). However, chain shuffling is dependent on the availability of large highly diverse variable domain libraries in order to supply sufficient sequence variation to improve binding, which is usually not obtained from hybridoma-derived antibody libraries.

1.15.2.2 *Error prone PCR*

Error prone PCR involves the random introduction of nucleotide substitutions (errors) throughout a gene by reducing the fidelity of Taq polymerase. The number of errors incorporated is usually kept low (1-2 substitutions) as introducing large numbers of mutations can be counterproductive in that highly defective antibodies (frameshifts, etc.) may bind non-specifically and out-compete improved variants (Johnson and Hawkins, 1996). Controlling the level of substituted errors can be difficult to optimise and often

be further enhanced by sequential mutagenesis of the remaining CDR regions also known as "CDR walking" (Takkinen *et al.*, 2001).

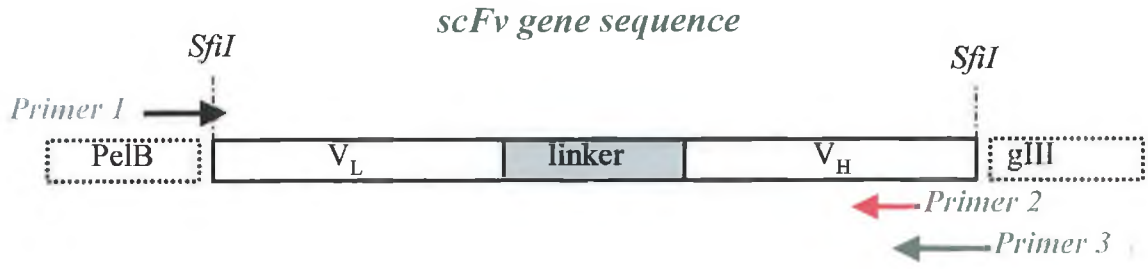


Figure 1.16: *Schematic illustration of an scFv sequence showing the primer positions for randomisation in the variable heavy chain CDR 3 region. Two PCR reactions are needed. The first reaction uses a primer homologous with the beginning of the variable light domain (primer 1) which incorporates the restriction enzyme site (e.g. SfiI) for subsequent cloning of the gene and the random primer (red arrow) introduces errors in the CDR 3 region and amplifies the rest of the V_H gene (minus the framework 4 region). A second PCR reaction is carried out using the new randomised PCR product as a template in which primer 1 amplifies the VL gene and primer 3, encoding the SfiI site and Framework 4 region, attaches to the end of the primer 2 sequence and amplifies up the whole scFv gene including the newly introduced errors.*

1.15.2.5 *Selection and screening of favourable affinity maturated antibodies*

As stated in section 1.15.2.4 above, mutagenesis using "spiked" PCR primers homologous with the variable domain CDR 3 regions, in conjunction with selection by phage display, possibly offers the greatest potential for the affinity maturation of recombinant scFv antibody fragments. In order to minimise the number of non-viable structures and to identify the amino acid residues which tend to have solvent accessible side chains from those with buried side chains (see section 5.3.4), molecular modelling of the most similiar variable domains (to that of the chosen antibody sequence(s)) in the PDB database should be carried out. The Protein Data Bank (PDB), established at Brookhaven National Laboratories (BNL) in 1971, is the single worldwide archive of structural data of biological macromolecules, which is now managed by the Research Collaboratory for Structural Bioinformatics (RCSB) (Berman *et al.*, 2000). The Protein Data Bank contains thousands (21,390 structures at the time of printing) of protein structures deposited from research groups throughout the world. Identifying important residues in the CDR regions will increase the chances of beneficial mutations and minimise selecting for the wild type antibody sequence. Designing random primers to different regions of the CDR sequences (varying the number and placement of targeted amino acids) can further enhance the potential for selecting favourable clones.

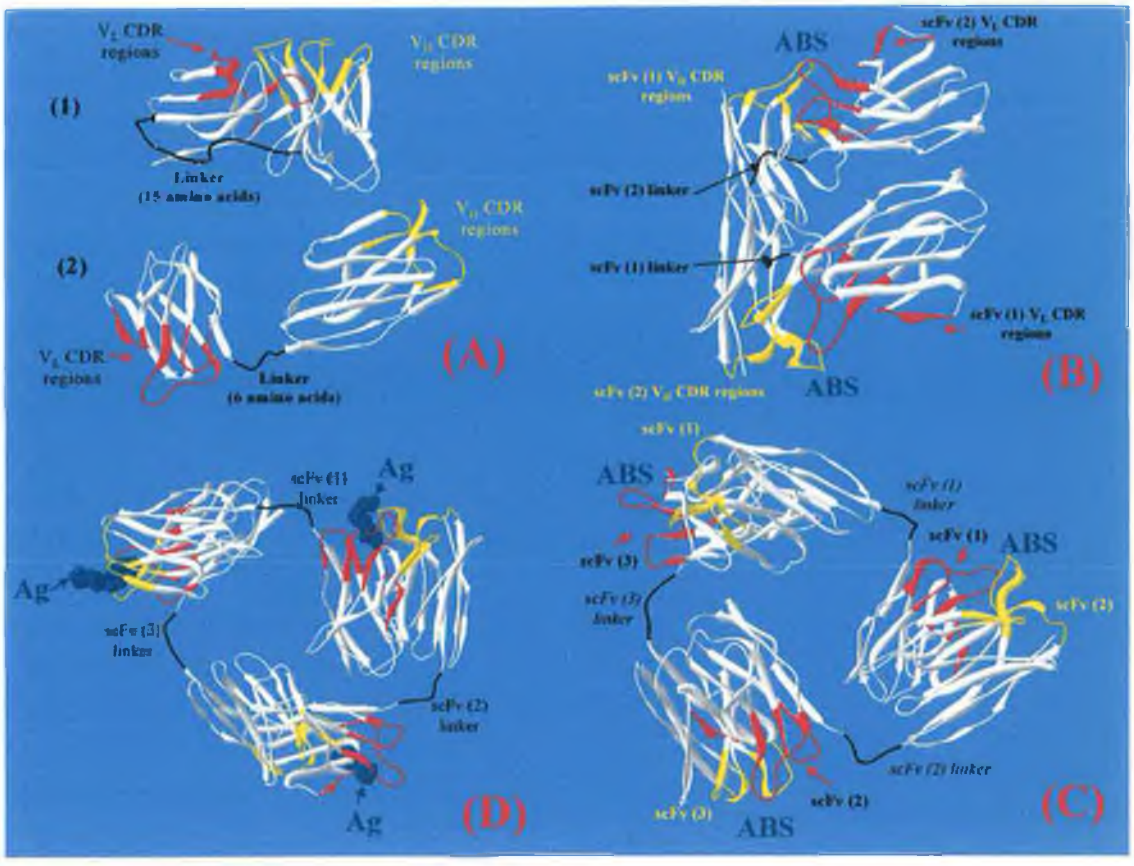
Independent of the affinity maturation technique used, selection of phage displaying scFv antibodies can be achieved on soluble or immobilised antigen. Antigen can be coated on immunotubes and randomised phage scFv antibodies allowed bind. Specific phage scFv antibodies can then be eluted from the tube and re-infected into *E. coli* for further amplification and selection. Alternatively specific phage scFv with improved affinities can be selected in solution using biotinylated antigen and recovered with streptavidin-coated magnetic beads. This method makes it easier to control the amount of antigen used (as the coating of certain antigen on plastic can be unpredictable) and, by lowering the concentration of antigen used, can allow improvements in the selection of high affinity antibodies. The choice of selection procedure will depend on the type of antigen and both methods should be evaluated.

Mutated phage-scFv antibodies can be screened by ELISA using 96 well plates coated with the desired antigen. Thorough screening of selected clones is of utmost importance in order to avoid re-selecting the wild type sequence and maximise the possibility of selecting the most favourable mutation. After selection, the most favourable clones should be sequenced to examine the diversity of the mutations and avoid wild type sequences. Affinity studies of selected clones can be performed by competition ELISA and by studying the dissociation rates using a BIAcore 3000^{TM} instrument. The clone(s) displaying the most improved affinities can then be used for the template for further mutagenesis (Nielson and Marks, 2001).

1.15.3 *Antibody multimer formation*

The Fv antibody fragment, (Figure 1.17) consisting of only the V_H and V_L domains, is the smallest immunoglobulin fragment containing the whole antigen-binding site. However, Fv antibodies appear to have a lower interaction energy between their two chains than Fab antibody fragments, which are also held together by the C_H1 and C_L constant domains (reviewed by Little *et al.*, 2000). To stabilise the association of the VH and VL domains, the domains have been linked with peptides (Huston *et al.*, 1988), disulphide bridges (Glockshuber *et al.*, 1990) and "knobs into holes mutations" (Zhu *et al.*, 1997). Peptide linkers of about 3.5 nm are required to span the distance between the C terminus of one domain and N terminus of the other (Hudson, 1998 and Huston, *et al.*, 1988). The most common scFv linker designs contain a combination of glycine and serine residues (usually 15 residues) providing flexibility and protease resistance (reviewed by Todorovska *et al.*, 2001). By shortening the peptide linker, monovalent scFv antibody fragments can be

forced to form antibody multimers (diabodies, triabodies and larger multimers). The most important advantage of multivalent scFvs over monovalent scFv and Fab fragments is the gain in functional binding affinity (avidity) to target antigens (Todorovska *et al.*, 2001). Intact antibodies are polyvalent molecules with generally enhanced functional affinity (avidity) relative to monovalent Fab fragments, particularly in the case of pentameric IgM molecules (Hudson, 1998). There is, therefore, a similar increase in functional affinity when Fab or scFv fragments are engineered into dimers, trimers or larger aggregates (Plückthun and Pack, 1997). Holliger *et al.* (1993a,b) have demonstrated that V_H and V_L domains within a scFv with a short polypeptide linker (0-10 amino acid residues) cannot pair to form a monomeric antibody fragment, but rather homodimerise to form a small bivalent antibody fragment termed "diabody". According to Todorovska *et al.*(2001), linkers greater than 12 residues in length provide enough flexibility for the V_L and V_H domain to assemble in natural Fv orientation (Figure 1.7). An scFv molecule with a linker of 3-12 residues cannot fold into a functional Fv domain and instead associates with a second scFv molecule to form a bivalent dimer (diabody, ~60 kDa). Reducing the linker length below 3 residues can force the scFv to associate into trimers (triabodies, ~90 kDa) or tetramers (tetrabodies, ~120 kDa) depending on linker length, composition and V-domain orientation. Also, the association of two different scFv molecules, each comprising a VH and VL domain derived from different parent immunoglobulin, can form a fully functional bispecific diabody (Holliger *et al.*, 1993a,b). Bispecific antibodies are hybrid molecules that are comprised of two specificities. Bispecific diabodies can be synthesised using a bicistronic expression vector that enables the two scFvs to associate *in situ*, in the *E. coli* periplasm (Todorovaska *et al.*, 2001). Although most applications of bispecific antibodies are aimed at therapeutic applications, they also show great potential as diagnostic and imaging reagents. Bispecific antibodies with a first binding specificity to a target antigen and a second to an enzyme (e.g. alkaline phosphatase) have great potential in improving and refining immunoassays as well immunohistochemical applications that employ antibodies that are chemically linked to radio-isotopes, enzymes or fluorochromes (Nolan and O'Kennedy, 1990; Azzazy and Highsmith, 2002 and Brennan *et al.*, 2003).



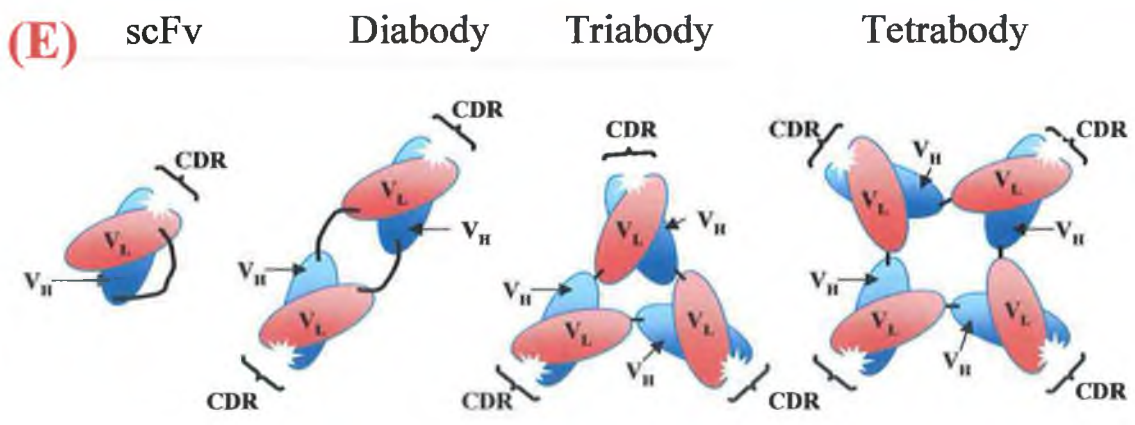


Figure 1.17: *Representation of various recombinant antibody constructs. As discussed in section 1.15.3, peptide linkers greater than 12 residues in length provide enough flexibility for the V_L and V_H domain to assemble in natural Fv orientation. Panel A shows the structures of two scFv antibodies with different linker lengths viewed as ribbon structures using the Swiss-PDB (Protein Data Bank) viewer program. The CDR regions of both antibodies are coloured red and yellow representing the light and heavy chain CDR regions, respectively. The linker region is coloured in black for both antibodies and shows the importance of linker length for the proper association of the heavy and light chain domains. The top antibody (labelled 1), a homology model of the anti-InlB scFv antibody described in section 5.5.4, has a 15 amino acid linker which permits proper folding of the scFv into a functional antibody. The bottom antibody (labelled 2), a*

scFv antibody obtained from the Protein Data Bank (accession code 1lmke), has only a 6 amino acid linker which does not allow the heavy and light chain domains to associate properly. Panel B is a representation of the structure of a diabody consisting of two identical scFv antibody molecules (scFv 1 and scFv 2). The scFv structure presented in the lower part of panel A was manually manipulated using the Swiss-PDB viewer program to form the possible diabody structure shown. The diagram shows how two scFv antibodies with short peptide linkers can associate to form a functional bivalent antibody. Using the Swiss-PDB viewer program the scFv antibody was further manipulated to form a triabody consisting of three scFv antibodies (Panel C). The associated scFv fragments form a trivalent functional antibody with the antigen binding sites (ABS) facing in opposite directions. Panel D shows a copy of the triabody presented in Panel C, binding a theoretical peptide antigen (Ag). The diagram illustrates how the increased valency of the scFv fragments can allow the binding of three antigens (shown here as a short peptide sequence) simultaneously, resulting in higher functional affinity (avidity). Note, the structures shown in Panels B, C and D were manually manipulated using the Swiss-PBS viewer for the purpose of illustration and, therefore, the exact orientation of the possible multimeric structures formed by the presented scFv (1lmke) may vary slightly. Panel E shows a diagram of the simplified structures of the various antibody fragments indicated.

1.15.4 *Ribosomal and mRNA Display*

As discussed in section 1.12, phage display offers a sophisticated method for the rapid identification, characterisation, and potential improvement of interaction partners. However, phage display is limited by the involvement of living cells in the process of library generation and screening. *In vitro* technologies, such as ribosomal and mRNA display technologies, are not limited by cellular transformation efficiencies and, thus, very large libraries of up to 10^{14} members can be built (reviewed by Amstutz *et al.*, 2001). Both methods also facilitate directed evolution of polypeptides as they have the potential for the generation of large libraries, contain fewer of the biases caused by cellular expression and allow the more facile application of round-by-round mutagenesis techniques (reviewed by Dower and Mattheakis, 2002). Ribosomal display, first developed by Mattheakis *et al.* (1994), relies on non-covalent ternary complexes of mRNA, ribosome and nascent polypeptide, ensuring the coupling of genotype and phenotype. For the display of proteins complexed with ribosomes, a DNA library is constructed with the gene of interest (usually an antibody fragment) fused to a C-terminal spacer region, allowing the peptide region to fold and be displayed clear of the ribosome (Figure 1.18). In ribosomal display this fusion construct lacks a stop codon at the mRNA level, thus preventing release of the mRNA and the polypeptide from the ribosome. Alternatively, the DNA construct can be fused to a sequence encoding the ricin A-subunit (RTA) via a spacer. Once translated, the nascent RTA will depurinate a specific site in the 28S rRNA of the large subunit of the nearby ribosome, blocking further elongation and producing a stable complex that is used for

selection on the immobilised target (Dower and Mattheakis, 2002). The related technology of mRNA display, predominately developed by Robert and Szostak (1997), relies on the covalent coupling of mRNA to the nascent polypeptide. The mRNA is first covalently linked to a short DNA linker carrying a puromycin moiety. As translation proceeds, the ribosome stalls at the RNA-DNA junction and the puromycin moiety enters the peptidyltransferase site of the ribosome. The ribosome catalyses the formation of an amide bond between puromycin and the carboxy-terminal residue of the nascent chain, forming a covalent bridge between RNA and protein (reviewed by Amstutz *et al.*, 2001 and Dower and Mattheakis, 2002).

In vitro display technologies have great potential for the maturation of high affinity protein binders. Hanes *et al.* (2000), described a process, performed entirely *in vitro*, for the affinity maturation of antibodies, resulting in the recovery of anti-insulin antibodies with K_d values as low as $\sim 10^{-10}$ M. Xu *et al.* (2002), constructed a library of $>10^{12}$ unique, covalently coupled mRNA-protein molecules by randomising three exposed loops of an immunoglobulin-like protein, the tenth fibronectin type III domain (^{10}Fn3). According to the authors, the large library size made possible by *in vitro* mRNA display technology allowed the randomisation of all three CDR-like loops of ^{10}Fn3 and the sampling of 104-fold more sequences than was possible in the phage-display selection reported by Koide *et al.* (1998). Using mRNA display, the authors selected ^{10}Fn3-based antibody mimics with higher affinity, increased specificity and with larger population variants with comparable properties. A common requirement for most biotechnological and medical applications of proteins is that they possess an intrinsic high stability against denaturation (Amstutz *et al.*, 2001). *In vitro* technologies have also shown great promise for the stability engineering of desired proteins. Matsuura and Plückthun (2003) used ribosome display to study the folding properties of proteins by selecting proteins on the bases of increased sensitivity to proteolysis and greater exposure of hydrophobic area. By targeting these properties the authors showed that compactly folded and soluble proteins can be enriched over insoluble and random coil proteins. Various evolutionary methods to perform stability engineering of proteins using ribosomal display were also described in the excellent review by Wörn and Plückthun (2001). Selection for scFv stability by simply using a destabilising strategy (adding increasing concentrations of DTT) of reducing the disulphide bonds during *in vitro* protein folding and antigen binding, and selection for scFv stability by functional cytoplasmic expression, were discussed. The work present in this review and in the more recent publication by Matsuura and Plückthun (2003) highlight the importance of *in vitro* technologies as sophisticated methods for the rapid identification, characterisation, and potential improvement of interaction partners.

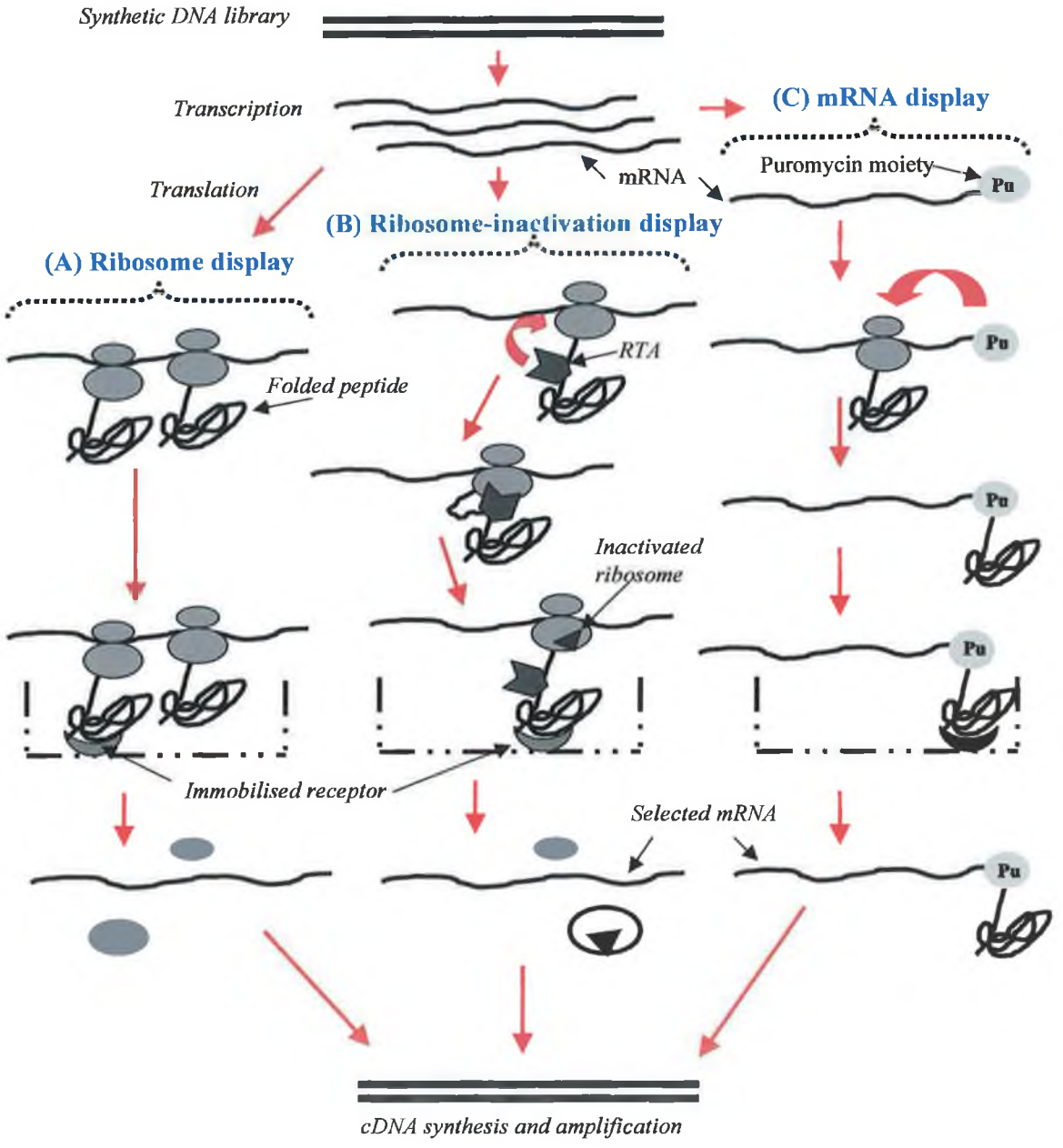


Figure 1.18: *Diagram showing different display formats adapted from Dower and Mattheakis (2002). For both the ribosomal display and the ribosomal-interaction display systems (RIDS), the DNA library is constructed with the gene of interest fused to a C-terminal spacer region, allowing the peptide product to fold and be displayed clear of the ribosome. A strong transcriptional promoter is installed upstream of the gene and in vitro transcription and translation performed. During translation, as one or more ribosomes travel down the RNA, the nascent polypeptide folds and becomes exposed. The release of the RNA and peptide from the ribosome can be prevented by (A) the absence of stop codons or by (B) by fusing a sequence encoding the ricin A-subunit (RTA) to the C-terminus of the gene of interest. Translated nascent RTA acts on the nearby ribosome to depurinate a specific site in the 28S rRNA of the large subunit. This modification blocks further elongation and as with the absence of stop codons method (A) produces a stable complex that is then used for selection on immobilised target. If nascent peptides bind to the target*

with sufficient affinity, they are not washed away. Bound complexes can be dissociated and cDNA reverse transcribed from the recovered mRNA. Recovered sequences can be sequenced or retranscribed, translated and screened in a subsequent selective round of enrichment. (C) In mRNA display, the transcription and translations steps are performed separately as modification of the RNA is required prior to translation. A segment of DNA carrying a 3'-puromycin moiety, is attached to the 3'-end of the RNA and the hybrid translated in vitro. When the transiting ribosome reaches the RNA/DNA junction, the ribosome stalls and the tethered puromycin enters the ribosomal A-site. The ribosome catalyses the covalent incorporation of the puromycin into the peptide chain, forming a covalent link between the RNA and peptide. The displayed peptides are then screened for binding to immobilised target and recovery, identification and amplification performed as described in the methods shown in (A) and (B) above.

1.16 *The use of biosensors for the detection of bacteria*

Even though both traditional antibody-based and nucleic acid-based detection have greatly decreased assay time after sample pre-enrichment compared to traditional culture techniques, they (with the exception of real-time PCR) still lack the ability to detect microorganisms in "real-time" taking up to several hours in some cases to yield confirmation of results. The need for a more rapid, reliable, specific and sensitive method of detecting a target analyte, at low cost, is the focus of a great deal of research, especially for applications outside the laboratory environment. Since its inception in the 1970's, Hazard Analysis at Critical Control Point (HACCP) methodology has evolved as the leading food safety strategy used by the food industry. HACCP identifies where potential contamination, time and temperature problems can occur (the critical control points). However, key technologies needed to successfully implement any HACCP program are "real-time" microbial detection, trace-ability and source identification. Biosensors offer the potential of detecting pathogens in "real time" (the microbial-antibody binding event is observed as it happens) but however still require the time consuming pre-enrichment in order to detect low numbers of pathogens in food and water. Advances in antibody production and the recent emergence of phage-displayed peptide biosensors (Chaplin and Bucke, 1990; Goldman *et al.*, 2000; Benhar *et al.*, 2001, and Goodridge and Griffiths, 2002) offer increased possibilities for the rapid detection of pathogens.

Biosensors use a combination of biological receptor compounds (antibody, enzyme, nucleic acid etc.) and the physical or physicochemical transducer directing, in most cases, "real-time" observation of a specific biological event (e.g. antibody-antigen interaction). They allow the detection of a broad spectrum of analytes in complex sample matrices, and have shown great promise in the areas such as clinical diagnostics, food analysis, bioprocess and environmental monitoring (Invitski *et al.*, 1999 and Fitzpatrick *et al.*,

2000). Biosensors may be divided into four basic groups, depending on the method of signal transduction: optical, mass, electrochemical and thermal sensors (Goepel, 1991; Seyhi, 1994, and Goepel and Heiduschka, 1995). Optical transducers are particularly attractive as they can allow direct "label-free" and "real-time" detection of bacteria. Current studies focusing on optically-based transduction methods aim to achieve a more robust, easy to use, portable, and inexpensive analytical system. The phenomenon of surface plasmon resonance (SPR) has shown good biosensing potential and many commercial SPR systems are now available (e.g. BIAcore^{TM}) (Homola *et al.*, 1999 and Quinn and O'Kennedy, 1999).

1.17 *Surface plasmon resonance biosensors*

Surface plasmon resonance (SPR) is an optical phenomenon which occurs as a result of total internal reflection (TIR) of light at a metal film-liquid interface (Anon., 2001 and Quinn and O'Kennedy, 1999) (Figure 1.19). The detection system of a SPR monitor essentially consists of a monochromatic and p-polarised (electrical vector parallel with plane of incidence) light source, a glass prism, a thin metal film in contact with the base of the prism, and a photodetector (Stenberg *et al.*, 1991). Total internal reflection is observed where light travels through an optically dense medium such as glass and is reflected back through medium at the interface with a less optically dense medium such as buffer, provided the angle of incidence is greater than the critical angle required for the pair of optical media (O'Shannessy *et al.*, 1993). Although light is totally reflected, an electromagnetic field component, termed the evanescent wave, penetrates a short distance of the order of one light wavelength into the less dense medium (buffer) (Dillon *et al.*, 2003). The evanescent wave will interact with free oscillating electrons, or plasmons, in the metal film surface, and therefore, when surface plasmon resonance occurs, energy from the incident light is lost to the metal film, resulting in a decrease in reflected light intensity (Leonard *et al.*, 2003). The resonance phenomenon occurs only at a precisely defined angle of the incident light which is dependent on the refractive index of the buffer solution (Karlsson *et al.*, 1991; O'Shannessy *et al.*, 1993 and Homola *et al.*, 1999). The effective penetration depth of the evanescent field which arises under conditions of total internal reflection is approximately 300 nm (O'Shannessy *et al.*, 1993 and Fratamico *et al.*, 1998), and therefore, only refractive index changes occurring within this distance from the surface will cause a change in the generated SPR signal. The interaction of macromolecules in the buffer solution causes a change in refractive index in close proximity to the metal-film surface which translates into a change in the resonance angle which is in turn detected and quantitated by the instrument. In the biosensor refractive changes are monitored continuously over time and presented in a sensorgram, where the y axis of the sensorgram

is the resonance signal and is expressed in resonance units (RU) (Karlsson *et al.*, 1991). A sensor response of 1000 RU corresponds to a shift of 0.1° in the SPR angle, which in turn correlates to a change of about 1 ng mm^{-2} in the surface protein concentration (Alfthan, 1998). SPR biosensors are potentially useful for environmental and food safety analysis because they are relatively easy to use, do not require labelling of either molecule in the reaction, and can assay crude samples without purification (Brigham-Burke *et al.*, 1992 and Rasooly, 2001).

Generally, an SPR optical sensor comprises an optical system, a transducing medium which interrelates the optical and (bio)chemical domains, and an electronic system supporting the optoelectronic components of the sensor and allowing data processing (Homola *et al.*, 1999). Recently, a number of surface plasmon resonance-sensing configurations and detection approaches have been used in SPR sensors. Surface plasmon resonance sensors using attenuated total reflection optical prism couplers, particularly the Kretschmann geometry of ATR method (Kretschmann, 1971), have been found to be very suitable for sensing and have become the most widely used geometry in SPR sensors. In this configuration a light wave is totally reflected at the interface between a prism coupler and the thin metal layer (of thickness of about 50 nm) and excites an SPW at the outer boundary of the metal by evanescently tunnelling through the thin metal layer (Homola *et al.*, 1999). All the main detection approaches have been demonstrated in SPR prism-based sensors including measurement of the intensity of reflected light wave (Nylander *et al.*, 1982; Leidberg *et al.*, 1983 and Leidberg *et al.*, 1995), measurement of the resonant angle of incidence of the light wave (Leidberg *et al.*, 1993), and measurement of the resonant wavelength of the incident light wave (Zhang and Uttamchandani, 1988). Surface plasmon resonance sensors using grating couplers and optical waveguides (Homola *et al.*, 1999) have also proven suitable for sensing.

The pioneers of commercial SPR-based biosensing were Pharmacia Biosensor AB, now BIAcore AB, who launched the original BIAcore system (see Figure 1.20 for a diagram of the detection method used in the BIAcore biosensor) in 1990 (Leidberg *et al.*, 1995). The company now has a large range of biosensors, which includes several generations of the original BIAcore (series 1000, 2000 and 3000) as well as the BIAlite™ (1994), BIAcore X™ (1996), BIAQuadrant™, BIAcore S51 and BIAcore J (2001) systems, offering varying degrees of automation and parameter specifications. Since the introduction of the original BIAcore in 1990, a number of commercial SPR based biosensors have become available (Table 1.5) utilising various sensing configurations and detection approaches (reviewed by Leonard, *et al.*, 2003). Although a number of SPR instruments are widely available, BIAcore instruments are the most commonly used with approximately 90% of the 1998

and 1999 commercial biosensor publications citing the use of a BIAcore instrument (Rich and Myszka, 2000).

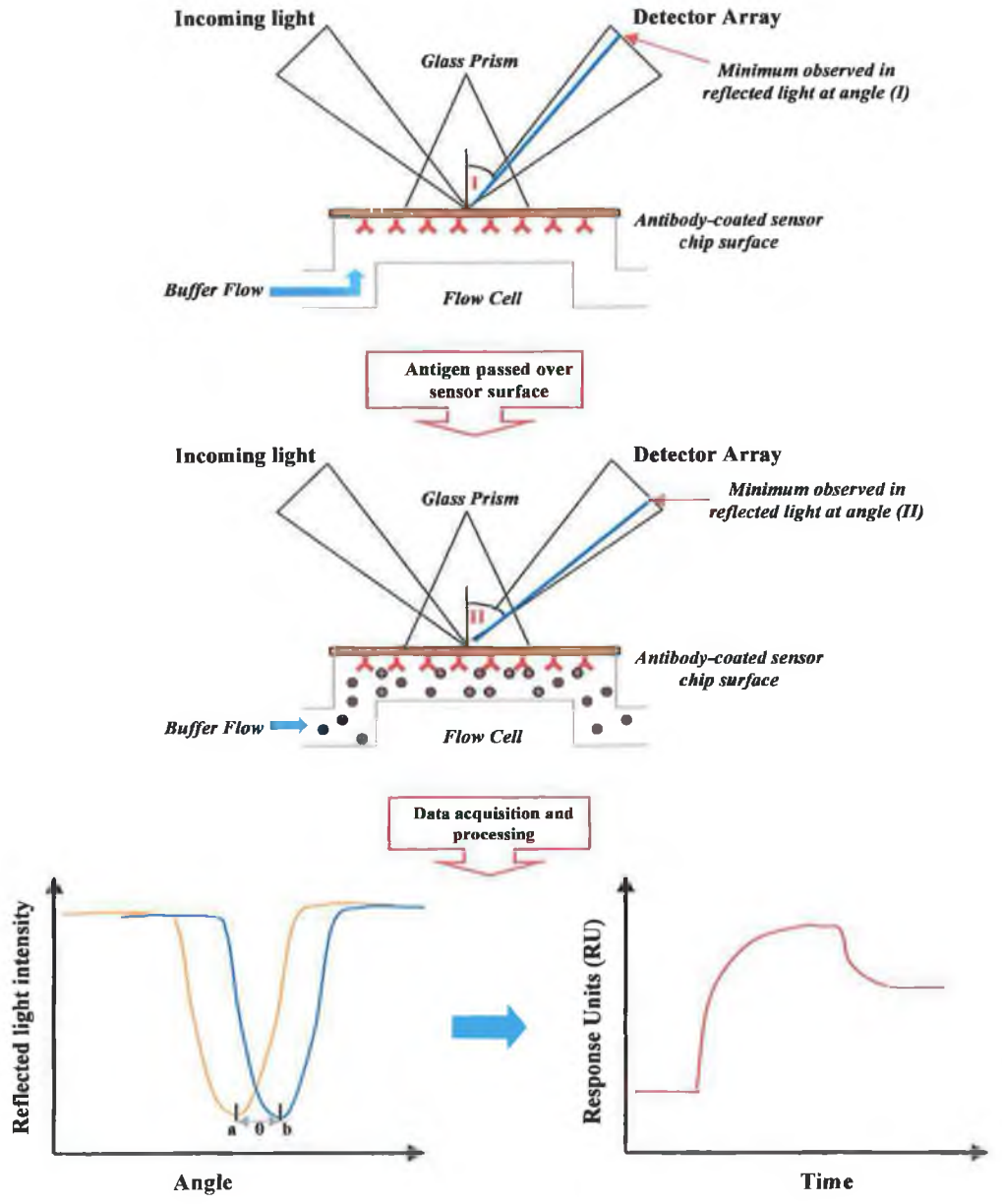


Figure 1.20: *Basis of the detection principle in the BIAcore biosensor instrument. Monochromatic and p-polarised light is focused onto a gold chip surface immobilised with an antibody (Y), by means of a prism under conditions of total internal reflection. Buffer is passed over the surface and the dip in reflected light intensity (blue line) recorded at an angle (I). As antigen is injected over the antibody immobilised surface, a change in refractive index near the sensor chip surface, results in a shift in the resonant angle of reflected light (II) which is measured and recorded. The shift in reflected light is measured constantly and the results presented as a sensorgram of the change in measured response over time. As changes in the resonant angle are a direct result of changes in the mass or concentration on the surface of the chip, the mass of the analyte specifically binding to the chip surface may be determined.*

Table 1.5: *List of six companies currently manufacturing a variety of SPR biosensor hardware.*

Company	Website
BIAcore AB	*http://www.biacore.com*
Affinity Sensors	*http://www.affinity-sensors.com*
Texas Instruments	*http://www.ti.com/spr*
BioTul AG	*http://www.biotul.com*
Windsor Scientific Limited	*http://www.windsor-ltd.co.uk*
Nippon Laser and Electronics Lab	*http://www.rikei.com*
Fairfield	*http://www.fairfields.co.uk*

1.18 *Detection of bacteria using SPR*

Biosensors offer the possibility of rapid and "real-time" detection of pathogens, and therefore a number of sensors and assay formats (Table 1.6) have recently been reported in the literature (Invitski *et al.*, 1999 and Leonard *et al.*, 2003). However, the vast majority of surface plasmon resonance-based biosensor publications have involved the detection of small molecules such as proteins (Nieba *et al.*, 1997), hormones (Gillis *et al.*, 2002 and Fitzpatrick *et al.*, 2003), toxins (Daly *et al.*, 2000) and drugs (Dillon *et al.*, 2003). One of the earliest attempts to use SPR for direct detection of microbes (Fratamico *et al.*, 1998) achieved a detection limit for *E. coli* O157:H7 of 5-7 x 10^7 CFU/ml. Fratamico and co-workers used a sandwich assay format in which they immobilised an anti-*E. coli* O157:H7 monoclonal antibody on the surface of the sensor chip. They then passed various concentrations of cells over the surface and amplified the signal with the injection of polyclonal anti-*E. coli* antibodies. Perkins and Squirrel (2000), described the detection of bacteria using light scattering in combination with a BIAcore 2000 surface plasmon resonance and reported that they could detect 10^7 bacterial spores ml^{-1}. Koubova *et al.* (2001) later described the detection of *Salmonella enteritidis* and *Listeria monocytogenes* at concentrations down to 10^6 cells/ml. Koubova and co-workers used a surface plasmon resonance biosensor-based on prism excitation of surface plasmons and spectral interrogation to directly detect cells on antibody immobilised surfaces. Various cell concentrations were injected over the surface and the shift in resonant wavelength recorded. Recently, Bokken *et al.* (2003) described the detection of *Salmonella* with a BIAcore surface plasmon resonance-based biosensor. Anti-*Salmonella* antibodies

immobilised to the biosensor surface were allowed bind injected bacteria followed by a pulse with soluble anti-*Salmonella* immunoglobulins to intensify the signal. The target organism was successfully detected at 1.7 x 10^5 CFU ml^{-1}. These recently reported publications highlight the potential of SPR-based biosensors for the detection of pathogens. Other sensor formats such as electrochemical sensors (listed in Table 1.6 below and reviewed more extensively by Leonard et al., 2003) have been reported in the literature with limits of detection as low as approximately 10^3 cells/ml (Susmel *et al.*, 2003 and Benhar *et al.*, 2001). However, further characterisation of these formats have not been evaluated and therefore the reproducibility of these assays and regeneration of the sensor surfaces would need to be determined before an accurate limit of detection can be assessed.

Table 1.6: *List of biosensor-based immunoassays for the detection of Listeria monocytogenes.*

Format	Limit of detection	Reference
SPR (IAsys)	Failed	Lathrop et al. (2003)
SPR (miniature prism)	10^6 cells/ml	Koubova et al. (2001)
SPR (BIAcore)	10^4 cells/ml	Haines and Patel (1995)
Modified electrochemical	10^5 cells/ml	Minett et al. (2003)
Electrochem. Redox probe	10^3-10^4/ml	Susmel et al. (2003)
Piezoelectric	10^7 cells/ml	Vaughan et al. (2001)
Piezelectric	10^5/crystal	Minunni et al. (1996)
Electrochemical	$2.5x10^3$/ml	Benhar et al. (2001)

1.19 *Thesis Outline*

As previously stated, the aim of this project was to develop a rapid immunoassay for the detection *Listeria monocytogenes* cells using the BIAcore optical biosensor.

The strategy presented in this thesis involved the generation of polyclonal and recombinant scFv antibodies to *Listeria monocytogenes* cells and invasion-associated proteins. Two polyclonal antibodies (described in chapter 3) were generated against heat-treated *Listeria monocytogenes* cells and internalin B (InlB) protein extract. Both antibodies were characterised by ELISA, SDS-PAGE and Western blotting. In order to safely generate large quantities of purified *Listeria monocytogenes* specific protein, the invasion associated Internalin B and p60 proteins were cloned into *E. coli* XL10 Gold cells (chapter 4). Both InlB and p60 are well characterised (see section 1.4), species specific, surface exposed (p60 is secreted) and are constitutively expressed making them excellent targets for antibody production. Expressed protein was purified by immobilised metal affinity

chromatography (IMAC) and the immunoreactivity of each protein determined with anti-*Listeria monocytogenes* derived monoclonal and polyclonal antibodies. Two combinatorial antibody libraries were developed from mice immunised with *Listeria monocytogenes* cells and InlB protein extract. Phage scFv antibodies were selected from both murine antibody libraries and from a large naïve human antibody library (chapter 5) against *Listeria monocytogenes* cells and invasion associated protein. A number of the selected phage-scFv antibodies could not be expressed as soluble scFv and also showed tendencies to cross react with various bacterial strains tested. A soluble scFv antibody was selected that recognised the *Listeria monocytogenes* invasion associated protein, Internalin B but did not recognise *Listeria monocytogenes* cells. The two polyclonal antibodies and the selected anti-InlB scFv antibody were used in the development of three biosensor-based immunoassays using a BIAcore 3000 instrument (chapter 6). Various assay formats and sensor chip surfaces were evaluated and intra- and interday assay variability studies performed to determine the precision and reproducibility of each assay. The assay developed with the polyclonal anti-InlB antibody proved to be most sensitive and cost effective while the assay developed with the anti-InlB scFv antibody fragment proved most specific.

Chapter 2

Materials & Methods

2.0 *Materials and Methods*

2.1 *General Formulations*

2.1.1 *Materials*

All standard laboratory reagents were purchased from Sigma Chemical Co., Poole, Dorset, England unless stated otherwise.

Reagent	**Supplier**
Acetic acid Hydrochloric acid	Riedel de-Haen AG, Wunstorfer, Strabe 40, D-30926, Hannover, Germany.
Bacteriological Agar Tryptone Yeast Extract PBS tablets	Oxoid, Basingstoke, Hampshire, RG24 8PW, England.
DNA ligase	Boehringer-Mannheim Ltd., East Sussex, England.
Trizol	Gibro BRL, Renfew Rd, Paisley PA49RF, Scotland.
HPLC-grade solvents	Lab-Scan, Stillorgan, Co. Dublin, Ireland.
PCR Optimizer kit TA cloning kit	Invitrogen, 9704-CH-Groningen, Netherlands.
PCR Primers	MWG-Biotech Ltd, Milton Keynes, MK12 5RD, UK.
Restriction enzymes	New England Biolabs, Hitchin, Herts., England.
VCSM13 Helper Phage	Stratagene, North Torrey Pines Rd. La Jolla, USA.
Wizard Plus Mini-prep kit PCR prep DNA purification kit Reverse-Transcription kit	Promega Corporation, 2800 Wood Hollow Rd., Madison, WI 53711-5399, USA.
Goat anti-rabbit (Fab portion) polyclonal antibody	Fitzgerald Industries International, Inc., Concord, MA 01742-3049, USA.

2.1.2 *Equipment*

Equipment	Supplier
BIAcore 1000™ BIAcore 3000™ CM5 chips	Pharmacia Biosensor AB, Uppsala, Sweden.
Eppendorf tubes Sterile universal containers	Sarstedt, Wexford, Ireland.
Heraeus Christ Labofuge 6000 Biofuge A Microcentrifuge	Heraeus Instruments Inc, 111-a Corporate Boulevard, South Plainfield, New Jersey, USA.
Titretek Twinreader Plus	Medical Supply Company, Damastown, Mulhuddart, Dublin 12, Ireland.
NUNC Maxisorb plates	NUNC, Kamstrup DK, Roskilde, Denmark.
3015 pH meter	Jenway Ltd., Essex, England.
Orbital incubator	Gallenkamp, Leicester, England.
RM6 Lauda waterbath T-Gradient BIOMETRA-PCR Millipore Filtration Apparatus	AGB Scientific Ltd., Dublin Industrial Estate, Glasnevin, Dublin 9, Ireland.
SB1 Blood tube rotator	Stuart Scientific, London, England.
Sorvall RC-5B refrigerated centrifuge	Du Pont instruments, Newtown, Connecticut, USA.
Sterile cell culture lab-wear	Brownes, Foxrock, Dublin, Ireland.
Stuart Platform Shaker STR6	Lennox, Naas Rd., Dublin, Ireland.
Ultrafiltration cell 8400	Amicon Inc. Beverly, Massachusetts 01915, USA.
UV-160A spectrophotometer	Shimadzu Corp., Kyoto, Japan.
UVP ImageStore 7500 gel documentation system	Ultra Violet Products, Upland, CA., USA.

2.1.3 *Culture media formulations*

2 x Tryptone and yeast extract (TY) medium	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
Luria Bertani broth (LB) medium	Tryptone	10 g/l
	Yeast Extract	5 g/l
	NaCl	5 g/l
Non-expression medium (NE)	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
	Glucose	1%, v/v
	Chloramphenicol	25 μg/ml
Low expression medium (LE)	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
	Glucose	1%, v/v
	Chloramphenicol	25 μg/ml
	IPTG	0.5 mM
SOB medium	Tryptone	20 g/l
	Yeast Extract	5 g/l
	NaCl	0.5 g/l
	KCl	2.5 mM
	$MgCl_2$	20 mM
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
Top Agar	Tryptone	10 g/l
	Yeast Extract	5 g/l
	NaCl	10 g/l
	Bacto agar	7 g/l

Solid medium was made by adding 15 g/l bacteriological agar to the media above.

2.1.4 *Buffer formulations*

2.1.4.1 *Phosphate buffered saline (PBS)*

One Dulbecco's A tablet (DA, Oxoid) was dissolved per 100 mls of distilled water according to the manufacturer's instructions. When dissolved, prepared PBS contained 0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, pH 7.4. This buffer will be referred to throughout as PBS.

2.1.4.2 *PBS-Tween (PBST)*

Tween 20 surfactant (Sigma) was added to PBS (described in 2.1.2.1 above) to a final concentration of 0.05% (v/v) and mixed.

2.1.4.3 *Tris buffered saline (TBS)*

Tris buffered saline containing 0.05 M Tris, 0.15 M NaCl, pH 7.4, was prepared using distilled water.

2.1.4.4 *Tris-acetic acid-EDTA buffer (TAE)*

A 50 X stock solution of TAE buffer was prepared in a final volume of 1 litre by dissolving 242 g Tris, and addition of 57.1 ml glacial acetic acid followed by 100 ml of 0.5 M EDTA. pH 8.0. All agarose gels were run in 1 X TAE by diluting the stock solution 1/50 with ultra pure water.

2.1.4.5 *Hepes Buffered Saline (HBS)*

Hepes buffered saline (BIAcore running buffer) containing 50 mM NaCl, 10 mM HEPES, 3.4 mM EDTA and 0.05% (v/v) Tween-20 was prepared by dissolving 8.76 g of NaCl, 2.56 g of HEPES, 1.27 g of EDTA and 500 µl of Tween 20 in 800 ml of distilled water. The pH of the solution was then adjusted to pH 7.4, by the addition of NaOH. The final volume was then made up to 1,000 ml in a volumetric flask. The solution was filtered through a 0.2 µm filter and degassed prior to use.

2.1.5 *Bacterial strains used*

Bacterial strain	Source	Comments
Heat-treated (HT) *L. monocytogenes* 4b (NCTC 4885)	Institute of Food Research (IFR) , Norwich, UK.	Cells were heat-inactivated by heating at 76°C for 20 minutes
L. monocytogenes 4b (NCTC 4885)	IFR	Cultured in Brain Heart Infusion (BHI) broth
L. monocytogenes 1/2a (NCTC 4886)	IFR	Cultured in Brain Heart Infusion (BHI) broth
L. monocytogenes EGDpERL3 50-1 $prfA_{EGD}$	Prof. Chakraborty, Institute of Med. Microbiology, University of Giessen, D-35392 Giessen, Germany	Overproduces PrfA regulated proteins
L. innocua 6a (NCTC 11288)	IFR	Cultured in BHI broth
L. innocua 6b (NCTC 11289)	IFR	Cultured in BHI broth
L. welshimeri (NCTC 11857)	IFR	Cultured in BHI broth
L. invanovii (-)	IFR	Cultured in BHI broth
L. seeligeri (ATCC 35957)	IFR	Cultured in BHI broth
Bacillus subtilis subsp. subtilis (NCTC 1650)	Dublin City University	Cultured in 2xTY
E. coli XL-1 Blue	Stratagene	Cultured in 2xTY broth
E. coli XL-10 Gold	Stratagene	Cultured in 2xTY broth
E. coli TG1	Stratagene	Cultured in 2xTY broth
E. coli INV αF'	Invitrogen	Cultured in 2xTY broth

Note: NCTC= UK National Collection of Type Cultures. Cells were obtained from Dr. Gary Wyatt, at the Institute for Food Research, Norwich, unless otherwise indicated. Cells that were not heat-treated will be referred to as "intact" in the remainder of this thesis. Cultures were stored at –80°C with 15-20% (v/v) glycerol.

2.2 *Production and characterisation of polyclonal antibodies*

2.2.1 *Antibody and antigen preparation*

2.2.1.1 *Preparation of bacterial stocks*

Stocks of *Listeria* cells used in this project (section 2.1.5) were prepared by growing the culture overnight in 50 ml BHI at 37^{0}C with constant shaking at 200 rpm, until the O.D.$_{600nm}$ value of between 0.5 and 0.8 was reached. The cultures were then centrifuged at 4000 rpm for 20 minutes and the pellet resuspended in 2xTY broth to 1/10 the original culture volume. Sterile glycerol was then added to the culture to give a final glycerol concentration of 20% (v/v). 1.2 ml aliquots of each stock culture were then placed in 2 ml cryovials and these were stored at –80^{0}C.

2.2.1.2 *Preparation of InlB Extract*

Purification of the InlB gene product of *Listeria monocytogenes* was performed as described by Müller *et al.* (1998b). Briefly, one 1.2 ml aliquot stock of *Listeria monocytogenes* EGD 1/2a pERL3 50-1 cells was thawed and inoculated into 50 mls of BHI containing 5 μg/ml erythromycin. The culture was incubated overnight for 12 hours at 37^{0}C and 200 rpm. The overnight culture was diluted 1/50 with fresh, filtered media and then incubated as above but at a lower rotational speed (120 rpm). When an O.D.$_{600nm}$ value of between 0.8 and 1.0 was reached, cell numbers were calculated by serially diluting a culture sample in media and plating the dilutions on BHI agar plates. The culture cells were removed by centrifugation at 4000 rpm for 20 minutes. The supernatant was decanted and the cell pellet washed x 2 with PBS at 1% (v/v) the original volume. The washed pellet was then re-suspended in 1 M Tris-HCl (Sigma), pH 7.5, and incubated on ice for one hour. The cells were then removed by centrifuging at 10000 rpm for 10 minutes. The supernatant (InlB extract) was retained and stored at –80^{0}C.

2.2.1.3 *Production of polyclonal antibodies*

All processes involving animals were licenced by the Department of Health and Children and care was taken to minimise the stress to the animals involved. An antigen solution (1 mg/ml solution of InlB extract or a 1x10^8 cells/ml *Listeria monocytogenes* solution) in PBS was added to an equal volume of Freund's Complete adjuvant. This solution was vortexed until an emulsion was formed. New Zealand White female rabbits were injected

subcutaneously at a number of sites with 1 ml of the emulsion. The immunisation was repeated 21 days later with Freund's Incomplete adjuvant. Bleeds were taken from the marginal ear vein of the animals between 10 and 15 days after immunisation, and the re-boosting and bleeding procedures repeated until an adequate antibody titre was obtained. Once a sufficient titre was obtained the animals were sacrificed and the serum removed.

2.2.2 *Purification of polyclonal antibody from whole serum*

Purification of polyclonal antibody from rabbit serum was carried out by precipitation with saturated ammonium sulphate, followed by affinity purification using a protein G column.

2.2.2.1 *Saturated ammonium sulphate precipitation*

The ammonium sulphate precipitation of antibody from rabbit serum was carried out according to the method of Hudson and Hay (1989). Saturated ammonium sulphate (SAS) was prepared by dissolving 500 g of ammonium sulphate in 500 ml distilled water at 50°C, leaving the solution overnight at 4°C and adjusting the pH to 7.2. 10 ml of cold saturated ammonium sulphate (100%, w/v) was added dropwise to an equal volume of rabbit serum on ice with stirring. The mixture was allowed to stir at room temperature for 30 minutes, followed by centrifugation at 3,000 rpm for 20 minutes. The supernatant was discarded and the precipitate washed twice in 10 ml of 45% (w/v) ammonium sulphate. The washed pellet was dissolved in 5 ml of PBS and dialysed in 5 L of PBS overnight at 4°C to remove residual ammonium sulphate.

2.2.2.2 *Protein G affinity chromatography*

A 2 ml protein G-sepharose column was poured and equilibrated with 50 ml of PBS, pH 7.4, containing 0.3 M NaCl (running buffer). 2 ml of the dialysed ammonium sulphate rabbit immunoglobulin fraction was applied to the column and the flow rate adjusted to 1 ml/min. The sample was then re-applied to the column twice followed by washing the column with 20 ml of PBS, pH 7.4, containing 0.5 M NaCl and 0.05% (v/v) Tween (wash buffer). Affinity captured-antibody was eluted with 0.1 M glycine/HCl, pH 2.2. 1 ml fractions were collected and immediately neutralised to pH 7.0 by addition of 100 µl of 1.5 M Tris/HCl, pH 8.7, to each fraction, in order to prevent denaturation of antibody. The optical density of each fraction was recorded at 280 nm, and those fractions containing

protein were pooled and dialysed overnight in PBS at 4°C, and sodium azide added to 0.05% (v/v). The sample was aliquoted (0.5 ml fractions) and stored at -20°C.

2.2.3 *Non-competitive ELISA for determination of polyclonal antibody titre*

96 well ELISA plates (Nunc) were coated with antigen (100 µl of $1x10^8$ cells/ml bacteria species, or 10-20 µg/ml protein) for 1 hour at 37°C or overnight at 4°C. The plates were incubated at 37°C for 1 hour, or at 4°C overnight and washed 3 times with PBS. The plates were then blocked with 200 µl of a 5% (w/v) Marvel milk powder solution in PBS and incubated at 37°C for 1 hour and washed as before. Dilutions of antibody were prepared in PBS-Tween (0.05% (v/v) Tween 20) containing 1% (w/v) milk Marvel and 100 µl of each dilution added to the plates in triplicate. The plates were then incubated at 37°C for 1 hour. Followed by 3 washes with PBS and 3 washes with PBS-Tween. A 1/3,000 dilution of secondary antibody (goat anti-rabbit horse radish peroxidase-labelled antibody) was prepared in PBS-Tween containing 1% (w/v) Marvel and 100 µl of this solution added to the plates and incubated at 37°C for 1 hour. The plates were washed 3 times with PBS and 3 times with PBST as before and 100 µl of freshly prepared OPD substrate added to each well. The absorbance was read at 450 nm after 30 minutes using a Titertek Plate Reader Plus®.

2.2.4 *Checkerboard ELISA for determination of optimal concentrations of immobilised interactant*

ELISA plates (Nunc) were coated with varying antigen concentrations ($1 x10^5$ to $2 x10^9$ cells/ml) with each antigen concentration added to a different row on the ELISA plate. Plates were blocked as described with 5% (w/v) milk Marvel and 100 µl samples of antiserum serially diluted in PBS-Tween containing 1% (w/v) milk Marvel added to each well and detected as in section 2.2.3.

2.2.5 *Inhibition ELISA for polyclonal antibody characterisation*

Nunc ELISA plates were coated with antigen and blocked as described in section 2.2.3. Dilutions of cells (*Listeria monocytogenes* 4b or cells listed in Table 3.10 for cross-reactivity studies) were made in PBS or chocolate milk and mixed with an equal volume of antibody. This mixture was incubated for 30 minutes at 37°C. The plates were washed as before and 100 µl of each sample added to the plate in triplicate and incubated at 37°C for 1 hour. Plates were washed three times with PBS and 3 times with PBS-Tween and 100 µl of 1/3000 dilution of anti-rabbit HRP-conjugated antibody in PBS-Tween

containing 1% (w/v) Marvel added to each well. After incubation for 1 hour at 37^{0}C, 100 µl of OPD substrate was added to each well and the plates incubated for 30 minutes to 1 hour at 37^{0}C. Absorbance was read at 450 nm.

2.2.6 *SDS polyacrylamide gel electrophoresis*

The following stock solutions were prepared: 30% (w/v) acrylamide containing 0.8% (w/v) bis-acrylamide, 1.5 M Tris-HCl, pH 8.8 (Tris base, rather than Tris-Cl, should be used for buffer preparation and pH adjustment made with HCl as use of Tris-Cl will result in higher ionic strength, poor migration and diffuse protein bands), 0.4% (w/v) SDS, 0.5 M Tris-HCl, pH 6.8, 0.4% (w/v) SDS and 10% (w/v) ammonium persulfate. The glass plates were washed with acetone and sealed with a gasket and grips. Free radical-induced polymerisation of the resolving gel acrylamide (Table 2.1) was catalysed by addition of ammonium persulphate and the accelerator TEMED and the gel added to the space between the plates and covered with a layer of distilled water. Once the gel had polymerised, the water was removed and the stacking gel was placed directly onto the resolving gel. A plastic comb was placed in this gel to create the wells for sample application. Once the gel had fully polymerised, the plates were then placed in an electrophoresis chamber, the comb was removed and the chamber and wells filled with electrophoresis buffer (25 mM Tris, 250 mM Glycine (electrophoresis grade), pH 8.3 and 0.1% (w/v) SDS).

Solution	Resolving Gel	Stacking Gel
Acrylamide (A)	10 ml	1.66 ml
Distilled Water	5.0 ml	5.75 ml
Resolving Gel Buffer (B)	5.0 ml	-
Stacking Gel Buffer (C)	-	2.5 ml
Ammonium Persulphate (D)	200 µl	70 µl
TEMED	20 µl	14 µl

Table 2.1: *Composition of Resolving and Stacking Gels. (A) 30% (w/v) Acrylamide solution containing 29.2% (w/v) acrylamide and 0.8% (w/v) Bis-acrylamide, (B) 4X resolving gel buffer consisting of Tris-HCl pH 8.8 (1.5M) and SDS (0.4% w/v) diluted in distilled water, (C) 4X stacking gel buffer consisting of Tris-HCl pH 8.8 (0.5M) and SDS (0.4%, w/v) diluted in distilled water and (D) 100 mg/ml ammonium persulphate diluted in water.*

Excess sample buffer (3:1 ratio of SDS to protein) is essential to maintain reduction of protein sulfhydryls and to prevent intramolecular disulfide bond formation through

oxidised cysteines. Therefore, samples were mixed 4:1 with sample buffer (60 mM Tris-HCl (pH 6.8), 25% (w/v) glycerol, 2% (w/v) SDS, 14.4 mM 2-mercaptoethanol and 0.1% (w/v) bromophenol blue) and heated for 4-5 minutes at 100°C. A Sigma wide range marker (product code M 4038), Sigma prestained marker (product code MW-SDS-Blue) or Pierce Blue Ranger prestained marker (product code 26681), was used for each gel. A sample volume of 20 µl was added to the respective wells on the gel. The gel was run at 30 mA until the sample had reached the bottom of the gel, at which time, the gels were stained with Coomassie blue for 1-2 hours followed by destaining in 30% (v/v) methanol:10% (v/v) acetic acid:60% (v/v) distilled water for 2-3 hours.

Protein standard	Sigma wide range marker	Sigma prestained marker	Pierce Blue Ranger marker
Aprotinin	6.5 kDa	-	-
α-Lactalbumin	14.2 kDa	-	-
Lysozyme	-	-	18 kDa
Trysin Inhibitor	20 kDa	-	26 kDa
Trypsinogen	24 kDa	-	-
Triosephosphate Isomerase	-	31 kDa	-
Carbonic Anhydrase	29 kDa	-	32 kDa
Glyceraldehyde-3-phosphate dehydrogenase	36 kDa	-	-
Lactic Dehydrogenase	-	36.5 kDa	-
Ovalbumin	45 kDa	-	48 kDa
Fumerase	-	54 kDa	-
Glutamic Dehydrogenase	55 kDa	-	-
Pyruvate Kinase	-	61.5 kDa	-
Albumin	66 kDa	-	85 kDa
Fructose-6-phosphate Kinase	84 kDa	85 kDa	-
Phosphorylase B	97 kDa	-	112 kDa
Galactosidase	116 kDa	116 kDa	-
α_2Macroglobulin	-	185 kDa	-
Myosin	205 kDa	-	208 kDa

Table 2.2: *Molecular weights of the protein standards used for SDS-PAGE and Western Blot analysis. The molecular weights of the Sigma prestained marker and Pierce Blue Ranger prestained marker indicate the apparent molecular weights of each protein after staining.*

2.2.7 *Western blotting*

Following electrophoresis, as outlined above (section 2.3.6), the gel was placed on top of three sheets of Whatman filter paper pre-soaked in transfer buffer. Generally, a Tris-glycine-methanol-SDS buffer is used to transfer separated proteins to nitrocellulose. At high concentrations, SDS actually seems to inhibit the binding of proteins to

nitrocellulose, and therefore in many labs, this component is omitted (Caponi and Migliorini, 1999). Without the addition of SDS, however, the gel may swell and partial refolding of protein may occur. Methanol helps to counteract swelling, but can diminish the transfer efficiency, since it also tends to fix the proteins on the gel. Therefore the composition of the transfer buffer (electrophoresis buffer (section 2.3.6) with 20% (v/v) methanol) represents a compromise between different requirements (Caponi and Migliorini, 1999). A sheet of Immobilon P^{TM} PVDF membrane or nitrocellulose membrane, also pre-soaked in transfer buffer, was carefully placed on top of the gel and any air bubbles removed by rolling a clean 10 ml pipette over the sheets. Three more sheets of pre-soaked filter paper were added on top of the gel and air bubbles removed. Proteins were transferred from the gel to the membrane using a Bio-Rad Wet Transfer system for 1 hour at 70V in a 4°C environment.

After transfer, the blotted membrane was washed 3 times with Tris-Buffered Saline (TBS; 10mM Tris-HCl, pH 8.0, 150mM NaCl), prior to overnight incubation in blocking buffer (5% (w/v) Marvel Milk Powder in TBS). Following blocking, the blot was washed 3 times in TBS buffer and then incubated with primary antibody (at the appropriate dilution in TBS containing 1% (w/v) Marvel Milk Powder, 0.05% (v/v) Tween-20) for 1.5 hours at room temperature. The blot was then washed 3 times in TBS and 3 times in TBS-T (TBS containing 0.05% Tween-20) prior to incubation with alkaline phosphatase-labelled secondary antibody (diluted 1/3000 in TBS-T containing 1% (w/v) Marvel Milk Powder) for 1.5 hours at room temperature. The blot was washed 3 times in TBS-Tween and rinsed in TBS, before incubation in substrate solution (175 μg/ml BCIP, 500 μg/ml NBT in alkaline phosphatase buffer). Colour development was allowed to proceed at room temperature until the desired bands were apparent, at which time the reaction was stopped by washing extensively with distilled water or by rinsing with 100mM EDTA.

2.3 *Cloning of the gene sequences encoding InlB and p60 into E. coli*

2.3.1 *Preparation of Listeria monocytogenes genomic DNA*

20 ml of a *L. monocytogenes* 1/2a culture was centrifuged at 4000 rpm for 10 minutes and the pellet resuspended in 1.5 ml TES (10 mM Tris-HCl, 1 mM Na_2-EDTA, 50 mM NaCl, pH 8). The 1.5 ml culture was centrifuged at 13,000 rpm for 5 minutes, the supernatant removed and pellet resuspended in another 1.5 ml TES. The 1.5 ml culture was re-centrifuged at 13,000 rpm for 5 minutes, the supernatant removed and the pellet resuspended in 0.7 ml TE (10 mM Tris-HCl, 1mM Na_2-EDTA, pH 8). 100 µl of lysozyme freshly prepared was added and incubated for 45 minutes at 30°C. When the solution turned viscous 50 µl of the Sarkosyl solution (10% (v/v) Sarkosyl in TE containing 5 mg/ml proteinase) was added and incubated at 37°C for 1 hour. After 1 hour 70 µl of a 3M sodium acetate solution was added and mixed. 600 µl of the Kirby mix (100 ml Phenol, 100 ml chloroform, 4 ml isoamylalcohol, 0.8 g 8-hydroxy Quinolone, stored under 100 mM Tris-HCl, pH 7.5 at 4°C) was then added and mixed. The mixture was then spun at 13000 rpm for 4 minutes and the aqueous phase decanted into a fresh eppendorf tube. A further 600 µl of the Kirby mix was added and mixed. The mixture was spun at 13000 rpm for 4 minutes and the aqueous phase again removed into a fresh eppendorf. 700 µl of chloroform:isoamylalcohol (24:1) was added and mixed. The mixture was then centrifuged at 13000 rpm for 4 minutes and the aqueous phase transferred to a fresh eppendorf tube as before. An equal volume of isopropanol was added to the aqueous phase and re-centrifuged at 13000 rpm (until a pellet was formed). The supernatant was removed and pellet was washed twice with 200 µl of 70% (v/v) ethanol and allowed air dry for 5 minutes. The pellet was then resuspended in 150-200 µl TE and stored at 4°C.

2.3.2 *Agarose gel electrophoresis for DNA characterisation*

DNA was analysed by running on agarose gels (containing 0.5 µg/ml ethidium bromide for staining of DNA) in a horizontal gel apparatus. Gels were prepared by dissolving agarose (typically 0.7-1.2% (v/v)) in 1 x TAE buffer by heating and 0.5 µg/ml ethidium bromide added to the cooled gel prior to setting. The gels were placed in the gel apparatus and 1 x TAE used as the running buffer. A tracker dye was incorporated into the sample (1 µl dye + 5 µl sample) to facilitate loading of samples. Where Sigma red *Taq* was used no tracking dye was added. Mini gels were frequently run at 100 Volts for 1 hour or until the tracker dye reached the end of the gel while maxi gels were frequently run at 40-80 Volts.

Gels were then visualised on a UV transilluminator and photographed using a UV image analyser.

2.3.3 *Design of p60 and InlB Primers*

Primers were designed (Table 2.2) for further ligation into the QIA*express*® Type ATG construct pQE-60 (QIAGEN). For both the p60 (accession number X52268) and InlB (accession number AJ012346) proteins the sequence coding for the signal peptides were deleted. The multi-cloning region of the pQE-60 vector contains a *NcoI* site at the N' end of the sequence and a *BamHI* site at the C' end. Therefore, forward primers were designed to include the *NcoI* binding site sequence and the reverse primers were designed to contain the *BamHI* sequence. Forward primers were designed using the same bases found in the starter sequence of the required protein and reverse primers were designed using the complementary bases from the 3' to 5' end of the sequences.

	Nco1
P60 forward primer	5'-c*ca tgg* gaa gca ctg tag tag tcg aag ctg g
P60 reverse primer	5'-*gga tcc t*ac gcg acc gaa gcc aac tag
	BamH1
	Nco1
InlB forward primer	5'-cc*a tgg* gag aga cta tca ccg tgc caa cgc
InlB reverse primer	5'-*gga tcc t*tt ctg tgc cct taa att agc tgc
	BamH1

Table 2.3: *Sequences of both p60 and InlB forward and reverse primers used for the amplification of p60 and InlB gene sequences by PCR.*

2.3.4 *PCR of p60 and InlB protein gene sequences*

PCR was used to amplify the DNA sequences for both proteins. A standard PCR reaction was set up as follows:

1 µl	dTNP mix (10mM)
5 µl	10x PCR buffer
0.5 µl (each)	Primers (0.5nM/µl) (total of 1 µl)
1 µl	Template DNA (approximately 20ng/reaction)
39.5 µl	Sterile H_2O
2.5 µl	Taq polymerase

A negative control was also set up as above except no Template DNA was added. The following gradient PCR cycle was used to determine the optimum annealing temperature for each protein:

Stage 1:	*Step 1:*	95°C for 10 minutes
Stage 2:	*Step 1:*	95°C for 1 minute
	Step 2:	58°C for 30 seconds or
		60°C for 30 seconds or
		62°C for 30 seconds or
		65°C for 30 seconds
	Step 3:	72°C for 1 minute

❖ **Stage 2** was repeated for 30 cycles

Stage 3:	*Step 1:*	72°C for 10 minutes

When the correct annealing temperature was optimised, the PCR was repeated as above except Stage 2 Step 2, was run only at the correct annealing temperature for 30 seconds.

2.3.5 *TA Cloning of p60 and InlB PCR products into E. coli INV αF' cells*

Cloning of p60 and InlB PCR products into *E. coli INV αF'* cells was carried out using a TA cloning kit (Invitrogen) under the recommended conditions. *Taq* polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in the kit has 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. For optimal ligation efficiencies, fresh PCR products (< 1 hour old) were used, as the single 3' A-overhangs on the PCR products will degrade over time, reducing ligation efficiency.

2.3.5.1 *Ligation of PCR Products into a pCR 2.1 Vector*

The ligation reaction (10µl) was set up as follows:

Sterile ultra pure water	5 µl
10x ligation buffer	1 µl
pCR 2.1 vector (25 ng/ µl)	2 µl
Fresh PCR product	1 µl
T4 DNA Ligase	1 µl
Total volume	10 µl

The ligation reaction was incubated at 14°C overnight and then used for transformation (If unable to do transformation immediately the ligation reaction can be stored at –20°C).

2.3.5.2 *Transformation of pCR 2.1 into E. coli INV αF' cells*

The vial containing the ligation reaction was centrifuged briefly and placed on ice. A 50 µl vial of frozen One-shot® competent cells was thawed on ice for each ligation/transformation. 2 µl of each ligation reaction was pipetted directly into the vial of competent cells and mixed by stirring gently with the pipette tip. The vial was incubated on ice for 30 minutes and the remaining ligation mixture stored at –20°C. Each Vial was heat shocked for exactly 30 seconds in a 42°C water bath and placed immediately back on ice. 250 µl of SOC medium (section 2.1.3) at room temperature was added to each tube. The vials were then shaken horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator. After 1 hour, 50 µl or 100 µl from each transformation vial was spread on separate, labelled LB agar plates (Luria-Bertani medium: 1% (w/v) Tryptone, 0.5 % (w/v) Yeast Extract, 1% (w/v) NaCl and pH 7.0) containing X-Gal (40 µl of the 40mg/ml X-Gal stock solution was pipetted onto LB plates, spread evenly, and let dry for 15 minutes) and 50 µg/ml of kanamycin. The plates were then inverted and placed in at 37°C for 18-24 hours before being shifted to 4°C for 2-3 hours to allow for proper colour development.

2.3.5.3 *Plasmid purification*

To determine the presence of a PCR insert, six (InlB and p60) colonies were picked from the plates and grown overnight in 3 ml LB broth containing 50 µg/ml kanamycin. A Promega Wizard® *Plus* Minipreps DNA purification system was used to purify the plasmid DNA as follows:

A 5 ml overnight bacterial culture was centrifuged for 5 minutes at 4,000 rpm, the supernatant decanted and tubes blotted dry on a paper towel. 250 µl of cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 10 µg/ml RNase A) was added and the cell pellet completely resuspended by vortexing or pipetting. The mixture was transferred to sterile 1.5 ml microcentrifuge tube(s), 250 µl of cell lysis solution (0.2 M NaOH and 1% w/v SDS) added and mixed by inverting the tube 4 times. The suspension was incubated for 1-5 minutes before 10 µl of alkaline protease solution was then added and mixed by inverting the tube 4 times (alkaline protease inactivates endonucleases and other proteins released during the lysis of the bacterial cells that can adversely affect the quality of the isolated DNA). After 5 minutes incubation at room temperature 350 µl of Wizard® *Plus* SV neutralisation solution (pH 4.2 buffer containing 4.09 M guanidine hydrochloride, 0.759 M potassium acetate and 2.12 M glacial acetic acid,) was added and

immediately mixed by inverting the tube 4 times. The bacterial lysate was then centrifuged at 14,000 rpm for 10 minutes at room temperature. The cleared lysate was transferred (~ 850 µl) to the prepared spin column by decanting. The supernatant was again centrifuged at 14,000 rpm for 1 minute at room temperature, the spin column was removed from the tube and the flow through discarded from the collection tube. 750 µl of column wash solution (162.8 mM potassium acetate, 22.6 mM Tris-HCl, pH 7.5 and 0.109 mM EDTA), previously diluted with 95% (v/v) ethanol, was then added to the reinserted spin column. The supernatant centrifuged at 14,000 rpm for 1 minute at room temperature and the flow through again discarded from the collection tube. The wash procedure was repeated using 250 µl of column wash solution. The supernatant was centrifuged at 14,000 rpm for 2 minutes at room temperature, and the spin column subsequently transferred to a new sterile 1.5 ml microcentrifuge tube taking care not to transfer any of the column wash solution with the spin column. The plasmid DNA was finally eluted by addition of 100 µl of sterile ultra pure water to the spin column and centrifuging the supernatant at 14,000 rpm for 1 minute at room temperature. After eluting the plasmid DNA, the spin column was discarded and DNA stored in the microcentrifuge tube at –20°C.

2.3.5.4 *Restriction Analysis of White Colonies from the X-Gal agar plates*

A restriction digest was carried out at 37°C for 1 hour on each of the twelve colony preparations as follows:

NE buffer 2 (10x)	5.0 µl
BSA (10mg/ml)	0.5 µl
Plasmid DNA (30ng/reaction)	10.0 µl
Up H_2O (sterile)	28.5 µl
BamH1	2.0 µl
Nco1	4.0 µl
Total volume	50.0 µl

Each digest was run on a 1% (w/v) agarose gel (section 2.3.2) and the restricted p60 insert and InlB insert cut from the gel using a sharp scalpel and placed in sterile 1.5 ml centrifuge tubes (section 2.3.6.3).

2.3.6 *Cloning of p60 and InlB gene sequences into a QIAGEN pQE-60 plasmid*

2.3.6.1 *Preparation of pQE-60 plasmid*

An *E.coli* colony was taken from a LB agar plate containing 100 µg/ml ampicillin and grown overnight in 5 ml LB broth containing 50 µg/ml ampicillin at 37°C. The pQE-60 plasmid was then purified using the Promega Wizard® *Plus* Minipreps DNA Purification System (section 2.3.5.3).

2.3.6.2 *Restriction of both pQE-60 and the pCR 2.1 plasmid containing the inserts*

A restriction digest was carried out on the purified pQE-60 plasmid, purified pCR 2.1- p60 plasmid and purified pCR 2.1-InlB plasmid as described in section 2.3.5.4.

2.3.6.3 *Purification of DNA from an agarose gel*

DNA was purified from the agarose gel using a Promega Wizard® PCR Preparation DNA Purification System as outlined below:

The DNA band of interest was excised using a clean, sterile scalpel blade and the agarose slice (<300 mg) transferred to a sterile 1.5 ml microcentrifuge tube. The tube was incubated at 70°C until the agarose melted (~10 minutes). 1 ml of resin was added to the melted agarose slice and mixed thoroughly for 20 seconds before being pipetted into a 5 ml sterile syringe attached to the Luer-Lok® extension of a Wizard® Minicolumn. The syringe plunger was then reattached and the DNA/resin gently pushed into the minicolumn. The syringe barrel was detached from the minicolumn, the plunger removed and the barrel reattached to the minicolumn. 2 ml of 80% (v/v) isopropanol was pipetted into the syringe, the plunger reattached and the solution gently pushed through the column. The syringe was again removed and the minicolumn transferred to a new sterile 1.5 ml microcentrifuge tube. The minicolumn was centrifuged at 10,000 rpm to dry the resin. The minicolumn was again transferred to a new microcentrifuge tube and 50 µl of sterile TE buffer added. After 1-2 minutes the minicolumn was centrifuged for 20 seconds at 10,000 rpm to elute the DNA fragment. The minicolumn was then discarded and the purified DNA stored at 4°C short term or at –20°C long term.

2.3.6.4 *Ligation of purified cut pQE-60 plasmid with purified protein inserts*

The ligation reaction was set up as follows and incubated at 14°C overnight

NcoI/BamHI digested pQE-60 (20ng/rxn)	4	µl
NcoI/BamHI digested gene insert (10ng/rxn)	4	µl
Ligase	1	µl
10x buffer	1	µl
Total volume	10	µl

The ligation mixture was stored at –20°C until needed.

2.3.6.5 *Preparation of competent cells*

High efficiency competent cells were prepared as previously described by Inoue *et al.* (1990). A stock of *E. coli* XL10 Gold was streaked on 2xTY agar and incubated at 37°C overnight. Approximately 10-12 large colonies were removed from the plate and inoculated into 250 ml of SOB medium in a 2L baffled flask. The culture was grown at 18°C with vigorous shaking (200-250 rpm) until an OD_{600} of 0.6 was reached. The culture was then placed on ice for 10 minutes before being transferred to two 250 ml centrifuge tubes and spun at 4,000 rpm at 4°C for 5 minutes in a Beckman J2-21 centrifuge. The pellets were resuspended in 40 ml of ice-cold TB buffer (10 mM Pipes, 15 mM $CaCl_2$, 150 mM KCl, pH adjusted to 6.7 with KOH and 55 mM $MnCl_2$, sterile filtered and stored at 4°C), placed on ice for 10 minutes, pooled and centrifuged as before. The cell pellet was gently resuspended in 20 ml of ice-cold TB buffer and DMSO added slowly with gentle swirling to a final concentration of 7%. After incubation in an ice bath for 10 minutes the cell suspension was dispensed in 1 ml aliquots into microcentrifuge tubes. The cells were then flash frozen in liquid nitrogen and stored at –80°C.

2.3.6.6 *Transformation of pQE-60 into E coli XL-10 Gold competent cells*

The transformation was carried out as described in section 2.3.5.2 except no X-Gal was added to the LB plates, as pQE-60 contains no *lacZα* gene.

2.3.7 ***Protein expression time course experiment***

5 mls of LB broth containing both ampicillin and kanamycin was inoculated with 100 µl of stock culture (culture with insert) and grown overnight at 37°C. The 5 ml culture was

then used to inoculate 50 mls LB broth containing both ampicillin and kanamycin and incubated at 37°C until an $O.D._{600}$ of 0.6 was reached. At this time a 1 ml sample was taken and placed at –20°C until needed. The culture was induced with IPTG to a final concentration of 1 mM and a 1 ml sample taken every hour. Each 1 ml sample was centrifuged at 14,000 rpm for 5 minutes and the supernatant discarded. The pellet was resuspended in 200 µl of appropriate buffer, PBS for native extract and lysis buffer (8 M urea, 10 mM Tris-Cl, 100 mM NaH_2PO_4, pH 8.0) for denatured extract. The samples were then sonicated for 45 seconds at an amplitude of 40 using a microtip Vibra Cell™ sonicator and the cell debris removed by centrifugation at 14,000 rpm for 5 minutes. The supernatant was mixed with SDS-PAGE sample buffer and SDS-PAGE analysis carried out as in section 2.2.6.

2.3.8 *Determination of protein solubility and cellular location*

To determine the solubility of each protein, a 5ml aliquot of a 50 ml induced protein culture (section 2.3.7), was centrifuged at 4000 rpm for 20 minutes and the pellet resuspended in 500 µl of PBS and sonicated. The remaining cell debris was resuspended in 8M Urea buffer and the insoluble matter separated by centrifugation. Both the soluble (PBS) and insoluble (Urea buffer) protein fractions were analysed by SDS-PAGE. To determine the cellular location of each protein 2 x 5 ml aliquots of a 50 ml induced protein culture (section 2.3.7) were centrifuged at 4000 rpm for 20 minutes and the supernatant discarded. One of the cell pellets was resuspended in 1 ml TES (200 mM Tris-HCl (pH 8.0), 0.5 M sucrose, 0.5 mM EDTA) buffer and left on ice for 1 hour. After 1 hour, the culture was centrifuged at 14,000 rpm for 5 minutes and the supernatant (periplasmic extract) removed to a fresh tube for further analysis. The remaining two pellets (pellet from periplasmic extract and original culture pellet) were resuspended in PBS (native extract) and sonicated as described in section 2.3.7. The periplasmic extract (pellet resuspended in TES), native cytoplasmic extract (pellet resuspended in PBS and sonicated) and cytoplasmic extract minus periplasmic extract (periplasmic lysate pellet resuspended in PBS and sonicated) were centrifuged and analysed by SDS-PAGE as described in section 2.2.6.

2.3.9 *Purification of recombinant protein by immobilised metal affinity chromatography*

1 ml of a Ni^{+}-NTA agarose resin (QIAgen) was added to a 20 ml universal tube and equilibrated with 20 ml of running buffer (PBST with 1% (v/v) Tween-20, 500 mM NaCl and 10 mM β-mercaptoethanol). The solution was centrifuged at 4000 rpm for 10 minutes

and the supernatant (running buffer) removed. 2-3 ml of InlB or p60 cytoplasmic extracts were added to the pellet (Ni^+-NTA resin) and mixed for 1-2 hours at room temperature on a rotating table ("belly dancer"). The mixture was then centrifuged at 4000 rpm for 10 minutes and the supernatant (unbound material) removed. The pellet (resin with bound protein) was washed 3 times with 20 ml of running buffer as before. The proteins were eluted with 2-3 ml of sodium acetate buffer, pH 4.5, and neutralised with 2 M Tris-base purified proteins were stored at –20°C.

2.3.10 *Identification of recombinant protein by Western blotting*

Purified proteins were analysed by SDS-PAGE and Western blotting as described in sections 2.2.6 and 2.2.7. Purified recombinant protein bands were detected with a mouse anti-His tag monoclonal antibody (Sigma) or with a mouse monoclonal antibody raised against the relevant protein, donated by Stephen Hearty, Dublin City University.

2.3.11 *Conjugation of HRP to the anti-InlB polyclonal antibody*

3 mg of peroxidase was dissolved in 250 µl freshly prepared 0.1 M $NaHCO_3$ in a glass test tube. 250 µl of a 8 mM $NaIO_4$ solution was added to the peroxidase, and incubated at room temperature for 2 hours in the dark. After 2 hours, 500 µl of 1 mg/ml antibody in PBS was added to the peroxidase solution and incubate for a further 2 hours in the dark. To stabilise the mixture, 100 µl of 100 mM $NaBH_4$, diluted in 100 mM NaOH, was added to the mixture and incubated for 1 hour at 4°C. The mixture was then dialysed overnight at 4°C in 5 L of PBS.

2.3.12 *Identification of recombinant protein by ELISA*

Recombinant p60 protein was identified by inhibition ELISA as described in section 2.2.5, while recombinant InlB was identified by a sandwich ELISA using HRP-conjugated anti-InlB polyclonal antibody. The sandwich ELISA was performed by coating an ELISA plate with 30 µg/ml anti-InlB polyclonal antibody for 1 hour at 37°C and blocking with 5% (w/v) milk marvel. Dilutions of purified recombinant InlB protein in PBS were added to the anti-InlB polyclonal antibody coated wells (in triplicate) and incubated for 1 hour at 37°C. Unbound recombinant InlB protein was washed from the plate with 3 washes with PBS and 3 washes with PBST. Bound protein was detected with the addition of 100 µl per well of HRP-conjugated anti-InlB polyclonal antibody.

2.3.13 *Alignment of protein gene sequences*

Purified DNA from section 2.3.6.1 was sent to MWG-Biotech Ltd for sequencing and linear nucleotide results were received via e-mail. Nucleotide sequences were translated to amino acid sequences by using the Expasy Translate tool (see section 2.4.3.8 for web addresses) and aligned with protein amino acid sequences given in EMBL (AJ012346 and X52268) using the clustalW program. Aligned sequences were imported into Genedoc for further sequence manipulation.

2.4 *Production of murine scFv antibody libraries*

Recombinant single chain variable fragment production provides an alternative source of high affinity *Listeria monocytogenes*-specific antibodies. Recombinant antibodies can be expressed in large quantities in *E. coli* and easily purified via engineered affinity tags, such as the polyhistidine tag or the Flag peptide tag. Antibody phage display has the advantage of a direct link between DNA sequence and protein function allowing modifications in the primary antibody sequence, often resulting in altered antigenic specificity and enhanced stability (Azzazy and Highsmith, 2002).

2.4.1 *Isolation of murine antibody genes from immunised mice*

2.4.1.1 *Immunisation procedure of BALB/c mice for the production of single chain Fv antibodies to Listeria monocytogenes cells and invasion-associated proteins.*

Solutions of $1x10^8$ heat-treated *Listeria monocytogenes* cells or 1 mg/ml InlB extract were prepared in PBS and added to an equal volume of Freund's Complete adjuvant. This solution was vortexed until an emulsion was present. 5-10 week old BALB/c mice were immunised subcutaneously with a total volume of 250 µl over several sites. Further intraperitoneal immunisations were carried out after 21 days using Freunds' Incomplete adjuvant. A tail bleed from the mice were taken 7 days later and the antibody titre against the specific drug determined. The mice were re-boosted and bleeding procedure repeated until an adequate antibody titre was obtained. The animals were then re-immunised 7-10 days prior to sacrifice. The mice were sacrificed by cervical dislocation and the spleens removed.

2.4.1.2 *Total RNA Extraction from the spleen of BALB/c mice*

The spleen from an immunised mouse (immunised with InlB extract or heat-treated *Listeria monocytogenes* cells) was removed aseptically and placed into a pre-weighed sterile universal. The spleen was weighed and therefore 1 ml of Trizol reagent per 50 mg of spleen added and homogenized using a sterile homogeniser that had been sterilised at

180°C overnight. The homogenized sample was aliquoted into 1 ml aliquots and placed in sterile 1.5 ml centrifuge tubes. These were then left for 5 minutes at room temperature before the addition of 200 µl of chloroform to each tube. The tubes were shaken for 15 seconds and incubated again at room temperature for 2-3 minutes. After incubation the tubes were centrifuged at 14,000 rpm for 15 minutes at 4°C. Three layers were observed, a lower red phenol/chloroform phase, a protein interphase and a colourless liquid upper phase containing the RNA. Transferring the aqueous phase to a fresh tube and adding 0.5 ml of isopropyl alcohol per tube precipitated the RNA. Samples were incubated at room temperature for 10 minutes and then centrifuged at 14,000 rpm for 10 minutes at 4°C. Small brown pellets were observed at the side of each tube. The supernatant was removed and the pellet washed by redissolving in 1 ml of 75% (v/v) ethanol per tube by gentle pipetting and inversion. Each tube was then centrifuged at 10,000 rpm for 5 minutes at 4°C, the ethanol decanted and the pellet dried at 37°C for 10 minutes. Each pellet was redissolved in 50 µl nuclease free water and pooled together in a fresh sterile centrifuge tube.

2.4.1.3 *Reverse transcription of mouse spleen mRNA*

PCR can be performed directly from RNA but it is more successful to synthesize complementary DNA (cDNA) from total RNA for use in PCR. Complementary DNA was synthesized using a Promega cDNA synthesis kit containing random hexamer primers. Random hexamer primers were used instead of poly T primers as it is possible that the mRNA could be sheared during the extraction process and the use of the poly T primer would reduce the amount of viable cDNA synthesized. The PCR reaction was set up as follows:

Component	**Working conc.**
$MgCl_2$	5mM
10X Buffer	1X
dNTP mix	1mM
RNase Inhib.	1U/µl
Random Primer	0.2 µg/rxn*
AMV RT	15U/rxn*
Sterile H_2O	Made up to 20 µl including RNA
RNA	7-10 µg RNA/rxn*

* total concentration per reaction (usually 20µl volume)

▼ Volume for one reaction (X1) or six reactions (X6)

All reaction components, except enzymes, were placed on ice and left to thaw slowly. Enzymes were only removed when required and immediately kept at –20°C after use, in order to preserve activity. A 6X master mix was made up and the required volume of enzymes added before pipetting the mixture in to pre-labelled eppendorfs. Finally the required volume of total RNA was added and mixed by pipetting.

2.4.2 *Amplification of antibody light and heavy chain genes*

2.4.2.1 *PCR Primers*

The listed PCR primers were obtained from Sigma-Genosys Ltd and designed to be The compatible with the pAK vector system as described by Krebber *et al.* (1997).

Variable light chain back primers

LB1 5′gccatggcggactacaaaGAYATCCAGCTGACTCAGCC3′

LB2 5′gccatggcggactacaaaGAYATTGTTCTCWCCCAGTC3′

LB3 5′gccatggcggactacaaaGAYATTGTGMTMACTCAGTC3′

LB4 5′gccatggcggactacaaaGAYATTGTGYTRACACAGTC3′

LB5 5′gccatggcggactacaaaGAYATTGTRATGACMCAGTC3′

LB6 5′gccatggcggactacaaaGAYATTMAGATRAMCCAGTC3′

LB7 5′gccatggcggactacaaaGAYATTCAGATGAYDCAGTC3′

LB8 5′gccatggcggactacaaaGAYATYCAGATGACACAGAC3′

LB9 5′gccatggcggactacaaaGAYATTGTTCTCAWCCAGTC3′

LB10 5′gccatggcggactacaaaGAYATTGWGCTSACCCAATC3′

LB11 5′gccatggcggactacaaaGAYATTSTRATGACCCARTC3′

LB12 5′gccatggcggactacaaaGAYRTTKTGATGACCCARAC3′

LB13 5′gccatggcggactacaaaGAYATTGTGATGACBCAGKC3′

LB14 5′gccatggcggactacaaaGAYATTGTGATAACYCAGGA3′

LB15 5′gccatggcggactacaaaGAYATTGTGATGACCCAGWT3′

LB16 5′gccatggcggactacaaaGAYATTGTGATGACACAACC3′

LB17 5′gccatggcggactacaaaGAYATTTTGCTGACTCAGTC3′

LBλ 5′gccatggcggactacaaaGATGCTGTTGTGACTCAGGAATC3′

Variable light chain forward primers

LF1 5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTGATTTCCAGCTTGG3′

LF2 5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTTATTTCCAGCTTGG3′

LF4 5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTTATTTCCAACTTTG3′

LF5 5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTCAGCTCCAGCTTGG3′

LFλ 5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACCTAGGACAGTCAGTTTGG3′

Variable heavy chain back primers

HB1 5′ggcggcggcggctccggtggtggtggatccGAKGTRMAGCTTCAGGAGTTC3′

HB2 5′ggcggcggcggctccggtggtggtggatccGAGGTBCAGCTBCAGCAGTC3′

HB3 5′ggcggcggcggctccggtggtggtggatccCAGGTGCAGCTGAAGSASTC3′

HB4 5′ggcggcggcggctccggtggtggtggatccGAGGTCCARCTGCAACARTC3′

HB5 5′ggcggcggcggctccggtggtggtggatccCAGGTYCAGCTBCAGCARTC3′

HB6 5′ggcggcggcggctccggtggtggtggatccCAGGTYCARCTGCAGCAGTC3′

HB7 5′ggcggcggcggctccggtggtggtggatccCAGGTCCACGTGAAGCAGTC3′

HB8 5′ggcggcggcggctccggtggtggtggatccGAGGTGAASSTGGTGGAATC3′

HB9 5′ggcggcggcggctccggtggtggtggatccGAVGTGAWGYTGGTGGAGTC3′

HB10 5′ggcggcggcggctccggtggtggtggatccGAGGTGCAGSKGGTGGAGTC3′

HB11 5′ggcggcggcggctccggtggtggtggatccGAKGTGCAMCTGGTGGAGTC3′

HB12 5′ggcggcggcggctccggtggtggtggatccGAGGTGAAGCTGATGGARTC3′

HB13 5′ggcggcggcggctccggtggtggtggatccGAGGTGCARCTTGTTGAGTC3′

HB14 5′ggcggcggcggctccggtggtggtggatccGARGTRAAGCTTCTCGAGTC3′

HB15 5′ggcggcggcggctccggtggtggtggatccGAAGTGAARSTTGAGGAGTC3′

HB16 5′ggcggcggcggctccggtggtggtggatccCAGGTTACTCTRAAAGWGTSTG3′

HB17 5′ggcggcggcggctccggtggtggtggatccCAGGTCCAACTVCAGCARCC3′

HB18 5′ggcggcggcggctccggtggtggtggatccGATGTGAACTTGGAAGTGTC3′

HB19 5′ggcggcggcggctccggtggtggtggatccGAGGTGAAGGTCATCGAGTC3′

Variable heavy chain forward primers

HF1 5′ggaattcggcccccgaggcCGAGGAAACGGTGACCGTGGT3′

HF2 5′ggaattcggcccccgaggcCGAGGAGACTGTGAGAGTGGT3′

HF3 5′ggaattcggcccccgaggcCGCAGAGACAGTGACCAGAGT3′

HF4 5′ggaattcggcccccgaggcCGAGGAGACGGTGACTGAGGT3′

2.4.2.2 *Components of PCR reaction for amplification of antibody light and heavy chain genes*

The concentration of stock primers varied. However, 0.1 nmol concentration of the forward and reverse primers were used per reaction.

Component	Working concentration
Forward Primers	0.1 nmol of each primer/reaction
Reverse Primers	0.1 nmol of each primer/reaction
10X Buffer	1 X
$MgCl_2$	1.0 mM
dNTP	0.4 mM
Ultrapure water	to 50 µl including *Taq* and DNA
cDNA	6-8 µg of cDNA/50 µl reaction
Taq polymerase	5 U/reaction

2.4.2.3 *PCR conditions for amplification of antibody light and heavy chain genes*

Typical thermal cycling conditions were as follows:
94°C X 5 min 'Hot-Start'
94°C X 1 min
63°C X 30 sec
58°C X 50 sec
72°C X 1 min
repeat X 7 cycles

94°C X 1 min
63°C X 30 sec
72°C X 1 min
repeat X 24 cycles
72°C X 10 min
All ramping rates were at 4°C/sec.

2.4.2.4 *Purification of PCR reaction products*

PCR purification was performed using the Wizard PCR prep DNA purification kit (Promega). An Atto AE-6100 gel electrophoresis system was used. The PCR product was run on a 1% (w/v) low melt agarose gel containing 0.5 µg/ml ethidium bromide. The 400 bp fragment was cut from the gel with a sterile scalpel and the DNA purified from the low melt agarose as follows. The sample was incubated at 70°C in a waterbath/heating block causing the agarose to melt within 5 minutes. 1 ml of resin was immediately added and mixed thoroughly by pipetting for 15 seconds. For each PCR preparation a plunger from a 5 ml syringe was attached to a Wizard Minicolumn and the resin was slowly

pushed through. The column was then washed with 2 ml of 80% (v/v) isopropanol. The column was centrifuged for 2 minutes at 14,000 rpm to remove any residual buffers. The minicolumn was placed in a new 1.5 ml eppendorf tube and 20 µl of autoclaved ultrapure water was applied to the minicolumn and allowed to incubate for 1 minute. The column was centrifuged at 14,000 rpm for 20 seconds to elute the DNA fragment and stored at –20°C until required.

2.4.2.5 *Quantification of PCR-purified products*

After purification the fragments were re-electrophoresed on an agarose gel with a quantitative Molecular Weight Marker (Promega, 100 bp Molecular Weight Markers), as the concentration must be determined prior to splice by overlap extension PCR (SOE-PCR). The 500 bp fragment of this marker was used as a reference for densiometric quantitation as 5 µl contains 150 ng of the 500 bp fragment and 50 ng of the other molecular weight fragments.

2.4.2.6 *Components of Splice by overlap extension (SOE) PCR*

Agarose gel-purified light and heavy chains were joined and amplified together to produce a 800 bp fragment by SOE PCR using components and PCR conditions outlined below.

SOE Primers

Single chain back 5′ttactcgcggcccagccggccatggcggactaccccg3′

Single chain forward 5′ggaattcggcccccgag3′

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

Component	**Working concentration**
10 X buffer	1 X
$MgCl_2$	1.0 mM
dNTP	0.4 mM
Ultrapure water	to 50 µl including *Taq*, DNA and scfor and scback
VH	10 ng/reaction
VL	10 ng/reaction
scfor	0.05 nmol /reaction
scback	0.05 nmol/reaction
Taq polymerase	5 U/reaction

SOE PCR buffer compositions: buffer A (7.5 mM $MgCl_2$, pH 8.5); buffer B (10 mM $MgCl_2$, pH 8.5); buffer C (12.5 mM $MgCl_2$, pH 8.5); buffer D (17.5 mM $MgCl_2$, pH 8.5); buffer E (7.5 mM $MgCl_2$, pH 9.0); buffer F (10 mM $MgCl_2$, pH 9.0); buffer G (12.5 mM $MgCl_2$, pH 9.0); buffer H (17.5 mM $MgCl_2$, pH 9.0); buffer I (7.5 mM $MgCl_2$, pH 9.5) and buffer J (10 mM $MgCl_2$, pH 9.5).

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

92°C X 1 min
45°C X 50 min
72°C X 1 min
repeat X 5 cycles
Add 0.05 mM scfor and scback primers:
5 U *Taq* Polymerase/rxn

92°C X 1 min
68°C X 30 sec
72°C X 1 min
repeat X 25 cycles

All ramping rates were at 4°C/sec.

2.4.2.7 *Preparation and purification of pAK100 vector using Wizard miniprep system (Promega).*

A single colony of *E.coli* XL1-Blue containing pAK100 (all vectors were kindly donated by Andreas Pluckthun, University of Zurich, Switzerland) was picked off an agarose plate and grown overnight at 37°C with vigorous shaking in 5 ml of 2 x TY supplemented with 30 μg/ml tetracycline. Purification of the plasmid was carried out using the Wizard miniprep system as described in section 2.3.5.3.

2.4.2.8. *Digestion of pAK100 and antibody light and heavy chain genes SOE PCR products*

Digestion of pAK100 and SOE products was performed using *SfiI* restriction enzyme. pAK100 requires digestion with *SfiI* restriction enzyme prior to ligation with light and heavy chain genes. Antibody light and heavy chain gene SOE products were also digested with *SfiI* as 4 bp are spliced off from either end. 2 U of *SfiI* enzyme was used to cut 30-50

ng/reaction SOE product or 50-100 ng/reaction pAK100 vector at 50°C overnight in a reaction volume of 10 µl.

2.4.2.9. *Ligation of antibody light and heavy chain genes into the pAK100 vector*

Agarose gel-purified SOE products were ligated into the pAK100 plasmid vector using a reaction mixture containing DNA ligase (Promega), reaction buffer, SOE V_L/V_H and pAK100 digests at a ratio of 1.5:1 and sterile ultrapure water to give a final volume of 10 µl. The reaction mixture was incubated at 15°C overnight and then transformed into *E.coli* XL1-Blue competent cells.

2.4.2.10 *Preparation of high efficiency competent cells*

High efficiency competent *E. coli* XL-1 Blue and JM83 cells were prepared as previously described in section 2.3.6.5

2.4.2.11. *Transformation of E.coli XL1-Blue supercompetent cells with pAK100 vector containing light and heavy chain genes and measurement of transformation efficiencies*

High efficiency competent *E. coli* XL1-Blue cells (prepared as described in section 2.4.2.10) were thawed gently on ice. Cells were gently mixed by hand and aliquoted into two 20 ml sterilin tubes (one tube was for the experimental transformation and the other tube was for the control transformation). 1.7 µl of β-mercaptoethanol was added to each of the two aliquots of bacteria, giving a final concentration of 25 mM in each tube. The contents of the tubes were stirred gently and incubated on ice for 10 min, swirling gently at 2 min intervals. 0.1-50 ng of vector, containing light and heavy chain genes was added to one of the tubes and swirled gently. 1 µl of pUC18 control vector was added to the second tube and also swirled. Both tubes were then incubated on ice for 30 min. Cells were heat-pulsed for 45 sec at 42°C followed by incubation on ice for 2 min. 0.9 ml of SOC medium, preincubated at 37°C, was then added and the tubes incubated at 37°C for 1hr with shaking at 225-250 rpm. pAK100 transformants were plated out on LB agar supplemented with 25 µg/ml chloramphenicol and 1% (v/v) glucose. Control transformants were plated out on LB agar supplemented with 20 µg/ml of ampicillin. Both sets of plates were allowed to grow overnight at 37°C. pAK100 transformed colonies were scraped off the plates and used as library stocks. These stocks were suspended in 15% (v/v) glycerol and stored at –80°C.

2.4.3 *Production of scFv antibodies to Listeria monocytogenes*

2.4.3.1 *Rescue of scFv displaying phage*

50 ml of non-expression (NE) medium (2xTY broth containing 1% (w/v) glucose and 25 μg/ml chloramphenicol) was inoculated with 100 μl of cells from a stock antibody library. The culture was shaken at 37°C until the $O.D._{550} = 0.5$, at which point it was incubated for 10 minutes at 37°C without shaking. 10^{11} cfu of helper phage (VCSM13) and 25 μl of 1M IPTG solutions were added to the culture, followed by incubation for 15 minutes at 37°C without shaking. The culture was then diluted in 100 ml of low-expression (LE) medium (NE medium containing 0.5 mM IPTG) and then shaken for 2 hours at 26°C to allow phage production. After 2 hours 30 μg/ml kanamycin was added to the culture and it was then incubated for a further 8 hours. Phage particles were purified and concentrated using PEG/NaCl precipitation and a phage titre carried out.

2.4.3.2 *PEG/NaCl Precipitation*

150 ml of the *E. coli* XL-1 blue/library was transferred to a sterile Sorvall 250 ml centrifuge tube and centrifuged at 10,000 rpm for 4°C for 10 minutes. Approximately 30 ml of PEG/NaCl (36.525g NaCl and 50g PEG 10,000 in 250 ml H_2O) was added to the supernatant, mixed and incubated at 4°C (on ice) for at least one hour. The mixture was then centrifuged at 10,000 rpm/4°C for 30 minutes. The pellet was re-suspended in 40 ml of sterile ultra pure water and 8 ml of PEG/NaCl, mixed and incubated at 4°C for 20 minutes. The mixture was then 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 a further 10 minutes. Any remaining supernatant was removed and the pellet re-suspended in 2 ml of sterile filtered PBS. The phage were then micro-centrifuged at 4,000 rpm for 4°C for 10 minutes to remove any bacterial debris. The phage precipitate was retained and stored at 4°C (short term) or at –80°C in a 15% (v/v) glycerol solution.

2.4.3.3 *Phage titre*

5 mls of 2xTY containing 30 μg/ml tetracycline was inoculated with a single colony of XL-1-blue overnight at 37°C. The culture was then diluted 1/100 in 5 mls of 2xTY containing 30 μg/ml tetracycline and left to grow shaking at 37°C until $O.D._{550}$ =0.5-0.6. It was then allowed stand at 37°C for 15 minutes without shaking. After this time serial dilutions (10^{-1}-10^{-11}) of phage were prepared using the bacterial culture (180 μl *E.coli* XL-

1 blue + 20 µl phage) and incubated for 30 minutes at 37°C without shaking. 100 µl of each dilution was spread on 2xTY agar plates containing 1% (w/v) glucose and 25 µg/ml chloramphenicol. The plates were allowed to grow overnight at 37°C. The colony forming units were counted in order to calculate the titre of the stock phage.

2.4.3.4 *Selection of antigen binders by bio-panning on Nunc immunotubes*

To allow selection, immunotubes were coated overnight at 4°C with Internalin B extract or whole heat-treated *Listeria monocytogenes* cells. Tubes were washed five times with PBS-Tween and five times with PBS. This was then blocked with 5% (w/v) milk marvel in PBS and incubated for 1 hour at 37°C. The tube was washed again as above and 1 ml of phagemid (10^{11} phage/ml) particles was added to 3 ml of PBS, which in total contained 2% (w/v) milk marvel. This was allowed to incubate, while gently shaking, in the immunotube for 2 hours at room temperature. After washing (as before) bound phage were eluted from the tube by adding 800 µl 0.1 M glycine/HCl, pH 2.2, for 10 minutes. This was then neutralised using 48 µl of 2 M Tris. A phage titre was then carried out as previously described in section 2.4.3.3, to determine the amount of phage recovered from the panning step. The titre plates were stored at 4°C for use in the phage ELISA.

2.4.3.5 *Re-infection of phage displaying scFv antibodies*

5 ml of 2xTY containing 30 µg/ml tetracycline was inoculated with 50 µl of XL-1 blue stock overnight at 37°C. The culture was then diluted 1/100 in 5 ml of 2xTY containing 30 µg/ml tetracycline and grown shaking until the $O.D._{550}$ =0.4-0.5. It was then allowed to stand for 10 minutes at 37°C. 700 µl of phage rescued from the panning step was added to the culture and allowed stand at 37°C for 30 minutes. This culture was then incubated shaking at 37°C for 1 hour before being plated out on 2xTY agar plates containing 25 µg/ml chloramphenicol and grown overnight at 37°C.

2.4.3.6 *ELISA of phage displaying scFv antibodies*

96 well culture plates were filled with 200 µl/well of NE medium containing 30 µg/ml tetracycline and 25 µg/ml chloramphenicol. Single clones from the phage titre plates were taken and put into each well, leaving 1 or 2 wells free as controls. These plates were allowed to grow, while shaking at 150 rpm overnight at 37°C. These were the master plates and were frozen with 15% (v/v) glycerol. A second set of plates were prepared with 180 µl/well of NE medium. 20 µl from each well on a master plate were transferred to the

corresponding well on the second plate and incubated shaking at 37°C for approximately 6-8 hours. 25 µl of solution X (5.75 ml 2xTY, with 1% (w/v) glucose, 25 µg/ml chloramphenicol, 1.5 mM IPTG and 5 x 10^9 phage/ml) was added to each well after 5 hours. The culture was then allowed to stand for 30 minutes at 37°C followed by shaking at 150 rpm for 1 hour. The plate was then centrifuged at 4000 rpm for 10 minutes. The supernatants were removed by inversion and the media was replaced with 200 µl of solution Y (20 ml 2xTY, 25 µg/ml chloramphenicol, 30 µg/ml kanamycin, 1% (w/v) glucose and 1.5 mM IPTG). The plate was then grown overnight shaking at 150 rpm at 26°C. An ELISA plate was coated with whole heat-killed *Listeria monocytogenes* cells overnight at 4°C. The following day the culture plates were centrifuged at 4000 rpm for 10 minutes and 75 µl of the supernatant was used for the phage ELISA. The coated ELISA plates were blocked using 5% (w/v) milk marvel in PBS. The plates were then washed 3 times with PBS and 75 µl of supernatant from the culture plates were added to the corresponding wells on the ELISA plates. 25 µl of 5% (w/v) milk marvel-PBS was then added in on top of each well and mixed gently. The phage were left to bind for 2 hours at room temperature and then washed with PBS-Tween. 100 µl/well of a 1/1000 dilution of anti-bacteriophage (Sigma rabbit antibody) polyclonal antibody was added and the plate was incubated for 1 hour at 37°C followed by the addition of anti-rabbit IgG-HRP (1/3000). This was again incubated for 1 hour at 37°C and OPD substrate was used. The plates were read at 450 nm after 30 minutes.

2.4.3.7 *Preparation of bacterial glycerol stocks*

Bacteria cells were grown in 2 xTY media containing the desired antibiotic at 37°C until the $O.D_{600}$ was 0.5-0.8. The cultures were then spun at 4,000 rpm for 10 minutes and the pellet resuspended in 2xTY with 20% (v/v) sterile glycerol to a volume of 1/4 the original culture volume. Cell/glycerol cultures were aliquoted into 0.5 ml aliquots in 1.5 ml sterile microcentrifuge tubes and flash frozen with liquid nitrogen. Cells stocks were stored at –80°C.

2.4.3.8 *Bioinformatics tools for analysis of variable domain genes*

Antibody heavy and light chain variable genes were analysed using the following web based programs found at the Expasy website on the following websites listed:

Expasy translate tool (http://www.expasy.org/)

SWISS Model (http://swissmodel.expasy.org/)

SWISS PDB viewer (http://www.expasy.org/)

ClustalW (http://www.ebi.ac.uk/clustalw/)

Genedoc (http://www.psc.edu/biomed/genedoc/)

Kabat rules for identifying antibody CDR regions from a linear amino acid sequence (http://www.rubic.rdg.ac.uk/abeng/cdrs.html)

The Protein Data Bank (PDB; http://www.rcsb.org/pdb/)

2.4.3.9 *Expression of soluble scFv antibodies from E. coli harbouring the pAK400 vector with scFv gene inserts*

E. coli JM83 cells harbouring the pAK400 vector with scFv gene inserts were grown in 2xTY media containing 25 μg/ml chloramphenicol at 37°C until the $O.D._{600}$ = 0.5-0.8. Expression of the scFv gene product was induced by the addition of 1 mM IPTG and incubated for a further 4-5 hours at 30°C with constant agitation. The cultures were later centrifuged at 4000 rpm for 15 minutes and supernatant analysed as described in section 2.4.3.10. The remaining cell pellets were resuspended in PBS to a volume of 1/10 the original culture volume and sonicated as described in section 2.3.7. The remaining cell debris and insoluble matter were pelleted by centrifugation at 14,000 rpm for 10 minutes and the supernatant analysed by ELISA as described in section 2.4.3.10.

2.4.3.10 *ELISA for the determination of soluble scFv*

A non-competitive ELISA was carried out with scFv antibody supernatants as described in section 2.2.3. In brief, dilutions of scFv antibody supernatants were added to HT-*Listeria monocytogenes* coated plates and detected using either a mouse anti-FLAG monoclonal antibody, a mouse anti-His tag antibody or a mouse anti-c-myc-HRP-labelled secondary antibody.

2.4.3.11 *Cross-reactivity studies of phage-scFv clones*

ELISA plates were coated overnight with $1x10^8$ cells/ml bacterial cells at 4°C and blocked with 5% (w/v) milk marvel for 1 hour at 37°C. Phage-scFv supernatant was added to each well and incubated for 1 hour at 37°C. After washing with three washes of PBS-Tween followed by three washes with PBS, bound phage-scFv antibodies were detected using a rabbit anti-bacteriophage fd antibody. Colour development occurred after the addition of a goat anti-rabbit-HRP conjugated antibody followed by the addition of OPD substrate. Absorbance values were read at 405 nm and background values subtracted.

2.4.4 *Bio-panning of a naïve human antibody library against HT-Listeria monocytogenes cells and invasion-associated proteins*

2.4.4.1 *Preparation of phage from library glycerol stocks*

An aliquot of a glycerol library stock was added to a 2 L flask, containing 500 ml of 2xTY supplemented with 2% (w/v) glucose and 100 μg/ml ampicillin (2xTYAG) and grown in a shaking incubator at 37°C at 200rpm for 1-2 hours or until $O.D._{600}$ = 0.5 to 1.0. M13K07 helper phage was then added to the culture to a final concentration of $5x10^9$ pfu/ml. After addition of helper phage, the cells were incubated at 37°C for 30 minutes without shaking and then for 30 minutes with moderate shaking (150 rpm) to allow infection. The culture was then centrifuged at 4000 rpm for 15 minutes and the cells resuspended in the same volume of 2xTY (no glucose) with kanamycin (50 mg/ml) and ampicillin (100 mg/ml) using a fresh 2 L flask. The culture was grown overnight with shaking (200 rpm) at 25°C.

2.4.4.2 *PEG/NaCl Precipitation*

The phage in the supernatant was concentrated by precipitation with polyethylene glycol as described in section 2.4.3.2.

2.4.4.3 *Selection of phagemid-antibody libraries by panning in immunotubes*

An immunotube was coated with 0.5 ml antigen ($1x10^8$ cells/ml *Listeria monocytogenes* or 10-15 μg/ml InlB extract) overnight at 4°C. After coating overnight, the immunotube was washed three times with PBS and blocked with 3% (w/v) skimmed milk powder in PBS for 1-2 hours at room temperature. Meanwhile a 20 ml culture of TG1 cells in 2xTY was setup to provide mid-log phase cells. For the first round of panning, phage concentrated by PEG precipitation was used. However, for subsequent rounds of panning supernatants were used without the need for PEG precipitation and concentration. For each round of panning, phage was pre-blocked in 3% (w/v) milk marvel powder in PBS to make a volume of 0.5 ml (Note: a few μl of input phage per round of panning were kept to determine the phage titre). Upon removal of the blocking solution from the immunotube, the tube was rinsed three times in PBS, 0.5 ml of pre-blocked phage added and incubated for 1.5 hours without agitation. After the 1.5 hour's incubation, the immunotube was washed ten times with PBST and ten times with PBS.

2.4.4.4 *Phage ELISA of phage displaying scFv antibodies*

A phage ELISA of phage displaying scFv antibodies was performed as described earlier in section 2.4.3.6.

2.4.4.5 *Inhibition ELISA of positive clones*

An inhibition ELISA was carried out as described in section 2.2.5 using a rabbit anti-bacteriophage Fd antibody for the detection of bound phage displaying scFv particles.

2.4.4.6 *Preparation of SDS-extracts of Listeria monocytogenes*

One millilitre of a $2x10^{10}$ cells/ml *Listeria monocytogenes* 4B solution was centrifuged at 8,000 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in 150 µl of a 2% (w/v) SDS solution and incubated for 1 hour at 37°C. After 1 hour, the cells were again centrifuged at 8,000 rpm for 10 minutes and the supernatant carefully decanted into a fresh micro-centrifuge tube. The pellet was discarded and the SDS extract (cell supernatant) stored at -20°C.

2.4.4.7 *SDS-PAGE and Western blot analysis of phage displaying scFv clones*

SDS-PAGE and Western blot analysis of phage displaying scFv clones were carried out as previously described in sections 2.2.6 and 2.2.7.

2.4.4.8 *ScFv gene sequence alignment and CDR region determination*

Sequence alignments and CDR region determinations were performed using the programs and web sites given in section 2.4.3.8.

2.4.4.9 *Expression of soluble scFv antibodies*

1 ml from an overnight culture of *E. coli* TG1 cells, containing the desired scFv gene sequences, was used to inoculate 50 ml of 2xTY media containing 100 µg/ml ampicillin. The cultures were grown at 37°C with shaking until the $O.D._{600}$ reached 0.5-0.8 at which time soluble antibody expression was induced with 1 mM IPTG for 4-5 hours at 30°C. After 4-5 hours both cell supernatant and cytoplasmic extract were analysed for antibody production by ELISA.

2.4.4.10 *Purification of recombinant InlB and p60 proteins*

Both recombinant proteins were purified by immobilised metal affinity chromatography as described in section 2.3.9.

2.4.4.11 *Preparation of bacterial cytoplasmic extracts*

Expression of scFv protein was induced as described in section 2.4.4.9. After 4-5 hours of expression, the cells were centrifuged at 4000 rpm for 20 minutes and the supernatant decanted into a fresh tube and stored for further analysis. The pellet was resuspended in PBS (native cytoplasmic extract) or denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-Cl, pH 8.0) to a volume of 1/10 the original culture volume. The resuspended pellets were then sonicated (section 2.3.7) and the bacterial debris removed by centrifugation at 14,000 rpm for 10 minutes. Cytoplasmic extracts (cell debris supernatant) were decanted into a fresh tube and stored at –20°C.

2.4.4.12 *Time course study for the expression scFv antibodies*

E. coli TG1 cells containing the desired scFv gene sequences were grown in 2xTY (200 ml with 100 µg/ml ampicillin) until the $O.D._{600}$ was between 0.5 and 0.8. When the cultures reached the desired O.D., a 1 ml sample was taken from each culture and stored immediately at –20°C. The remaining culture was induced with IPTG at a final concentration of 1 mM and scFv antibodies expressed at 30°C. 1 ml aliquots were taken from the culture every hour and stored at –20°C. Stored aliquots were later centrifuged at 14,000 rpm for 10 minutes and the supernatant decanted into a fresh tube. Cytoplasmic extracts were prepared from the sample pellet as described in section 2.4.4.11. Supernatants and cytoplasmic extracts were later analysed by ELISA or SDS-PAGE.

2.4.4.13 *Preparation of Tris-Cl extracts from bacterial cells*

Tris-Cl extracts were prepared from bacterial cells as described by Müller *et al.* (1998), and as outlined in section 2.2.1.2.

2.4.4.14 *Inter- and Intraday assay reproducibility studies*

An inhibition ELISA was performed as described in section 2.2.5. Briefly, ELISA plates were coated with 20 µg/ml purified recombinant InlB protein and blocked with 5% (w/v)

dried milk powder. Clone G7 scFv supernatant, diluted 1/4 in PBS, was added to doubling dilutions of InlB extract diluted in PBST and incubated for 1 hour at 37°C. Antibody and InlB extract were added to recombinant InlB-coated ELISA wells (in triplicate) and incubated for a further hour at 37°C. After washing with PBS and PBST, bound scFv antibodies were detected with a mouse monoclonal anti-c-myc-HRP-labelled antibody and colour development observed upon the addition of OPD substrate. Results were plotted with a 4 parameter equation using BIAevaluation software and the plot accuracy observed by substituting the actual absorbance values for each standard into the equation of the line and expressing the observed cell concentrations as a percentage of the actual cell concentrations. The coefficients of variation (CV's) were determined to assess the precision of the analytical method, expressing the standard deviation as a percent function of the mean.

2.4.4.15 *Cross reactivity of scFv antibody clone G7 against Tris-Cl extracts from various bacterial cells*

Tris-Cl extracts from each of the bacterial strains to be tested were produced as described in section 2.4.4.13 and an inhibition ELISA performed as described in section 2.4.4.14 except that dilutions of InlB extracts were replaced with dilutions of Tris-Cl extracts from each bacterial strain tested. The decrease in binding observed for the recombinant InlB-coated wells, at a equivalent cell concentration of $1x10^7$ cells/ml from each bacterial strain was recorded. The observed binding was expressed as a percentage of the decrease in binding observed at the same cell concentration (concentration at half the maximum inhibition observed for InlB extract) for that of the *Listeria monocytogenes* InlB extract.

2.5 *Development of SPR-based immunoassay using a BIAcore 3000™ instrument*

Analysis was carried out on a BIAcore 3000™ instrument using a range of available sensor chips. The running buffer for all BIAcore experiments was HBS buffer, pH 7.4, containing 10 mM HEPES, 150 mM NaCl, 3.4 M EDTA, and 0.05% (v/v) Tween 20. The running buffer was filtered (pore size of 0.22 μm) and degassed using a millipore filtration apparatus (millipore sintered glass filtration unit) immediately before use.

2.5.1 *Preconcentration studies*

In order to optimise the conditions needed for the immobilisation of desired proteins on the sensor chip surface, an initial "preconcentration" step, resulting from electrostatic binding of protonated amine groups on the biological component to negatively-charged carboxyl groups on the chip surface, was performed. Preconcentration of a desired protein on the sensor surface be can be facilitated by adjusting the pH below the isolectric point (pI) of the protein. Therefore, protein solutions were prepared in 10 mM sodium acetate at a range of different pHs and these solutions passed over an underivatised chip surface, with the degree of electrostatic binding monitored. The pH at which highest preconcentration of protein on to the underivatised surface was observed, was chosen as the pH for immobilisation.

2.5.2 *General immobilisation conditions*

The carboxymethylated dextran matrix was activated by mixing equal volumes of 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride), and injecting the mixture over the sensor chip surface for 7 minutes at a flowrate of 5 μl/minute. The desired protein to be immobilised was diluted in 10 mM sodium acetate at the optimised pH, and at a typical concentration of 50-100 μg/ml. This solution was then injected over the derivatised chip surface for 30-45 minutes at 5 μl/minute. Unreacted NHS groups were capped, and non-covalently bound protein removed by injection of 1 M ethanolamine hydrochloride, pH 8.5, for 7 min.

2.5.3 *Regeneration studies*

To assess the stability of immobilised interactant (antibody, peptide or peptide conjugates) surfaces, a known concentration of antibody was passed over the chip surface, and the surface regenerated by passing over various concentrations of NaOH and HCl ranging from 1-30 mM. This cycle of 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 the development of a sensitive and reproducible assay.

2.5.4 *Subtractive inhibition assay for the detection of Listeria monocytogenes cells using an anti-Listeria monocytogenes polyclonal antibody*

Approximately 20,000 RUs of commercial goat anti-rabbit (Fab portion) polyclonal antibody (section 2.1.1) was immobilised on a CM5 sensor chip surface. 300 µl of a 1/250 dilution of protein-G purified anti-*Listeria monocytogenes* whole cell polyclonal antibody solution diluted in ultra pure water (Millipore) was added to 300 µl of decreasing numbers *Listeria monocytogenes* cells diluted in PBS. Each mixture was incubated for 20 minutes at 37°C, inverting the mixture occasionally to allow good mixing of cells and antibodies. After incubation, the cells were centrifuged in a stepwise fashion separating cells and bound antibody from unbound antibody in the supernatant. Gradually increasing the centrifugation speed seemed less abrasive on the antibodies bound to cells, forming pelleted layers in which the antibodies would not dissociate from the cells. Centrifugation at higher speeds seemed to shear the antibodies from the cells resulting in no inhibition being observed. The supernatants from each sample were then randomly passed over the sensor surface in triplicate and an average value taken.

2.5.5 *Inhibition assay for the detection of Listeria monocytogenes cells using an anti-InlB extract polyclonal antibody*

Approximately 10,000 response units (RU) of purified recombinant InlB protein (section 2.3.9) was immobilised on the surface of a CM5 sensor chip as described in section 2.5.2. 250 µl of a 1/30 dilution of an anti-InlB extract polyclonal (described in chapter 3) diluted in Millipore ultra pure water was added to 250 µl of various concentrations of HT-*Listeria monocytogenes* cells and incubated for 1 hour at 37°C. After 1 hour, the antibody-cell solutions were randomly passed over the recombinant InlB surface in four replicates with the value obtained from the first replicate discarded (see section 6.3.3). A decrease in binding response was observed with increasing cell concentrations and the response at each concentration recorded.

2.5.6 *Inhibition assay for the indirect detection of Listeria monocytogenes cells using an anti-InlB protein scFv antibody*

Approximately 1,100 response units (RU) of purified recombinant InlB protein (section 2.3.9) was immobilised on the surface of a C1 sensor chip as described in section 2.5.2 except the surface was doubly activated with two 7 minute injections of EDC/NHS and "over capped" with a 7 minute injection of 1M ethylene-diamine, pH 8.5. 250 µl of a 1/5 dilution of an anti-InlB protein scFv antibody supernatant (described in section 5.5.0) diluted in PBS was added to 250 µl of various concentrations of *Listeria monocytogenes* Tris-Cl extracts and incubated for 1 hour at 37°C. After 1 hour, the antibody-protein solutions were randomly passed over the recombinant InlB surface in triplicate. A decrease in binding response was observed with increasing extract concentrations and the response at each concentration recorded.

Chapter 3

Characterisation of polyclonal antibodies against *Listeria monocytogenes*

3.1 *Introduction*

With the increased demand on production, in not only the food but clinical and pharmaceutical industries, the need for a rapid, reliable, specific and sensitive method of detecting a target analyte is of key significance. Conventional methods for the detection and identification of bacteria mainly rely on specific microbiological and biochemical identification. While these methods can be sensitive, inexpensive and give both qualitative and quantitative information on the number and the nature of the microorganisms tested, they are greatly restricted by assay time. For example, with some standard methods, such as, the NF EN ISO 11290-1 method for the detection of *Listeria monocytogenes,* up to seven days is required to generate results, as such tests rely on the ability of microorganisms to multiply to visible colonies (De Boer and Beumer, 1999; Artault *et al.*, 2001). Immunological detection with antibodies is perhaps the only technology that has been successfully employed for the detection of cells, spores, viruses and toxins alike (Iqbal *et al.,* 2000). Polyclonal antibodies can be raised quickly and cheaply and do not require the time or expertise associated with the production of monoclonal or recombinant antibodies (Stapleton *et al.,* 2003). During the past decade, immunological methods for detection of microbial contamination have become more sensitive, specific, reproducible and robust with many commercial immunoassays available.

Listeria monocytogenes is regarded as a very important food borne pathogen, due to its ability to grow at refrigeration temperatures, in high salt concentrations and at a wide pH range. *Listeria monocytogenes* has been isolated from a variety of foodstuffs ranging from an array of dairy products, meat, fish and ready-to-eat-foods stored at 4°C. Rapid detection of *Listeria* in ready-to-eat-foods such as cheese, coleslaw, and chocolate milk, is of utmost importance, as these foodstuffs are not usually heated before consumption.

The aim of this chapter was to develop an immunoassay to detect *Listeria monocytogenes* in food. Two polyclonal antibodies were produced, one raised against heat-treated *Listeria monocytogenes* cells and the other against *Listeria monocytogenes* Internalin B (InlB)-protein extracts. These antibodies were crudely purified by saturated ammonium sulphate precipitation followed by affinity purification by protein-G affinity chromatography. Purified antibodies were characterised by ELISA, SDS-PAGE and Western blotting. Both purified antibodies were used in the development of immunoassays to detect *Listeria monocytogenes* cells in an inhibition ELISA format. Intra- and interday studies were performed with both immunoassays to determine the accuracy and precision of the assays. Interday calibration curves were constructed by plotting the mean (3 replicates) change in

response for each *Listeria monocytogenes* cell standard against the known concentration of *Listeria monocytogenes* cells over a five separate assay runs. A four parameter equation was fitted to each calibration curve and the percentage accuracies calculated to evaluate if the curves provided an accurate representation of the sigmoidal relationship between the measured response and the logarithm of concentration observed for the immunoassay.

The feasibility of detecting *Listeria monocytogenes* cells in chocolate milk, a food matrix which has been reported to have been the cause of a well known *Listeria monocytogenes*-associated food poisoning outbreak (Dalton *et al.*, 1997), was also examined. An inhibition ELISA was developed with each antibody for the detection of *Listeria monocytogenes* in spiked chocolate milk samples. Intra- and interday assay variability studies were performed to evaluate the accuracy and reproducibility of each assay.

To determine the potential cross reactivity of each antibody, inhibition ELISAs were performed with a number of known bacterial strains. Two strategies for calculating the percentage cross reactivity values were compared. Both antibodies showed tendencies to cross react with the other bacteria tested from the genus *Listeria* but showed little or no cross reactivity with *B. subtilis* or *E. coli*. It was concluded that both antibodies can be used as valuable tools for the genus-specific detection of *Listeria* cells, but were severely limited for the species-specific detection of *Listeria monocytogenes* cells.

3.2 *Production and characterisation of polyclonal antibodies to heat-treated (HT) Listeria monocytogenes cells and the invasion-associated protein internalin B (InlB)*

The production, purification and characterisation of two polyclonal antibodies raised against *Listeria monocytogenes* 4b cells and Internalin B extract is described in the following sections. When the rabbit antibody titre had reached a sufficiently high level (Figures 3.1 and 3.2), the blood was harvested by cardiac puncture and the rabbit serum purified by saturated ammonium sulphate precipitation followed by protein G affinity chromatography (section 3.2.1). SDS-PAGE gel electrophoresis (section 3.2.2) was used to assess the purity of the antibodies. The optimal coating concentration of *Listeria monocytogenes* cells and optimal working dilution of the each antibody for use in an inhibition assay (section 3.2.3) were calculated by performing a checkerboard ELISA of different antibody dilutions and cell concentrations as described in section 3.2.4.

3.2.1. *Purification of polyclonal antiserum*

Initial purification of the rabbit serum was performed by saturated ammonium sulphate precipitation, as described in section 2.2.2.1. Protein G affinity chromatography (section 2.2.2.2) was then carried out to further purify the antibodies. Protein G is a cell wall protein (originally isolated from type G Streptococci) that binds the Fc region of immunoglobulin G (IgG) with high affinity. In addition to the Fc receptor, intact Protein G has membrane spanning regions as well as specific binding sites for albumin and for the Fab region of immunoglobins (Bjorck and Kronvall, 1984; Akerstrom *et al.*, 1985 and Akerstrom and Bjorck, 1986). The protein G resin used in this section (Sigma product code P-3296) is prepared from a truncated recombinant protein G produced by Pharmacia, Inc., that retains the two high affinity Fc binding sites and also binds the Fab region with low affinity (Sigma product information sheet). Partially purified saturated ammonium sulphate precipitated-antibody was passed through the column allowing IgG to bind to protein G. After extensive washing of the column to remove non-specifically bound proteins, the antibodies were eluted with glycine buffer, pH 2.2, and neutralised using 2 M Tris base as described in section 2.2.2.2. Fractions were analysed spectrophotometrically at 280 nm and the fractions with the highest absorbance pooled. Pooled fractions were then dialysed in PBS buffer overnight and the titre of protein G-purified fractions determined by ELISA.

3.2.2 *Characterisation of purified antibody by SDS-PAGE and Western blotting*

The purity of the anti-*L. monocytogenes* cells and anti-InlB extract antibodies were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blotting using a polyclonal anti-rabbit AP-labelled antibody as described in section 2.2.6. SDS-PAGE allows the separation of proteins according to their size. Proteins can be subsequently transferred to a nitrocellulose membrane (Western blotting) and probed with a specific antibody, allowing the specific identification of the sample protein. A picture of the SDS-PAGE gels and Western blots of the affinity-purified antibodies with molecular weight bands at 50 kDa and 25 kDa representing the antibody heavy and light chains, respectively, are presented in Figure 3.3 and 3.4.

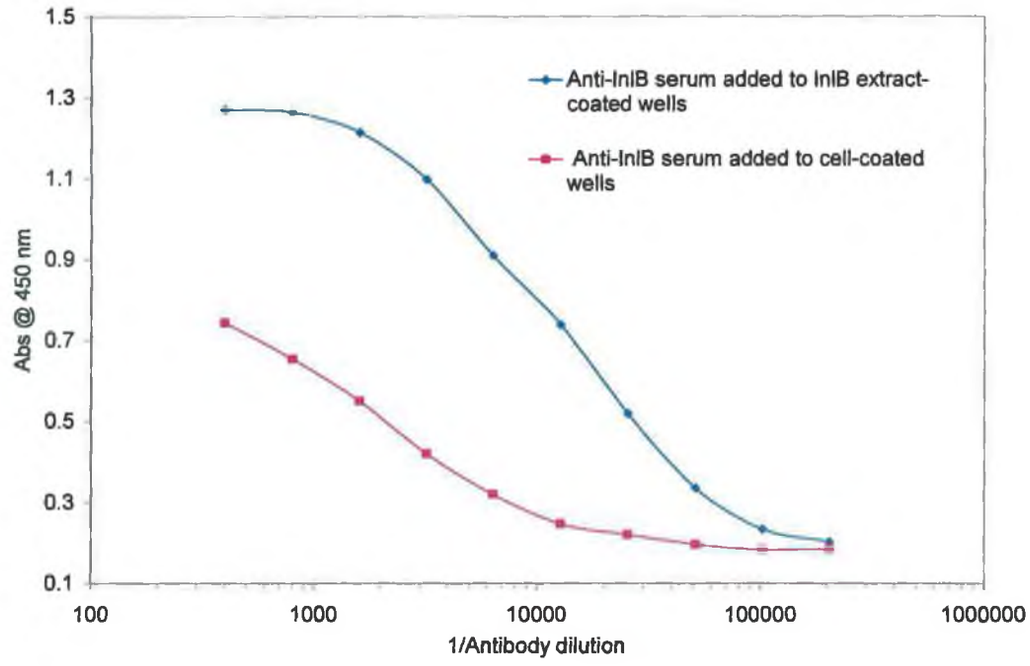


Figure 3.1: *A plot showing the titre of the anti-InlB extract polyclonal serum diluted in PBS, added to ELISA plate wells coated with 15 µg/ml InlB extract and 1 x 10^8 cells/ml heat-killed Listeria monocytogenes 4b cells. Serial dilutions of serum was added to the wells and detected with an anti-rabbit HRP-conjugated antibody. A significant antibody titre of over 1/100,000 serum dilution was observed against InlB extract, while a lower titre of approximately 1/25,000 –1/50,000 was observed against whole cell-coated surfaces.*

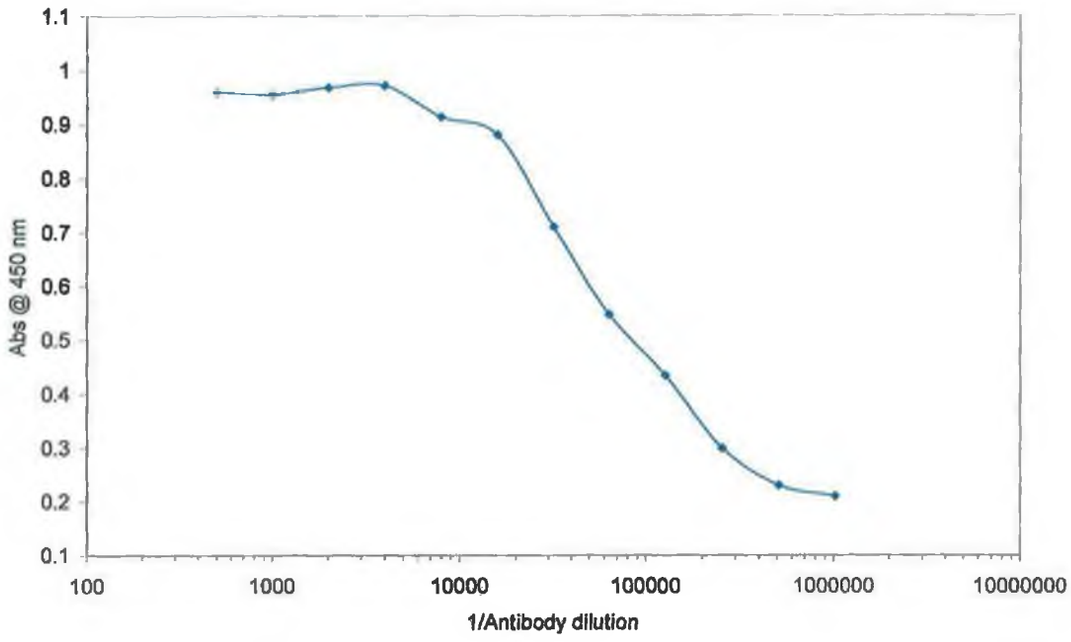


Figure 3.2 *A plot showing the titre of the anti-Listeria monocytogenes 4b serum added to wells coated with 1 x 10^8 cells/ml heat-killed Listeria monocytogenes 4b cells. Serial dilutions of serum were added to the wells and detected with an anti-rabbit HRP-conjugated antibody. A significant antibody titre of over 1/1,000,000 serum dilution was observed against whole Listeria monocytogenes cell-coated plate wells.*

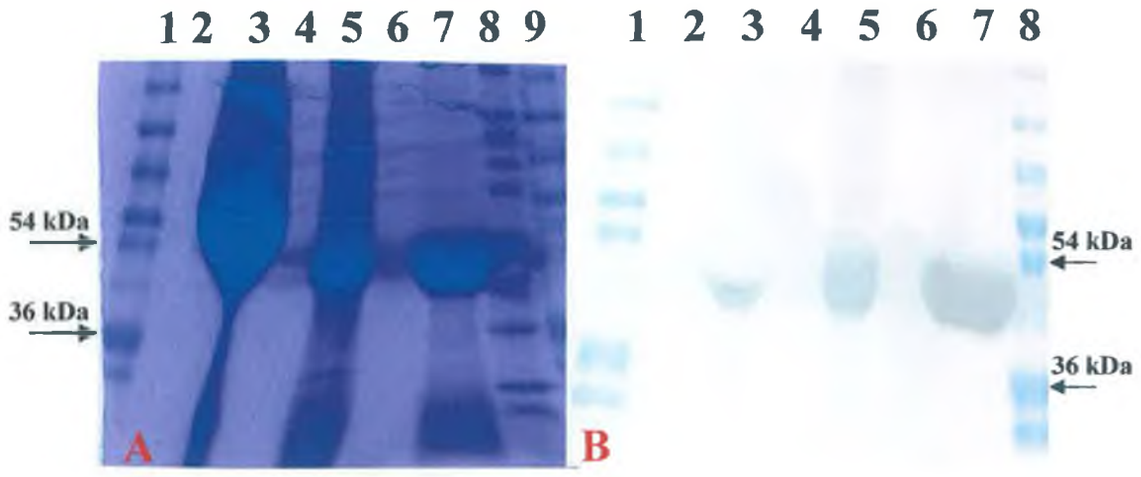


Figure 3.3: *(A) SDS-PAGE gel of the protein G sepharose affinity-purified anti-InlB extract polyclonal antibody. Lanes 1 and 9 contain pre-stained markers and lane 8 contains a wide range marker. Lanes 2, 4 and 6 are blank lanes containing no protein. Lanes 3 contains a 1/5 dilution of serum with PBS, lane 5 contains rabbit serum purified by saturated ammonium sulphate precipitation while lane 7 represents affinity purified saturated ammonium sulphate (SAS)-precipitated antibody on a protein G sepharose column. (B) Western blot of serum, SAS-precipitated antibody and protein G sepharose-purified antibody. Lanes 1 and 8 contain a pre-stained protein marker (Sigma) while lanes 2, 4 and 6 contain no protein (blank lanes). Lanes 3, 5 and 7 contain serum (1/5 dilution in PBS), SAS-precipitated antibody and protein-G sepharose purified-antibody, respectively. IgG heavy chain bands were detected using an anti-rabbit AP-conjugated antibody as described in section 2.2.7.*

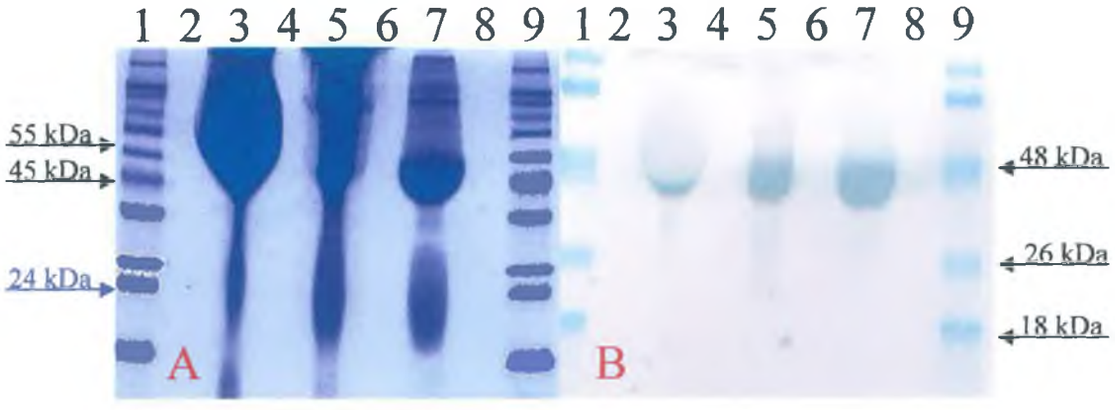


Figure 3.4: *(A) SDS-PAGE gel of the protein G sepharose affinity-purified anti-Listeria monocytogenes polyclonal antibody. Lanes 1 and 9 contain wide range markers. Lanes 2, 4 , 6 and 8 are blank lanes containing no protein. Lanes 3 contains a 1/5 dilution of serum with PBS, lane 5 contains rabbit serum purified by saturated ammonium sulphate-precipitation followed by lane 7 representing affinity-purified saturated ammonium sulphate (SAS)-precipitated antibody on a protein G sepharose column. (B) Western blot of serum, SAS-precipitated antibody and protein G sepharose-purified antibody. Lanes 1 and 8 contain a Pierce pre-stained protein marker while lanes 2, 4, 6 and 8 contain no protein (blank lanes). Lanes 3, 5 and 7 contain serum (1/5 dilution in PBS), SAS-precipitated antibody and protein G sepharose-purified antibody, respectively. IgG heavy chain bands were detected using an anti-rabbit AP-conjugated antibody.*

3.2.3 *Development of an inhibition enzyme-linked immunosorbent assay (ELISA) for the detection of heat treated-Listeria monocytogenes 4b in phosphate buffered saline (PBS)*

Inhibition ELISA's were developed for the detection of free *Listeria monocytogenes* cells in solution using both the affinity-purified anti-InlB extract antibodies and the affinity-purified anti-HT *Listeria monocytogenes* antibodies.

3.2.4 *Checkerboard ELISA for determination of optimal loading ratios of cell coating and optimal antibody dilution for the anti-InlB extract polyclonal antibody*

In an inhibition ELISA format, the degree of sensitivity in terms of detection limits, is a function of the antibody affinity (or range of affinities as is the case with polyclonal antibodies), and the equilibrium between both free and immobilised antigen. Consequently, utilising too high a coating concentration of antigen will shift the binding equilibrium in favour of binding to the ELISA plate and cause reduced sensitivity to free antigen in solution. Similarly, utilising too high an antibody concentration, will require high concentrations free antigen in solution to inhibit antibody binding to the ELISA plate, and therefore result in decreased assay sensitivity.

In order to determine the optimal working dilution of the purified antibody and optimal cell coating concentration, a checkerboard ELISA was performed as described in section 2.2.4. Varying dilutions of a 2×10^{10} cells/ml *Listeria monocytogenes* stock were coated on a microtitre plate and dilutions of affinity-purified antibody added to each cell dilution. The results were plotted in Figures 3.5 and 3.6. The optimal coating concentration of *Listeria monocytogenes* cells was approximately a 1/200 dilution representing 1×10^{8} cells/ml of whole cells. The optimal anti-InlB extract antibody dilution, which gave the greatest change in absorbance per change in antibody dilution, was determined to be 1/300, while the optimal anti-*Listeria monocytogenes* antibody dilution determined to be approximately 1/12,500.

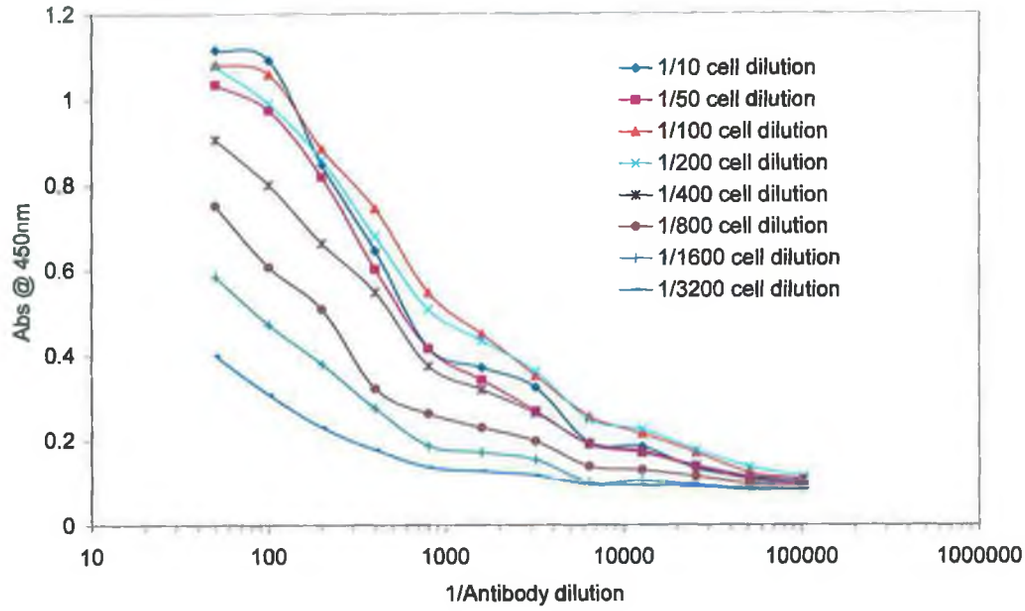


Figure 3.5: *Checkerboard ELISA for the determination of the optimal cell coating concentration and antibody dilution for the development of an inhibition assay with the anti-InlB polyclonal antibody. Dilutions of the anti-InlB polyclonal antibody were added to the wells of an immunoplate containing varying concentrations of immobilised Listeria monocytogenes cells, as described in section 2.2.4.*

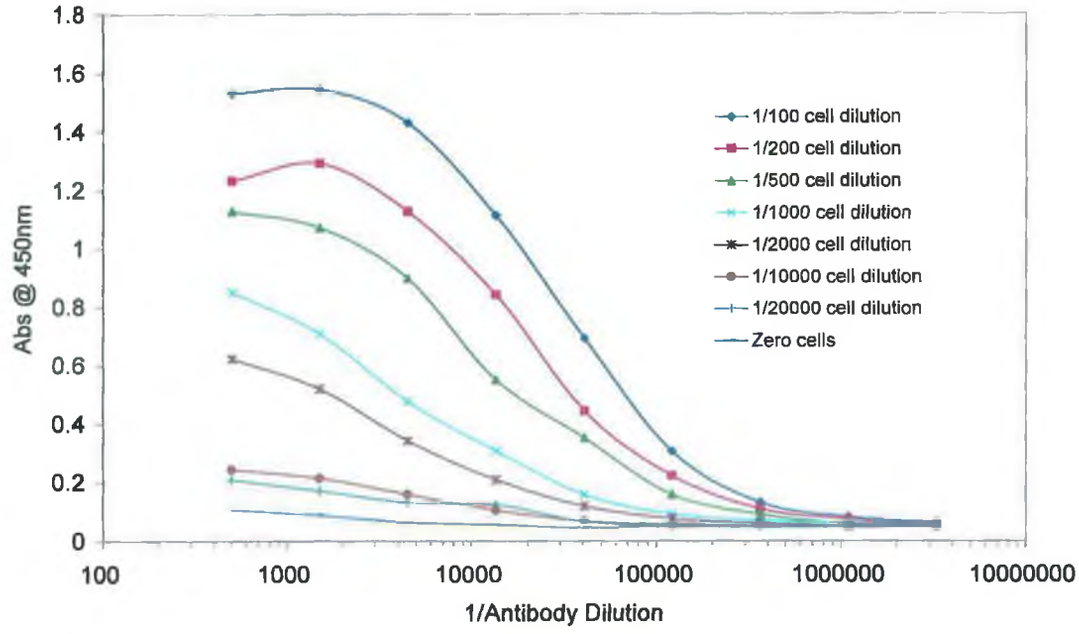


Figure 3.6: *Checkerboard ELISA for the determination of the optimal cell coating concentration and antibody dilution for the development of an inhibition assay the anti-L. monocytogenes polyclonal antibody. Dilutions of antibody were added to the wells of an immunoplate containing varying concentrations of immobilised Listeria monocytogenes cells, as described in section 2.2.4.*

3.2.5 *Intra- and interday inhibition assay variability studies of each polyclonal antibody in PBS*

An inhibition ELISA was developed with each antibody as described in section 2.3.5, for the detection of *Listeria monocytogenes* 4b in solution. A microtitre plate was coated with 1 x 10^8 cells/ml heat-killed *Listeria monocytogenes* cells and blocked with 5% (w/v) milk marvel. *Listeria monocytogenes* cell standards were prepared, mixed with antibody diluted in PBST containing 1% (w/v) milk marvel and incubated at 37°C for 30 minutes. The number of standards used in assays developed with both antibodies, varied in order to obtain the maximum number of assayed data points (*Listeria monocytogenes* cell concentrations) within the range of detection of each assay. Twenty two standards ranging from 1 x 10^{10} to 2 x 10^6 cells/ml were used with the anti-InlB antibody while 19 standards ranging from 1 x 10^{10} to 3.8 x 10^4 cells/ml were used with the anti-*Listeria monocytogenes* antibody. The cell-antibody mixtures were then added to the microtitre plates, incubated and detected with an anti-rabbit HRP antibody after washing. The observed results were plotted using BIAevaluation software and a four parameter equation fitted to the data. The four parameter function provides an accurate representation of the sigmoidal relationship between the measured response and the logarithm of concentration observed for many immunoassays (Findlay *et al.*, 2000).

In order to measure the accuracy of the immunoassay, intra-day assay variability studies were performed. Five sets of standards were prepared and incubated with antibody before being assayed. The results obtained for the intra-day assay studies for both the anti-InlB extract antibody and the anti-*Listeria monocytogenes* antibody are shown in Tables 3.1 and 3.2, respectively. The coefficients of variation (CV's) were determined to assess the precision of the analytical method, expressing standard deviation as a percent function of the mean. These values ranged from 0.65 to 9.83% and 0.85 to 5.32% as observed with the anti-InlB extract and anti-*Listeria monocytogenes* antibodies, respectively, indicating that both assays had a high degree of precision.

To measure the intermediate precision (precision of repeated measurements, taking into account sources of variation such as runs, days, reagents etc.), inter-day assay variability studies were performed with each antibody. Three sets of standards were prepared and incubated with antibody before being assayed five times over five days. The inter-day assay study for the anti-InlB extract antibody, shown in Figure 3.7, had a limit of detection of approximately 7 x 10^6 cells/ml (concentration at below which no significant decrease in binding could be observed). The coefficients of variation were determined and ranged

from 1.37 to 15.70% as shown in Table 3.1, where it can be seen that there was a much greater degree of variance with very high concentrations of cells in solution when compared to lower concentrations. The use of high concentrations of *Listeria monocytogenes* can sometimes lead to the formation of cell aggregates resulting in increased inaccuracies, which are not evident at low cell concentrations. A graphical representation of the relationship between the coefficient of variance and the nominal concentration of *Listeria monocytogenes* cells is presented in Figure 3.8. From the graph, it can be seen that CV's of less than 10% were observed with all cell concentrations less than 8 $x10^8$ cells/ml. A general increase in precision is observed with decreasing cell concentrations, with only a slight increase in percentage CV's observed between 7.76 x 10^7 and 3.45 x 10^7 cells/ml probably due to insufficient sample mixing. The percentage accuracies of the curve (Figure 3.7) were good (Table 3.1) especially in the linear range of the assay, which indicates that the curve provides an accurate representation of the sigmoidal relationship between the measured response and the logarithm of concentration observed for the immunoassay.

The interday assay study for the anti-*Listeria monocytogenes* antibody, shown in Figure 3.9, had a lower limit of detection of approximately 1 x 10^6 cells/ml. The coefficients of variation (CV's) were determined and ranged from 2.44 to 10.52% as shown in Table 3.2. A graphical representation of the relationship between the coefficient of variance and the nominal concentration of *Listeria monocytogenes* cells is presented in Figure 3.10. Unlike that observed with the anti-InlB Extract antibody (Figure 3.8) no pattern of increased precision with decreasing cell concentrations was observed. The calculated percentage coefficient of variation values obtained varied throughout the *Listeria monocytogenes* cell range studied. However, all the % CV values obtained were well within the 20% limits suggested by Findlay *et al.* (2000). The percentage accuracies of the curve (Figure 3.9) were good in the linear range of the assay, while large inaccuracies were observed with values found in the non-linear parts of the curve (Table 3.1).

	Intraday			*Interday*		
Concentration (cells/ml)*	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**
1.00×10^{10}	6.88×10^{9}	7.03	131.15	-	14.28	-
6.67×10^{9}	7.63×10^{9}	4.03	85.64	5.29×10^{9}	15.71	120.64
4.45×10^{9}	3.92×10^{9}	4.87	111.86	4.20×10^{9}	15.07	105.54
2.97×10^{9}	3.45×10^{9}	9.49	83.81	2.57×10^{9}	13.86	113.52
1.98×10^{9}	2.11×10^{9}	9.83	93.59	1.85×10^{9}	11.71	106.68
1.32×10^{9}	1.17×10^{9}	4.13	111.13	1.54×10^{9}	10.55	83.17
8.81×10^{8}	1.11×10^{8}	1.96	74.46	7.41×10^{8}	9.75	115.81
5.87×10^{8}	6.49×10^{8}	2.45	89.49	6.49×10^{8}	9.27	89.57
3.92×10^{8}	3.79×10^{8}	5.92	103.28	3.74×10^{8}	5.45	104.46
2.61×10^{8}	2.75×10^{8}	2.00	94.85	2.65×10^{8}	4.76	98.54
1.74×10^{8}	1.72×10^{8}	1.13	101.56	1.67×10^{8}	3.55	103.91
1.16×10^{8}	9.56×10^{7}	7.30	117.79	1.19×10^{8}	5.00	97.41
7.76×10^{7}	8.16×10^{7}	1.34	94.72	9.21×10^{7}	6.87	81.26
5.17×10^{7}	4.60×10^{7}	0.72	111.03	4.88×10^{7}	4.32	105.70
3.45×10^{7}	3.65×10^{7}	1.93	94.18	3.37×10^{7}	5.17	102.30
2.30×10^{7}	2.25×10^{7}	5.19	102.17	2.09×10^{7}	3.54	109.16
1.53×10^{7}	1.64×10^{7}	0.65	93.13	1.54×10^{7}	3.57	99.47
1.02×10^{7}	1.15×10^{7}	2.60	87.19	1.06×10^{7}	2.68	96.50
6.82×10^{6}	7.15×10^{6}	3.96	95.28	5.50×10^{6}	2.10	119.49
4.55×10^{6}	4.55×10^{6}	5.42	100.10	4.39×10^{6}	1.37	103.58
3.03×10^{6}	2.27×10^{6}	4.62	125.12	3.77×10^{6}	1.79	76.04
2.02×10^{6}	2.31×10^{6}	1.43	86.01	1.89×10^{6}	1.44	106.77

Table 3.1: *Intra- and interday assay coefficients of variation (CV's) and percentage accuracies for the anti-InlB extract polyclonal antibody in PBS. Intra- and interday studies were performed as described in section 2.3.5 and 3.2.5. Coefficients of variance (a quantitative measure of precision) was calculated using the equation % CV = (S.D./Mean) x 100 where for intraday studies, the standard deviation (S.D.) is computed from replicate (5 replicates) analyses within a single validation run and for intermediate precision (interday), the S.D. is computed from replicate (3 replicates) analyses over 5 validation runs on 5 separate days. Cells containing a dash indicate that no value could be obtained as the data point did not fit the calibration curve.*

** The cell numbers presented represent the calculated cell concentrations by performing serial cell dilutions from a 1 x 10^{10} cells/ml Listeria monocytogenes cell stock. 2/3 dilutions were performed and the cell numbers calculated to 2 decimal places.*

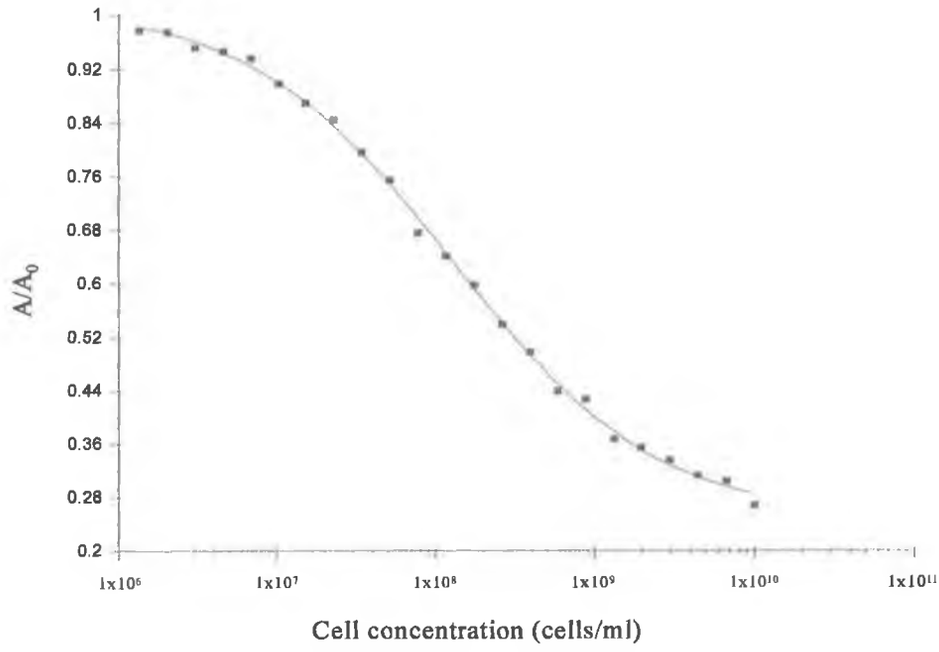


Figure 3.7: *Interday assay calibration curve for the anti-InlB extract antibody in PBS. A 4-parameter equation was fitted to the data set using BIAevaluation 3.1 software. The inter-assay means and coefficients of variance are tabulated in Table 3.1. Each point on the curve is the mean of five replicate measurements analysed over five days.*

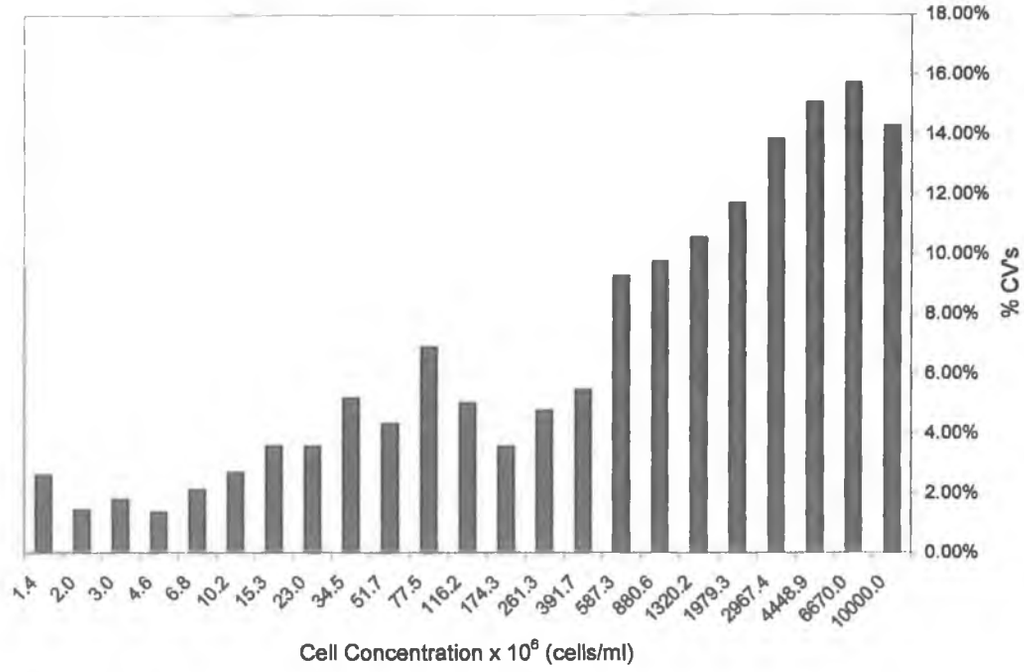


Figure 3.8: *Precision profile for the interday inhibition ELISA assay using the anti-InlB extract polyclonal antibody. This figure denotes a graphical representation of the relationship between the coefficient of variation (CV) in analytical measurements and the concentrations of cells in each sample.*

	Intraday			*Interday*		
Concentration (cells/ml)*	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**
1.00×10^{10}	3.17×10^{9}	1.30	168.30	4.87×10^{9}	5.20	151.27
5.00×10^{9}	3.66×10^{9}	1.02	126.81	5.15×10^{9}	6.26	97.03
2.50×10^{9}	2.34×10^{9}	3.36	106.48	2.05×10^{9}	10.52	118.15
1.25×10^{9}	1.45×10^{9}	1.20	83.66	1.22×10^{9}	9.56	102.19
6.25×10^{8}	8.37×10^{8}	3.92	66.01	6.08×10^{8}	9.02	102.72
3.13×10^{8}	3.80×10^{8}	4.10	78.29	3.50×10^{8}	3.46	87.94
1.56×10^{8}	1.80×10^{8}	4.13	84.86	1.69×10^{8}	7.96	91.84
7.81×10^{7}	7.52×10^{7}	3.70	103.80	8.47×10^{7}	8.98	91.63
3.91×10^{7}	3.42×10^{7}	3.38	112.55	3.73×10^{7}	9.44	104.49
1.95×10^{7}	1.47×10^{7}	4.63	124.76	1.80×10^{7}	2.97	107.74
9.77×10^{6}	9.59×10^{6}	1.70	101.79	8.79×10^{6}	2.50	110.03
4.88×10^{6}	6.67×10^{6}	3.98	63.36	5.03×10^{6}	2.44	96.99
2.44×10^{6}	3.99×10^{6}	2.50	36.65	3.49×10^{6}	3.95	56.90
1.22×10^{6}	1.19×10^{6}	2.83	102.40	1.31×10^{6}	5.47	92.70
6.10×10^{5}	5.28×10^{5}	4.58	113.47	6.23×10^{5}	3.47	97.87
3.05×10^{5}	5.66×10^{5}	5.32	14.62	1.18×10^{5}	5.01	161.17
1.53×10^{5}	-	0.85	-	2.34×10^{5}	5.71	46.88
7.63×10^{4}	1.53×10^{4}	2.80	179.95	2.08×10^{4}	5.06	172.78
3.81×10^{4}	-	3.41	-	-	6.18	-

Table 3.2: *Intra- and interday assay coefficients of variation (CV's) and percentage accuracies for the anti-Listeria monocytogenes polyclonal antibody in PBS. Intra- and interday studies were performed as described in section 2.3.5 and 3.2.5. Coefficients of variance (a quantitative measure of precision) was calculated using the equation % CV = (S.D./Mean) x 100 where for intraday studies, the standard deviation (S.D.) is computed from replicate (5 replicates) analyses within a single validation run and for intermediate precision (interday), the S.D. is computed from replicate (3 replicates) analyses over 5 validation runs on 5 separate days. Cells containing a dash indicate that no value could be obtained as the data point did not fit the calibration curve.*

** The cell numbers presented represent the calculated cell concentrations by performing serial cell dilutions from a 1 x 10^{10} cells/ml Listeria monocytogenes cell stock. Serial doubling dilutions were performed and the cell numbers calculated to 2 decimal places.*

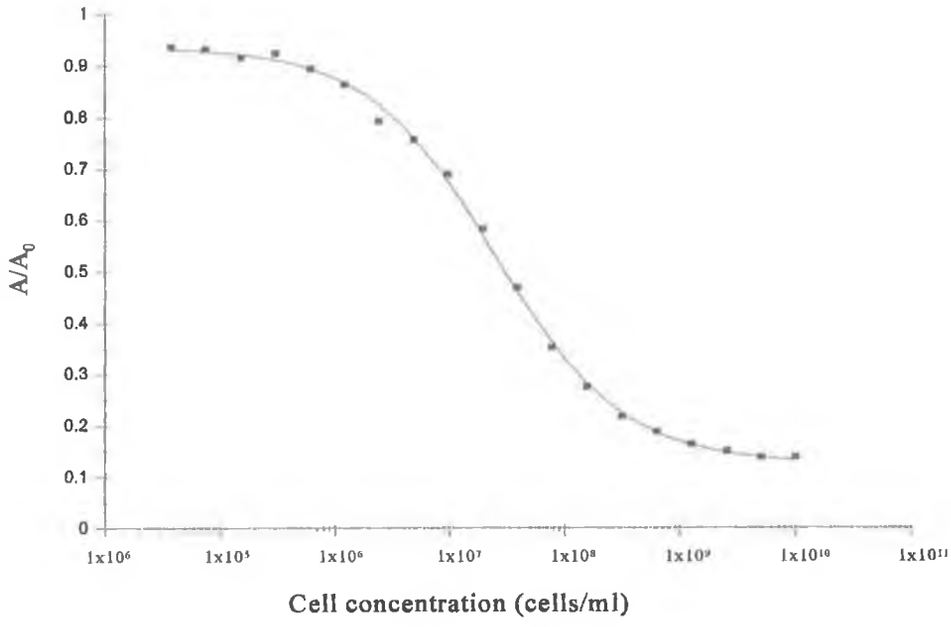


Figure 3.9: *Interday assay calibration curve for the anti-Listeria monocytogenes antibody in PBS. A 4-parameter equation was fitted to the data set using BIAevaluation 3.1 software. The inter-assay means and coefficients of variance are tabulated in Table 3.2. Each point on the curve is the mean of five replicate measurements analysed over five days.*

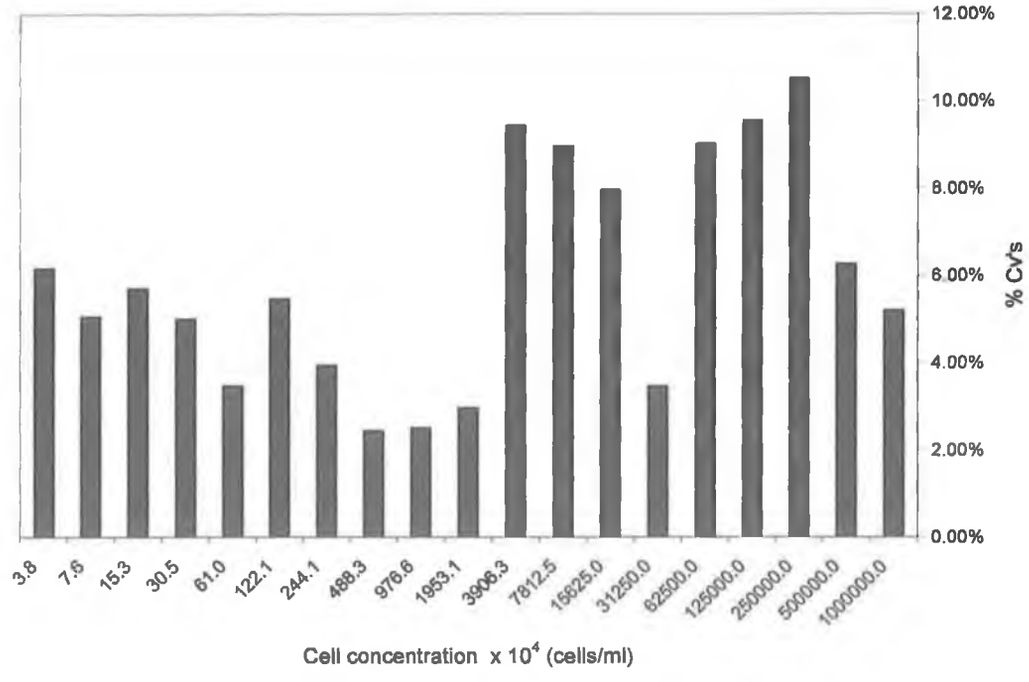


Figure 3.10: *Precision profile for the interday inhibition ELISA assay using the anti-Listeria monocytogenes polyclonal antibody in PBS. This Figure denotes a graphical representation of the relationship between the coefficient of variation (CV) in analytical measurements and the concentrations of cells in each sample.*

3.2.6 *Intra- and interday inhibition assay variability studies of each polyclonal antibody in chocolate milk*

As discussed in section 3.1, an outbreak of gastroenteritis and fever among people at a picnic, in Illinois, on July 9th 1994, was later traced to chocolate milk consumed at the picnic (Dalton *et al.* 1997). To determine the feasibility of detecting *Listeria monocytogenes* in a food matrix such as chocolate milk, widely available Yazoo™ chocolate milk (Campina UK, Denne Road, Harsham, RH12 1JF) was purchased, aliquoted into 10 ml aliquots and stored at -20°C. For each assay, an aliquot was thawed and spiked with known cell concentrations of heat-killed *Listeria monocytogenes* cells.

An inhibition ELISA was developed with each antibody as described in sections 2.3.5 and 3.2.5, for the detection of *Listeria monocytogenes* 4b in chocolate milk. In order to determine the accuracy of the immunoassay, intra- and interday assay variability studies were performed as discussed in section 3.2.5. The results obtained for the intra- and interday assay studies for both the anti-InlB extract antibody and the anti-*Listeria monocytogenes* antibody are shown in Tables 3.3 and 3.4, respectively. The coefficients of variation (CV's) were determined to assess the precision of the analytical method. These values ranged from 0.78 to 5.81% and 1.75 to 12.70% as observed with the anti-InlB antibody, for the intra- and interday studies, respectively, indicating that the assay had a good degree of precision. Both intra- and interday precision were increased when the assay was carried out in chocolate milk compared to PBS with highest %CV values of 5.81 and 12.70% (Table 3.3 and Figure 3.12), respectively, compared to 9.83 and 15.70% observed in PBS. Also, the sensitivity of the assay increased in chocolate milk, with a limit of detection of approximately 4-5 x 10^6 cells/ml (Figure 3.11) compared to 7 x 10^6 cells/ml in PBS. The coefficients of variance values obtained with the anti-*Listeria monocytogenes* antibody ranged from 1.30 to 5.72% and 1.41 to 8.69% (Table 3.4) for the intra- and interday studies, respectively, which are comparable to those values observed in PBS (Table 3.2).

	Intraday			*Interday*		
Concentration (cells/ml)	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**
1.00×10^{10}	8.16×10^{9}	1.67	118.37	7.77×10^{9}	12.70	122.34
6.67×10^{9}	6.88×10^{9}	3.06	96.86	7.04×10^{9}	12.58	94.40
4.45×10^{9}	4.24×10^{9}	4.96	104.61	6.02×10^{9}	10.53	64.62
2.97×10^{9}	2.82×10^{9}	5.30	104.81	2.99×10^{9}	8.78	99.31
1.98×10^{9}	2.68×10^{9}	2.55	64.83	1.79×10^{9}	8.72	109.53
1.32×10^{9}	1.17×10^{9}	4.10	111.29	1.40×10^{9}	8.49	94.19
8.81×10^{8}	9.50×10^{8}	3.09	92.07	8.29×10^{8}	9.71	105.87
5.87×10^{8}	6.69×10^{8}	2.42	86.07	5.54×10^{8}	6.63	105.62
3.92×10^{8}	4.15×10^{8}	4.46	94.10	3.95×10^{8}	6.47	99.29
2.61×10^{8}	2.22×10^{8}	5.73	114.98	2.56×10^{8}	8.33	101.91
1.74×10^{8}	1.72×10^{8}	2.29	101.32	1.81×10^{8}	5.08	96.23
1.16×10^{8}	9.72×10^{7}	2.03	116.39	1.37×10^{8}	8.63	82.16
7.76×10^{7}	1.00×10^{8}	5.79	70.42	7.72×10^{7}	4.19	100.39
5.17×10^{7}	4.84×10^{7}	0.78	106.46	4.97×10^{7}	8.70	103.95
3.45×10^{7}	2.79×10^{7}	5.81	119.22	2.64×10^{7}	6.07	123.47
2.30×10^{7}	2.71×10^{7}	2.63	82.33	2.24×10^{7}	5.69	102.80
1.53×10^{7}	1.68×10^{7}	1.52	90.76	1.83×10^{7}	4.81	80.47
1.02×10^{7}	9.27×10^{6}	1.89	109.43	1.03×10^{7}	3.35	99.68
6.82×10^{6}	6.13×10^{6}	4.40	110.28	8.28×10^{6}	1.75	78.65
4.55×10^{6}	5.82×10^{6}	1.53	72.20	4.03×10^{6}	2.49	111.61
3.03×10^{6}	3.01×10^{6}	2.65	100.82	3.20×10^{6}	3.89	94.81
2.02×10^{6}	1.82×10^{6}	3.50	110.18	2.03×10^{6}	3.16	99.74

Table 3.3: *Intra- and interday assay coefficients of variation (CV's) and percentage accuracies for the anti-InlB extract polyclonal antibody in chocolate milk. Intra- and interday studies were performed as described in section 2.3.5 and 3.2.5. Coefficients of variance (a quantitative measure of precision) was calculated using the equation % CV = (S.D./Mean) x 100 where for intraday studies, the standard deviation (S.D.) is computed from replicate (5 replicates) analyses within a single validation run and for intermediate precision (interday), the S.D. is computed from replicate (3 replicates) analyses over 5 validation runs on 5 separate days.*

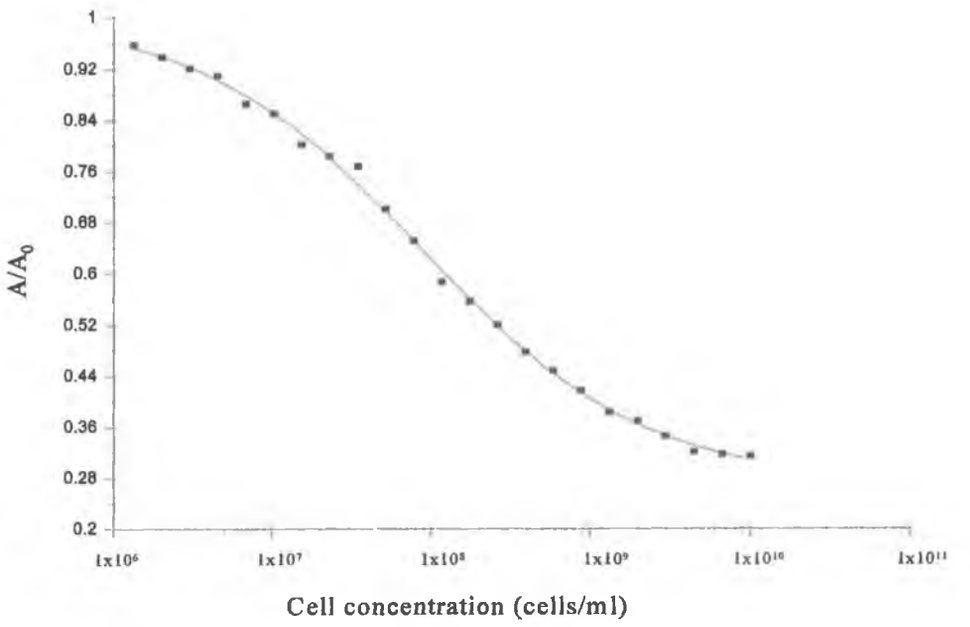


Figure 3.11: *Interday assay calibration curve for the anti-InlB extract antibody in chocolate milk. A 4-parameter equation was fitted to the data set using BIAevaluation 3.1 software. The inter-assay means and coefficients of variance are tabulated in Table 3.3. Each point on the curve is the mean of five replicate measurements analysed over five days.*

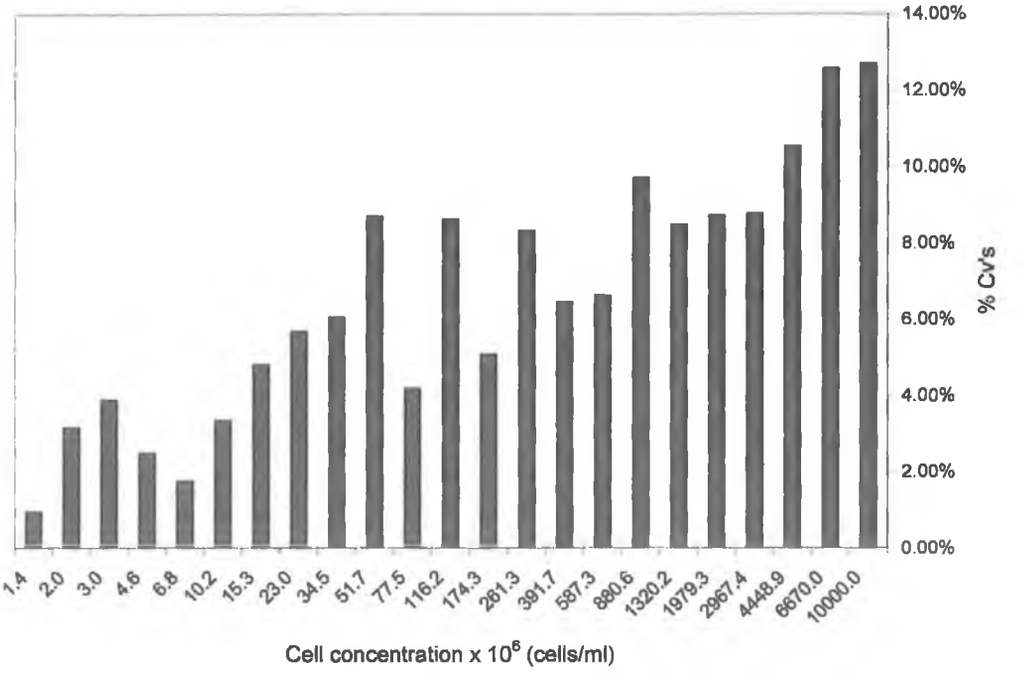


Figure 3.12: *Precision profile for the inter-day inhibition ELISA assay using the anti-InlB extract polyclonal antibody in chocolate milk. This figure denotes a graphical representation of the relationship between the coefficient of variation (CV) in analytical measurements and the concentrations of cells in each sample.*

	Intraday			*Interday*		
Concentration (cells/ml)	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**
1.00×10^{10}	6.37×10^{9}	3.76	136.34	5.77×10^{9}	8.16	142.26
5.00×10^{9}	5.20×10^{9}	3.16	96.07	5.27×10^{9}	7.51	94.65
2.50×10^{9}	2.50×10^{9}	3.68	99.87	2.51×10^{9}	7.66	99.59
1.25×10^{9}	1.54×10^{9}	3.36	76.93	1.32×10^{9}	7.28	94.20
6.25×10^{8}	7.02×10^{8}	3.97	87.71	6.66×10^{8}	6.48	93.43
3.13×10^{8}	2.87×10^{8}	2.87	108.28	3.04×10^{8}	6.78	102.56
1.56×10^{8}	1.69×10^{8}	3.81	91.99	1.56×10^{8}	8.69	100.28
7.81×10^{7}	6.54×10^{7}	3.20	116.31	7.87×10^{7}	4.65	99.21
3.91×10^{7}	3.99×10^{7}	5.72	97.91	3.96×10^{7}	5.06	98.60
1.95×10^{7}	2.35×10^{7}	3.80	79.84	1.97×10^{7}	3.60	99.30
9.77×10^{6}	8.19×10^{6}	2.83	116.17	8.60×10^{6}	4.18	111.97
4.88×10^{6}	5.74×10^{6}	3.45	82.52	5.14×10^{6}	4.72	94.69
2.44×10^{6}	2.38×10^{6}	3.25	102.54	3.20×10^{6}	4.96	68.88
1.22×10^{6}	6.78×10^{5}	4.37	144.46	1.29×10^{6}	4.04	94.72
6.10×10^{5}	6.04×10^{5}	1.30	101.09	4.01×10^{5}	3.79	134.28
3.05×10^{5}	5.18×10^{5}	5.11	30.31	4.72×10^{5}	3.57	45.23
1.53×10^{5}	2.29×10^{5}	3.11	49.98	4.04×10^{4}	5.57	173.51
7.63×10^{4}	6.94×10^{4}	2.30	199.09	9.47×10^{4}	3.01	75.90
3.81×10^{4}	-	1.41	-	-	1.41	-

Table 3.4: *Intra- and interday assay coefficients of variation (CV's) and percentage accuracies for the anti-Listeria monocytogenes polyclonal antibody in chocolate milk. Intra- and interday studies were performed as described in section 2.3.5 and 3.2.5. Coefficients of variance (a quantitative measure of precision) was calculated using the equation % CV = (S.D./Mean) x 100 where for intraday studies, the standard deviation (S.D.) is computed from replicate (5 replicates) analyses within a single validation run and for intermediate precision (interday), the S.D. is computed from replicate (3 replicates) analyses over 5 validation runs on 5 separate days. Cells containing a dash indicate that no value could be obtained as the data point did not fit the calibration curve.*

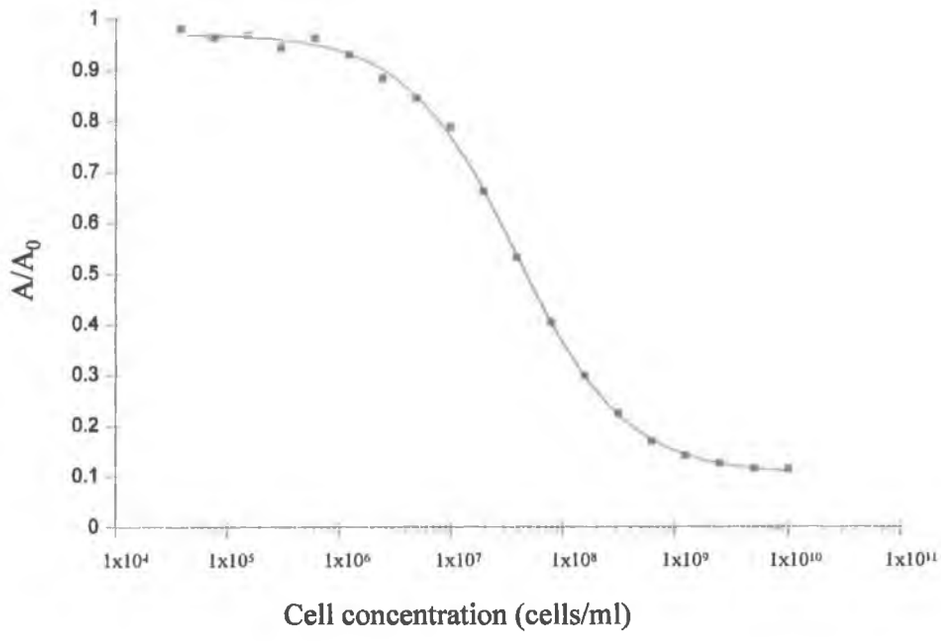


Figure 3.13: *Interday assay calibration curve for the anti-Listeria monocytogenes antibody in chocolate milk. A 4-parameter equation was fitted to the data set using BIAevaluation 3.1 software. The inter-assay means and coefficients of variance are tabulated in Table 3.4. Each point on the curve is the mean of five replicate measurements analysed over five days.*

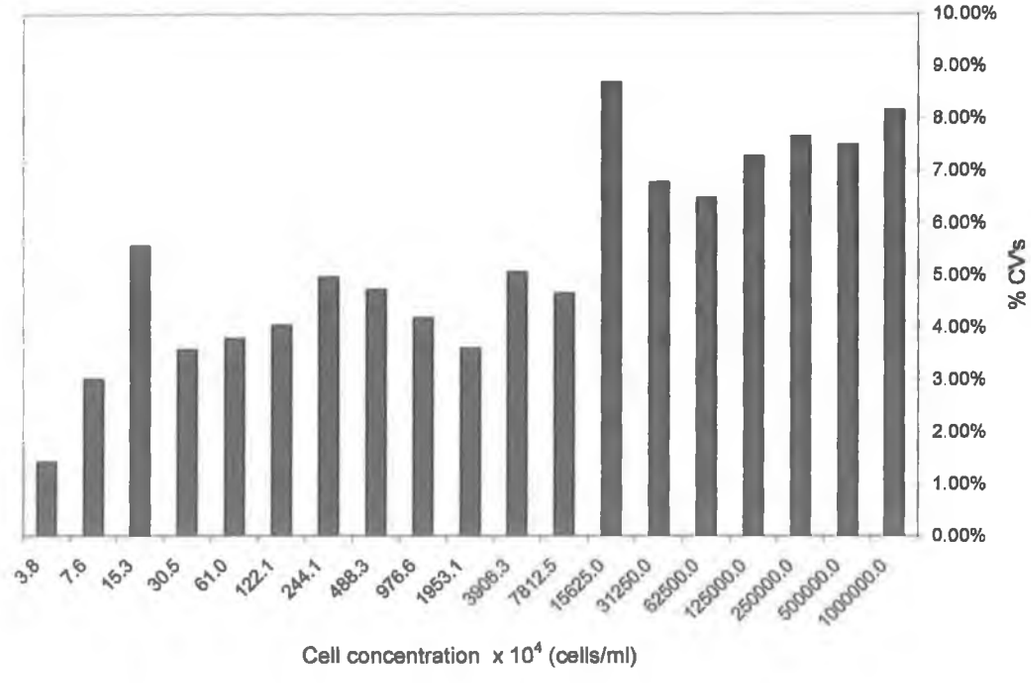


Figure 3.14: *Precision profile for the inter-day inhibition ELISA assay using the anti-Listeria monocytogenes polyclonal antibody in chocolate milk. This Figure denotes a graphical representation of the relationship between the coefficient of variation (CV) in analytical measurements and the concentrations of cells in each sample.*

3.2.7 *Cross reactivity studies*

Cross reactivity may be defined as a measure of the antibody response to structurally related molecules, as a result of shared epitopes. A specific immunoassay should have the ability to measure the antigen unequivocally in the presence of other components, either exogenous or endogenous (Findlay, *et al.*, 2000). The degree of cross reactivity of polyclonal antibodies can be assessed by performing the assay (described in section 2.3.5) with serial dilutions of competing antigen in parallel with similar dilutions of specific antigen. The cross-reactivity of an immunoassay is often conveniently defined as the point where the reduction in signal recorded in the presence of a particular analyte concentration (C_x) gives a 50% reduction in the signal in the presence of zero analyte (C_0) (i.e. C_x/C_0=50%). Therefore, the antigen concentration value at 50% inhibition (EC_{50}) can be expressed as a percentage of the cross reactant concentration (Figure 3.15) giving the same decrease in signal where the percentage cross reactivity will be defined as:

$$\% \text{ cross reactivity} = \frac{\text{Concentration of analyte giving 50\% decrease in signal}}{\text{Concentration of cross reactant giving 50\% decrease in signal}} \times 100$$

However, the percentage cross reactivity can also be expressed as a ratio of the inhibition observed with the antigen at the EC_{50} concentration to the inhibition observed with the cross reactant at the same concentration, whereby the percentage cross reactivity will be defined as (Figure 3.16):

$$\% \text{ cross reactivity} = \frac{\text{inhibition value of cross reactant at } EC_{50} \text{ concentration}}{\text{inhibition value of antigen at } EC_{50} \text{ concentration}} \times 100$$

Table 3.5 contains the percentage cross reactivity values obtained by both the above methods against various bacterial strains. Each antibody was incubated with decreasing concentrations of intact bacterial cross reactants and added to ELISA plate wells coated with intact *Listeria monocytogenes* cells. A striking difference is observed between the two methods with much lower calculated cross reactivities obtained when using ratio of concentrations rather than ratio of inhibitions. Figures 3.17 and 3.18 show the actual cross reactivity plots obtained with both antibodies against the various bacterial strains. From analysing the plots, it can be deduced that both antibodies cross react to various degrees with different bacteria tested from the genus *Listeria* and thus using the ratio of the inhibitions observed is probably a more accurate method of determining the percentage cross reactivity of both immunoassays.

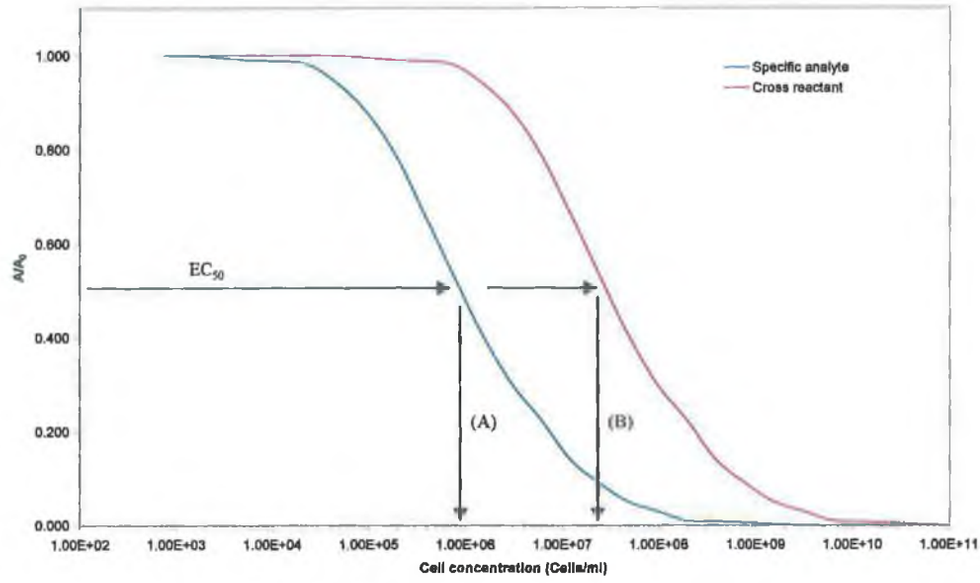


Figure 3.15: *Typical plot showing the potential cross reactivity of a particular antibody preparation towards a structurally related compound using the ratio of concentrations of each reagent that gives 50% inhibition (EC_{50}) as discussed in section 3.2.7. The % cross reactivity is calculated by expressing the antigen concentration (A) as percentage of the cross reactant concentration (B).*

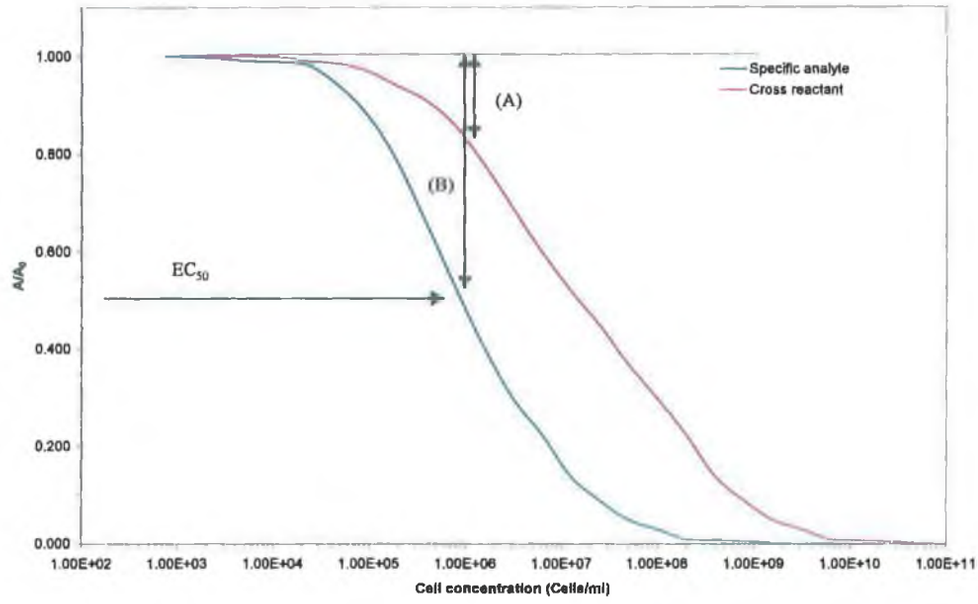


Figure 3.16: *Typical plot showing the potential cross reactivity of a particular antibody preparation towards a structurally related compound using the ratio of inhibition values of each reagent at the concentration of specific analyte that gives 50% inhibition (EC_{50}) as discussed in section 3.2.7. The % cross reactivity is calculated by expressing the cross reactant inhibition value (A) as percentage of the antigen inhibition value (B).*

	Percentage cross reactivity with anti-InlB polyclonal antibody (%)		Percentage cross reactivity with anti-L. monocytogenes polyclonal antibody (%)	
Bacterial strain	**Ratio of inhibitions**	**Ratio of concentrations**	**Ratio of inhibitions**	**Ratio of concentrations**
L. monocytogenes	100	100	100	100
Listeria innocua 6a	36.7	6.3	14.6	-
Listeria innocua 6b	55.8	4.5	39.3	1.0
Listeria welshimeri	63.8	25.0	61.7	6.7
Listeria ivanovii	36.0	3.5	19.0	-
Listeria seeligeri	51.8	10.5	29.8	-
Bacillus subtilis	11.2	-	8.3	-
Escherichia coli	6.7	-	Neg.	-

Table 3.5: *Cross reactivity of the affinity purified rabbit anti-InlB extract and anti-Listeria monocytogenes polyclonal antibodies with various bacterial strains. Percentage cross reactivity values were calculated using both the ratio of concentrations (shown in Figure 3.15) and ratio of inhibition values (shown in Figure 3.16) as discussed in section 3.2.7. From the results tabulated, there is a large difference between both methods in defining the % cross reactivity with the ratio of inhibition values method generating much larger % cross reactivity values. Cells containing a dash indicate that the cross reactant did not cross react with the antibody (i.e. did not give a decrease in inhibition of 50% over the range of concentrations tested). The actual overlay plots for each antibody are shown in Figures 3.17 and 3.18.*

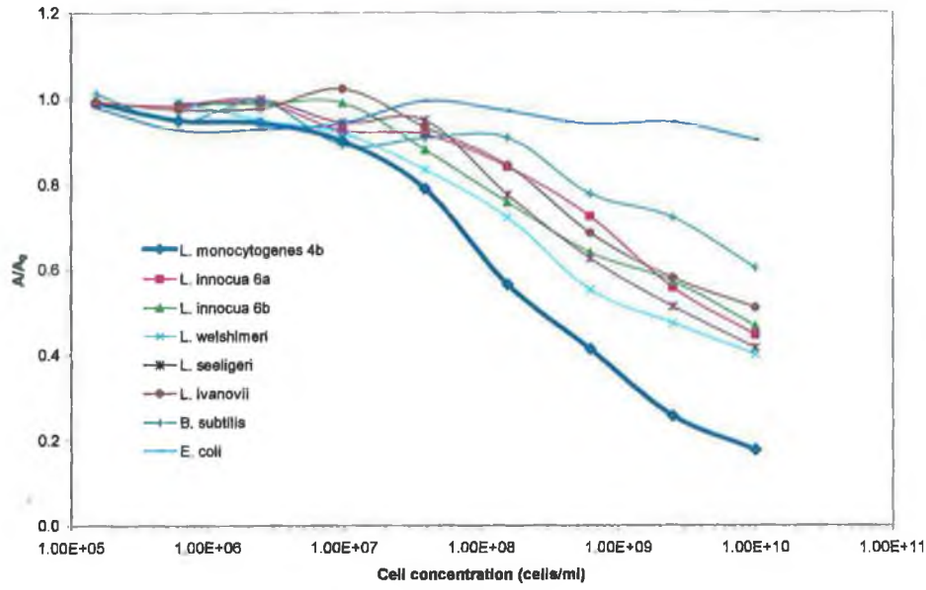


Figure 3.17: *Overlay plot showing the cross reactivity of the anti-InlB Extract polyclonal antibody with various bacterial strains using the inhibition ELISA format described in section 2.2.5. Antibody was mixed with decreasing concentrations of intact bacterial cells and added to ELISA wells coated with intact Listeria monocytogenes 4b cells in triplicate.*

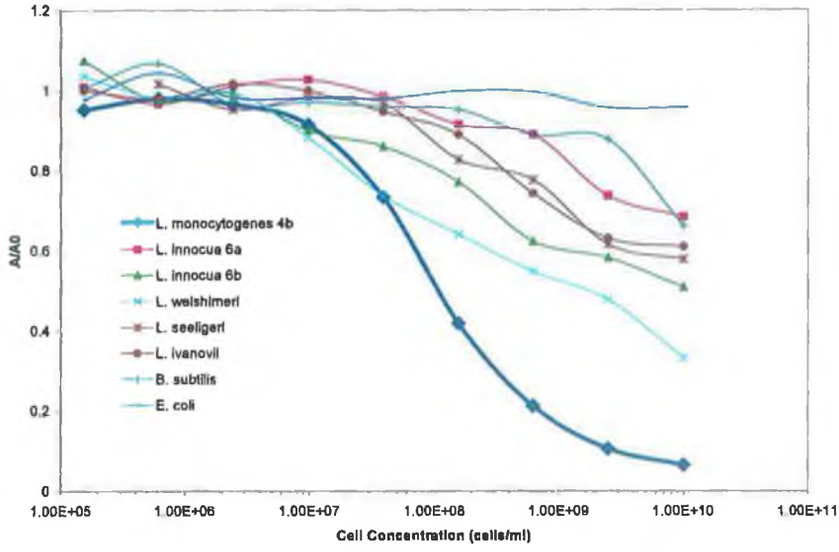


Figure 3.18: *Overlay plot showing the cross reactivity of the anti-Listeria monocytogenes polyclonal antibody with various bacterial strains using the inhibition ELISA format described in section 2.2.5. Antibody was mixed with decreasing concentrations of intact bacterial cells and added to ELISA wells coated with intact Listeria monocytogenes 4b cells in triplicate.*

3.3 *Discussion*

This chapter describes the production and characterisation of polyclonal antibodies to Internalin B protein extract and heat-treated *Listeria monocytogenes* cells. Both antibodies were crudely purified by saturated ammonium sulphate precipitation and later further purified on a protein G affinity chromatography column. The purity of each antibody was assayed by SDS-PAGE analysis (Figures 3.3 and 3.4) and purified antibody detected by Western blotting using an anti-rabbit AP-conjugated antibody. Purified antibody was subsequently used in the development of an inhibition ELISA immunoassay for the detection of heat-treated *Listeria monocytogenes* cells in solution. Due to the danger of using live *Listeria monocytogenes* cells in an uncontrolled laboratory, optimisation and development of the inhibition ELISA immunoassays were performed with heat-treated *Listeria monocytogenes* cells, obtained from the Institute for Food Research, in Norwich. However, intact (not heat-treated) cells were used to determine the cross reactivities of each antibody (section 3.2.7) with no obvious decrease in sensitivity observed (Figures 3.17 and 3.18).

In order to determine the optimal working dilution of the purified antibody and optimal cell coating concentration, a checkerboard ELISA was performed as described in section 2.2.4. Varying dilutions of a 2 x 10^{10} cells/ml *Listeria monocytogenes* stock were coated on a microtitre plate and dilutions of affinity purified antibody added to each cell dilution on the microtitre plate. The optimal coating concentration of *Listeria monocytogenes* cells was determined to be approximately 1 x 10^{8} cells/ml and the optimal antibody dilutions determined to be 1/300 and 1/12,500, for the anti-InlB extract and anti-*Listeria monocytogenes* polyclonal antibodies, respectively. An inhibition ELISA was developed with each antibody as described in sections (2.3.5 and 3.2.5) and a four parameter equation fitted to the data using BIAevaluation 3.1 software. Intra- and interday studies were carried out with both antibodies to determine the accuracy and precision of repeated measurements of both assays. The coefficients of variance values of each assay provide a quantitative measure of precision of the analytical method. The percentage accuracies values obtained in the linear part of both interday assay plots (Figures 3.7 and 3.7) indicate the fitted four parameter curves provide an accurate representation of the sigmoidal relationship between the measured response and the logarithm of concentration observed for both immunoassays. Limits of detection of approximately 7 x 10^{6} and 1 x 10^{6} cells/ml were observed for the anti-InlB extract and anti-*Listeria monocytogenes* antibodies, respectively.

To assess the feasibility of detecting *Listeria monocytogenes* in a food matrix such as chocolate milk, the widely available Yazoo™ chocolate milk was purchased and spiked with known concentrations of *Listeria monocytogenes* cells. An inhibition ELISA was developed for both antibodies and intra- and interday assay variations analysed (Tables 3.3 and 3.4). Using chocolate milk as a sample matrix increased the precision of the anti-InlB extract antibody assay (section 3.2.6) and seemed to cause a slight shift in the limit of detection from approximately 7 x 10^6 cells/ml in PBS (Figure 3.7) to approximately 4-5 x 10^6 cells/ml in chocolate milk (Figure 3.11). These findings suggest the antibody can be reliably used for the detection of *Listeria monocytogenes* chocolate milk samples without the need for prior sample preparation. The anti-*Listeria monocytogenes* antibody coefficient of variance values observed in chocolate milk were comparable with those observed in PBS, however, the limit of detection of the assay decreased slightly from 1 x 10^6 cells/ml in PBS to 2 x 10^6 cells/ml in chocolate milk. Overall, the results obtained for both antibodies in chocolate milk suggest that no sample preparation is required for the detection of *Listeria monocytogenes* cells in chocolate milk samples.

The level of cross reactivity observed with both antibodies was determined against a number of bacterial cross contaminants. Two strategies for calculating the percentage cross reactivity of each antibody with the bacterial cross contaminants were examined. Expressing the antigen concentration value at 50% inhibition (EC_{50}) as a percentage of the cross reactant concentration giving the same decrease in signal (Figure 3.15) was compared to strategy of expressing the inhibition observed with the antigen at the EC_{50} value to the inhibition observed with the cross reactant at the same concentration (Figure 3.16). The calculated percentage cross reactivity's observed using both strategies are tabulated in Table 3.5 and show a considerable difference between the two methods. The ratio of inhibition values yield a lot higher cross reactivity values which fit the observed results (Figures 3.17 and 3.18) more accurately. The calculated ratio of concentration values observed for the anti-*Listeria monocytogenes* antibody indicate that there is little cross reactivity of the antibody with *L. innocua, L. ivanovii, L. seeligeri* and *B. subtilis* which is not the case observed in Figure 3.17. Using the ratio of concentration values of each cross contaminant can falsely over exaggerate the specificity of an immunoassay and can lead to the continued detection of false positive samples. Both the antibodies discussed in this chapter cross reacted with the other *Listeria* strains to different degrees, albeit at high cells numbers (no significant cross reactivity observed at less than 5 x10^7 cells/ml). These findings indicate that neither antibody can be used for the specific detection of *Listeria monocytogenes* cells from a heterogeneous population of bacterial cells. However, by varying the assay format, and by utilising either of the antibodies as a

capture antibody in conjunction with a detection antibody with a more refined specificity (monoclonal antibody), both antibodies can be valuable reagents for the development of an immunoassay for the reliable detection of *Listeria monocytogenes* cells from a mixed population.

Overall the two immunoassays presented in this chapter do not reach the sensitivities for *Listeria* sp. reported for other assays such as the *Listeria* Rapid Test; Oxoid, Unipath ($5x10^4$ to 10^5 bacteria/ml, Roberts, 1994), VIDAS *Listeria* kit (10^4-10^5 cfu/ml, Sewell *et al.*, 2002) and a microsphere-associated fluorescence assay (<1000 organisms/ml, Dunbar *et al.*, 2003). Both antibodies have been further utilised for the identification of *Listeria monocytogenes* recombinant invasion-associated proteins (chapter 4) and for the rapid genus specific detection of *Listeria* cells using surface plasmon resonance (SPR) described in chapter 6.

Chapter 4

Cloning, expression and purification of *Listeria monocytogenes* invasion-associated protein gene sequences in *E. coli*

4.1 *Introduction*

This chapter involves the cloning and expression of the *Listeria monocytogenes* invasion associated proteins, Internalin B (InlB) and p60 (also known as iap), gene sequences in *E. coli* XL10 Gold cells and the purification of each protein by immobilised metal affinity chromatography (IMAC). Expressing *Listeria monocytogenes* invasion-associated proteins in *E. coli* allows the safe and efficient production of high quantities of pure protein for use in the generation of *Listeria monocytogenes* specific antibodies and immunoassay development, without the need for culturing large volumes of this important food borne pathogen. The fusion of a polyhistidine tag at the C terminus of each protein also allows the easy purification and detection of the expressed gene products.

The InlB and p60 gene sequences (accession numbers AJ012346 and X52268, respectively), lacking the signal sequences for secretion, were amplified from purified *Listeria monocytogenes* genomic DNA and directly cloned into an Invitrogen TA cloning vector, pCR 2.1, and ligated into *E. coli* INV αF' cells (Figure 4.1 shows an outline of the steps involved in the cloning, expression and purification of each protein). TA cloning (section 4.2.2) allows the simple one step cloning of amplified DNA and facilitates the screening of positive clones containing the desired PCR products. Most restriction enzymes require additional DNA flanking the restriction site in order to sufficiently digest DNA. Cloning of the InlB and p60 gene sequences into a TA cloning vector also provides the additional DNA needed for the efficient digestion of the PCR products. However, TA cloning does not allow directional cloning of PCR products which can result in the desired genes being present in the wrong orientation or in the wrong reading frame. In order to express high levels of the correct InlB and p60 gene products, the InlB and p60 gene sequences were directionally cloned into the QIAgen pQE-60 expression vector (Figure 4.4) as described in section 2.3.6.

In *E. coli*, the promoter region consists of a hexanucleotide sequence located approximately 35 bp upstream of the transcription initiation base (-35 region) separated by a short spacer from another hexanucleotide sequence (-10 region) (Makrides, 1996). The ribosome binding site in an optimised-expression vector extends from the −10 region of the promoter to the beginning of the coding sequence. The consensus Shine-Delgarno sequence (AGGA) is contained within the ribosome binding site and interacts with the 3' terminus of the ribosomal RNA during translation initiation (Hannig and Makrides, 1998). Transcription initiation in *E. coli* begins with binding of *E. coli* RNA polymerase to the promoter; however, the successful transition from transcription initiation to transcription

elongation can be influenced by downstream elements. Many popular bacterial expression systems contain components of the *lac* operon. Binding of the *lac* repressor protein to the *lac* operator, a specific sequence of DNA to which the *lac* repressor binds, greatly decreases the frequency of successful transcription elongation events by the RNA polymerase (Novy and Morris, 2001). Inducers of the *lac* operon, such as IPTG, bind to the *lac* repressor and substantially decrease its binding affinity to the *lac* operator, thereby permitting transcription elongation. However, efficient transcription initiation also requires the presence of cyclic AMP (cAMP) and the cyclic AMP receptor protein, called CRP or CAP. The CAP/cAMP complex binds just upstream of the *lac* promoter and directly stimulates transcription by RNA polymerase. However, cAMP levels are strongly influenced by the carbon source present in the medium. In the presence of glucose, (an easily metabolised monosaccaride), cAMP levels are low, so transcription from the *lac* promoter is low and this phenomenon is known as the glucose effect or catabolite repression (Novy and Morris, 2001). The *lac* repressor protein can be coded upstream from the promoter region or as is the case of the pQE-60 vector, the *lac* repressor protein can be coded on a separate plasmid (Figure 4.4 shows the major components of the pQE-60 plasmid).

QIAgen pQE vectors contain an optimised promoter-operator element consisting of a phage T5 promoter (recognised by *E. coli* RNA polymerase) and two *lac* operator sequences which increase *lac* repressor binding and ensure efficient repression of the powerful T5 promoter. Each vector also contains a synthetic ribosomal binding site for high translation rates, a 6xHis tag (5' or 3' to the multiple cloning site) for convenient detection and purification, strong transcriptional terminators to prevent read-through transcription and ensure stability of the expression product, a β-lactamase gene (bla) conferring resistance to ampicillin and a Col E1 origin of replication (QIAgen Ltd, 2000). Regulation of expression from the pQE-60 vector can be controlled by the addition of glucose (catabolite repression) and by co-expressing the plasmid in cells containing the pREP4 plasmid, a low copy number plasmid that expresses the *lac* repressor protein encoded by the *lacI* gene (QIAgen Ltd, 2000). Also *E. coli* strains that harbour the $lacI^q$ mutation, such as XL1 Blue, TG1 and XL10 Gold, produce enough *lac* repressor to efficiently block transcription, and are ideal for storing and propagating pQE plasmids (QIAgen Ltd, 2000).

Expressed recombinant proteins were extracted from cell cytoplasmic extracts and purified by immobilised metal affinity chromatography using a Ni-NTA (nickel-nitrilotriacetic acid) agarose resin (QIAgen, UK) and the purity determined by SDS-PAGE

analysis. The immunoreactivity of the recombinant antigens with anti-*Listeria monocytogenes*-related antibodies was further examined. Both proteins were probed with protein specific monoclonal antibodies by Western blotting and detected with anti-*Listeria monocytogenes* polyclonal antibodies by ELISA. Both proteins were also used for the selection of specific anti-*Listeria monocytogenes* antibodies (chapter 5) and for the development of an immunoassay to detect *Listeria monocytogenes*.

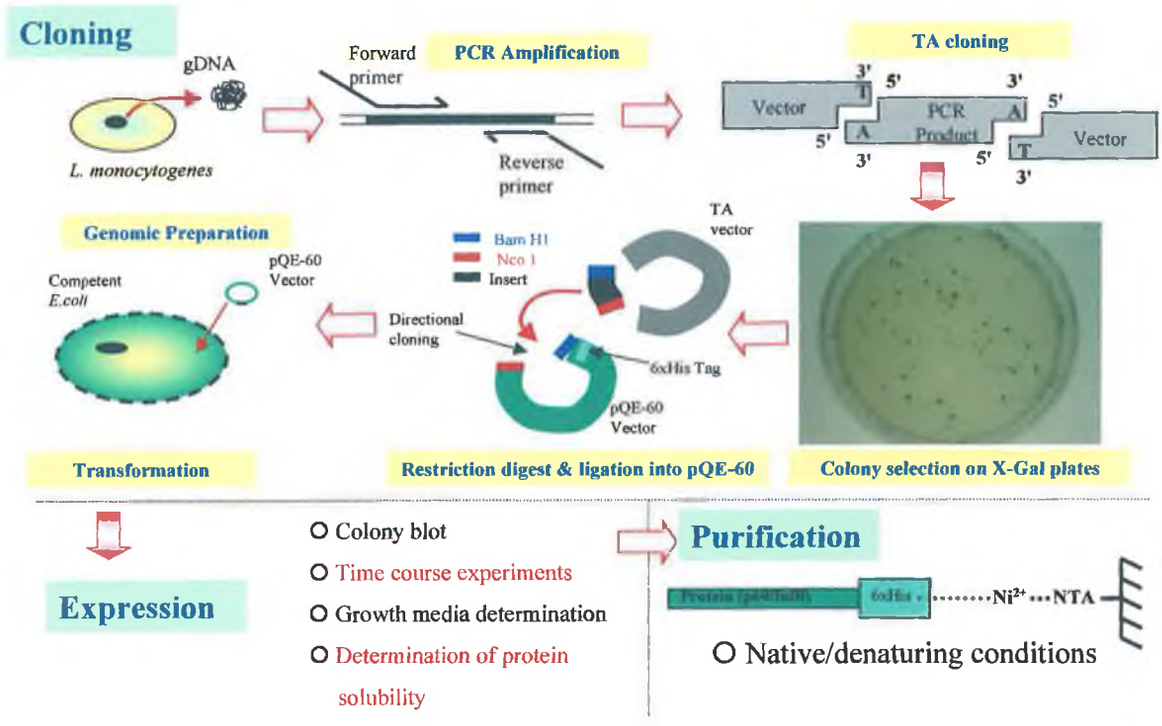


Figure 4.1: *Overview of the processes involved in the cloning, expression and purification of the InlB and p60 gene sequences.*

Cloning: *Genomic DNA from Listeria monocytogenes was prepared (described in section 2.3.1) and the InlB and p60 gene sequences amplified by specific PCR primers (sections 2.3.3 and 2.3.4). Fresh PCR product (PCR A overhangs degrade over time) was ligated into a pCR 2.1 vector (TA cloning) and transformed cells spread on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) for the blue-white screening of positive clones. Plasmid DNA from selected clones was then purified (section 2.3.5.3) and the gene insert directionally subcloned into the pQE-60 expression vector by digestion with NcoI and BamHI restriction enzymes (section 2.3.5.4). The pQE-60 vectors containing gene inserts (pQE-InlB and pQE-p60 vectors) were finally transformed into E. coli XL10 Gold cells (section 2.3.6).*

Expression: *A number of strategies, such as colony blotting, time course experiments, growth media determination and determination of protein solubility by SDS-PAGE, can be utilised to examine and optimise the expression of recombinant protein in E. coli. In this chapter, time course experiments (section 2.3.7) were performed to determine the expression of each protein and determination of protein solubility (section 2.3.8) carried out by SDS-PAGE analysis of cell lysates.*

Purification: *Both proteins were purified by immobilised metal affinity chromatography (section 2.3.9) using a Ni-NTA agarose resin (QIAgen) and the purity of each protein assessed by SDS-PAGE analysis.*

4.2 *Cloning of the InlB and p60 gene sequences into E. coli*

Due to the obvious dangers of culturing high numbers of *Listeria monocytogenes* cells in an uncontrolled environment and with inherent difficulties in extracting large quantities of purified invasion-associated protein from *Listeria monocytogenes* cultures, it was decided to clone two *Listeria monocytogenes* invasion-associated proteins in *E. coli*. The gene sequences (lacking the signal sequences for secretion) coding for the *Listeria monocytogenes* invasion-associated proteins, Internalin B (InlB) and p60 (discussed in Chapter 1), were cloned into *E. coli* as described in Figure 4.1 above.

4.2.1 *Amplification of the InlB and p60 gene sequences by PCR*

Listeria monocytogenes 1/2a (serovar 1/2a was used as the protein sequences obtained from accession numbers AJ012346 and X52268 were sequences from *Listeria monocytogenes* 1/2a cells) genomic DNA was extracted as described in section 2.3.1 and used as the template for the amplification of the InlB and p60 genes. Primers were designed to incorporate the sequences for *NcoI* and *BamHI* digestion for the subsequent directional cloning into the pQE-60 plasmid (section 2.3.3). The use of the *NcoI* site necessitated the incorporation of two additional bases immediately following the *NcoI* site in order to maintain the correct reading frame. This resulted in the addition of a glycine codon between the ATG initiation codon and the first codon of the gene. A standard PCR reaction was used to amplify the DNA sequences (section 2.3.4) and the optimum annealing temperature determined to be 65°C (Figure 4.2) for each protein.

4.2.2 *TA cloning of amplified gene sequences*

Thermostable polymerases like *Taq* DNA polymerase, which lack 3' to 5' exonuclease activity, leave 3' A-overhangs in amplified products. Therefore, PCR products generated with *Taq* DNA polymerase have a high efficiency of cloning in the TA Cloning system. Cloning in the TA vector allows the distinction between transformants bearing plasmids with inserts from transformants bearing recircularised plasmids without inserts by plating on media containing X-Gal to test for α-complementation of β-galactosidase. Transformants harbouring plasmids with inserts do not express a functional *lacZα* gene product, which is the α peptide of β-galactosidase, and therefore appear white on X-Gal plates because α-complementation cannot occur. Transformants harbouring recircularised plasmid DNA without any inserts express a functional *lacZα* gene product and thus appear blue on X-Gal plates because α-complementation of β-galactosidase can occur resulting in

the cleavage of X-Gal. Figure 4.3 shows a picture of a X-Gal agar plate containing transformants harbouring the InlB gene sequence inserts. From Figure 4.3 it can be seen that blue and white colonies can be easily differentiated and the plate contains an even spread of blue and white colonies. The high number of blue colonies can be the result of degradation of either the plasmid or insert overhangs or be due to the ligation of the pCR 2.1 with very small or truncated inserts. DNA inserts less than 500 bp in length may produce light blue colonies and therefore some blue colonies should be checked.

4.2.3 *Cloning of amplified inserts into the pQE-60 expression vector*

TA cloning pCR 2.1 plasmids from selected colonies harbouring a PCR product insert were purified (section 2.3.5.3) and digested with *NcoI and BamHI* as described in section 2.3.5. The InlB and p60 gene sequences were purified from an agarose gel (Figure 4.5) and ligated into a QIAgen pQE-60 vector. Ligated vectors were transformed into *E. coli* XL10 Gold cells (section 2.3.6.6) and expression of InlB and p60 gene products determined as described in section 4.2.4.

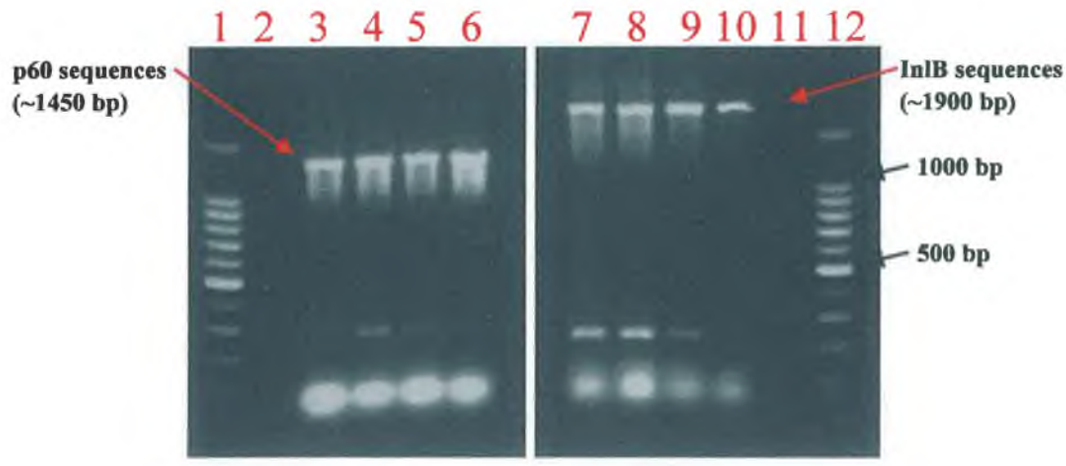


Figure 4.2: *Gradient PCR to determine the optimum annealing temperature for each protein gene sequence. Lanes 1 and 12 contain a Promega 100 bp marker; lanes 2 and 11 are blank wells; lanes 3-6 contain the p60 amplification products at 58°C, 60°C, 62°C and 65°C annealing temperatures, respectively. Lanes 7-10 contain the InlB amplification products at 58°C, 60°C, 62°C and 65°C annealing temperatures, respectively. One clear band (no non-specific bands as observed with lower temperatures) was obtained at 65°C for each protein and therefore this temperature was chosen for subsequent amplification reactions.*

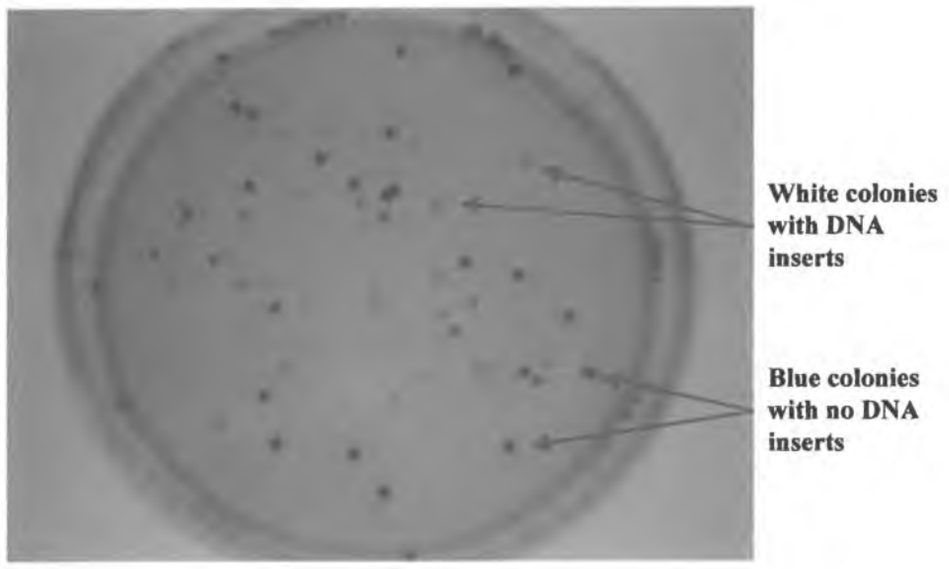


Figure 4.3: *Typical X-Gal agar plate used for the identification of clones containing PCR inserts after TA cloning. After transformation E. coli cells were spread on agar plates containing the required antibiotics and 40 µl of a 40 mg/ml X-Gal stock spread evenly over the top of the plates. Colonies with no inserts turned blue on the plate after incubation at 4°C for 3 hours. The picture shown here was taken from an agar plate containing transformants with the InlB gene sequence.*

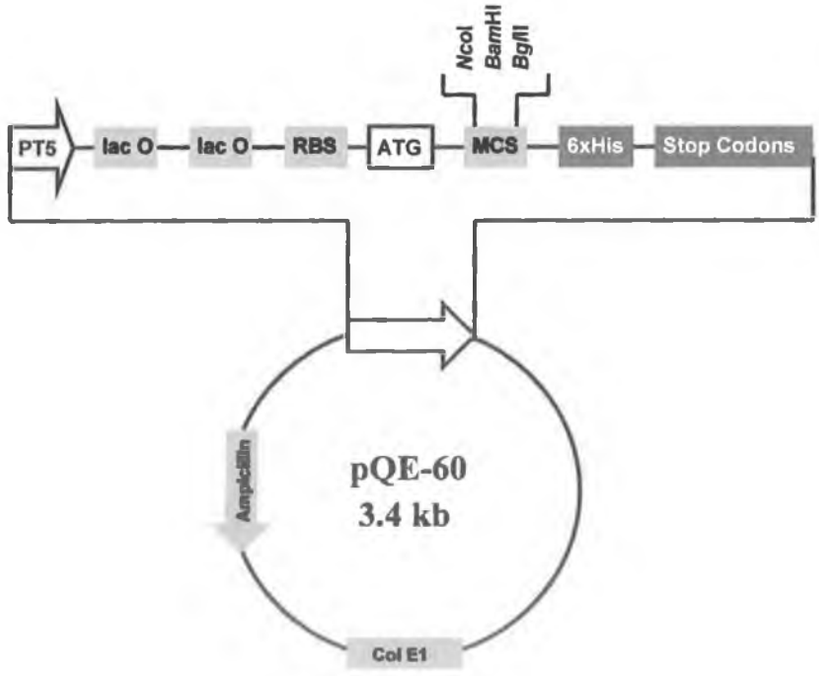


Figure 4.4: *Diagram of the pQE-60 vector used for the cloning of the InlB and p60 gene sequences. The plasmid contains a T5 promoter (PT5), lac operators (lac O), a ribosome binding site (RBS), ATG start codon, 6xHis tag sequence, multiple cloning site (MCS), stop codons in all three reading frames (stop codons), a Col E1 origin of replication and an ampicillin resistance gene. The expression levels can be regulated by addition of glucose (catabolite repression) and by co-expressing the plasmid in cells containing the pREP4 plasmid, a low copy number plasmid that*

expresses the lac repressor protein encoded by the lacI gene. Also E. coli strains that harbour the lacI^q mutation, such as XL1 Blue, TG1 and XL10 Gold, produce enough lac repressor to efficiently block transcription, and are ideal for storing and propagating pQE plasmids (QIAgen Ltd, 2000).

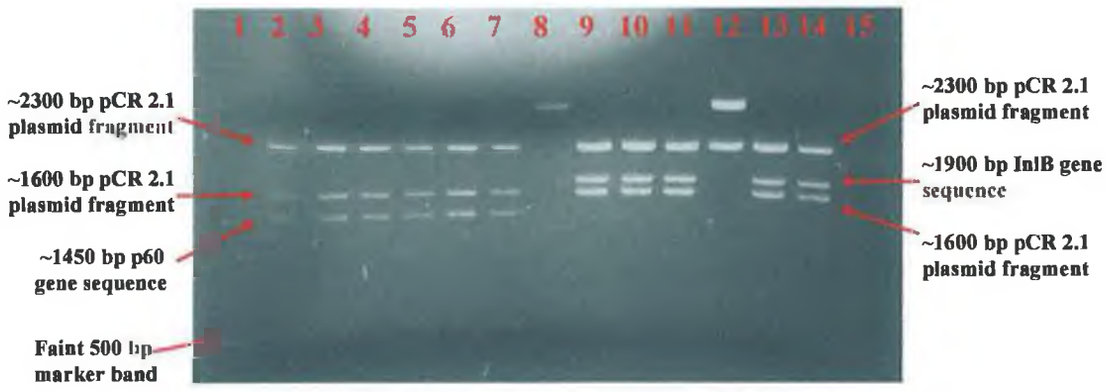


Figure 4.5: *Restriction of pCR 2.1 plasmid clones containing an InlB or p60 gene insert. Plasmid DNA was purified as described in section 2.3.5.3 and the purified plasmid digested with NcoI and BamHI restriction enzymes as described in section 2.3.5.4. The pCR 2.1 vector contains a NcoI site 1878 bp downstream from the promoter and BamHI site 253 bp downstream from the promoter (first base in promoter region is base number 1). Digestion with NcoI and BamHI (sites also now present in the MCS as amplified with the InlB and p60 genes) results in the generation of four DNA fragments. The first fragment, an approximately 2300 bp pCR 2.1 vector fragment, results from digestion from the NcoI site at position 1878 through the promoter to the BamHI site in position 253. The second fragment, an approximately 1600 bp pCR 2.1 vector fragment, results from digestion from the MCS, roughly in position 280, to the NcoI site in position 1878. The third fragment, an insert fragment of ~1450 bp for the p60 gene sequence and ~1900 bp for InlB gene sequence results, from the digestion of the PCR product while the fourth fragment, a tiny pCR 2.1 plasmid fragment of approximately 30 bp, is not present in the gel picture described here. Lanes 1 and 15 contain a Promega 100 bp marker, with only the 500 bp band visible on the gel. Lanes 2-7 represent digested pCR 2.1 vector harbouring a p60 gene sequence and lanes 9-14 represent digested pCR 2.1 vector harbouring an InB gene sequence. Lane 8 represents an undigested pCR 2.1 vector harbouring no insert. The high band present in lane 12 is probably an InlB gene sequence-pCR 2.1 vector fragment (1600 bp) fusion caused by restriction with NcoI only. The InlB and p60 gene fragments indicated were purified from the gel and ligated into the pQE-60 expression vector as described in sections 2.3.6.*

4.2.4 *Time course experiments to determine the expression gene products*

E. coli cells harbouring either the InlB or p60 gene sequences were induced with IPTG as described in section 2.3.7 and samples taken every hour. Cell samples were resuspended in lysis buffer and sonicated. The resulting denatured cytoplasmic extracts were analysed by SDS-PAGE as shown in Figure 4.6 and 4.7. Both the InlB (Figure 4.6) and p60 (Figure 4.7) gene products were observed at the expected molecular weights (indicated). The level of expression of each protein varied considerably with the expression of the p60 gene product being vastly superior. Both gene products showed optimum expression between 5 and 6 hours of induction.

4.2.5 *Determination of protein solubility and cellular location*

To determine the solubility of each protein, *E. coli* cultures harbouring either an InlB or p60 gene sequence were induced with IPTG and grown for 5 hours. Culture cell pellets were then resuspended in PBS (referred to as native or soluble cytoplasmic extract) and sonicated as described in section 2.3.8. The remaining insoluble matter was resuspended in lysis buffer and the cell debris removed by centrifugation. The soluble and insoluble extracts were analysed by SDS-PAGE as shown in Figure 4.8. As observed from the gel, the majority of the expression gene products are expressed in the soluble form (lanes 1 and 3) with some protein expressed an insoluble inclusion bodies (lanes 2 and 4). Running both gene products on the same gel indicates the different molecular weights of each protein and highlights the presence of either an InlB or p60 expressed protein band not present in the alternative lysate.

By comparing periplasmic and cytoplasmic expression (section 2.3.8) it was determined that each protein is expressed mainly in the cytoplasm with small amounts of protein (mainly p60) being expressed in the periplasm (Figure 4.9). *E. coli* cell pellets expressing either an InlB or p60 gene product were either resuspended in PBS and sonicated (native sonicated cytoplasmic extract) or resuspended TES buffer (section 2.3.8) for 1 hour at 4°C (native periplasmic extract). The remaining pellet from the periplasmic extract was also resuspended in PBS and sonicated (native cytoplasmic extract minus periplasmic extract). Each fraction was analysed by SDS-PAGE as shown in Figure 4.9. The large protein bands present in lanes 2 and 4 (InlB) and 5 and 7 (p60) indicate that both proteins are expressed in the cytoplasm. The expression levels of both proteins were observed to differ significantly. The p60 protein was expressed at approximately 18mg/l while InlB was observed at approximately 7mg/l.

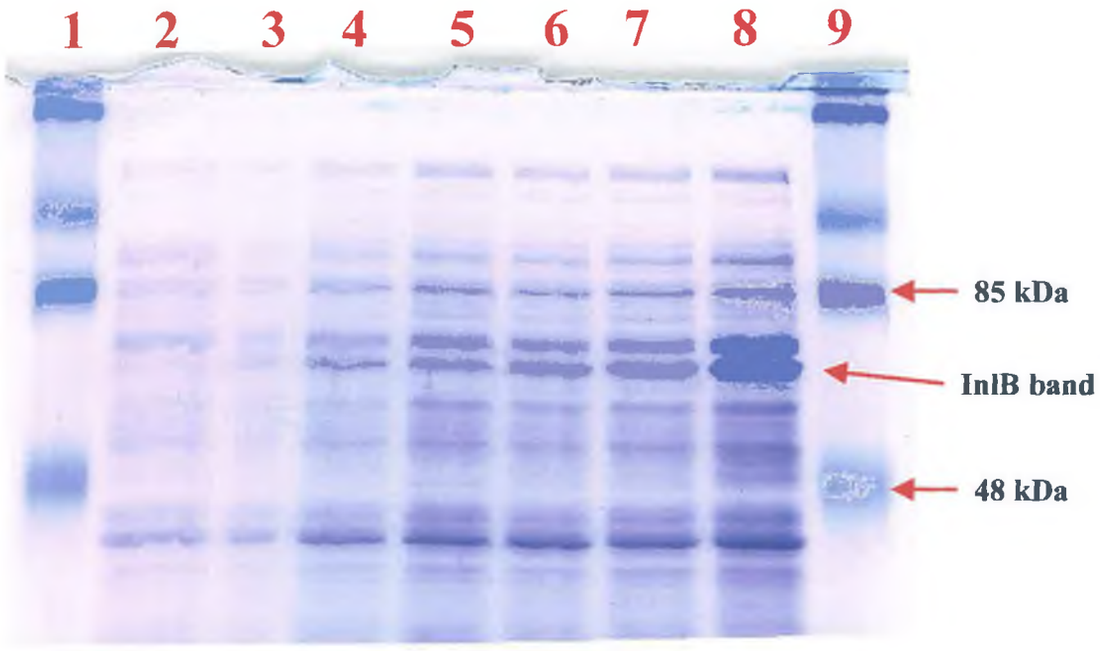


Figure 4.6: *Time course experiment for the expression of the InlB gene product. Lanes 1 and 9 represent a Pierce Blue Ranger prestained molecular weight marker and lanes 2-8 represent E. coli cell lysates taken after induction with IPTG at 0, 1, 2, 3, 4, 5 and 6 hours, respectively. 1 ml samples from the growing induced culture were taken at 1 hour intervals, centrifuged and the pellet resuspended in urea buffer as described in section 2.3.7. 20 µl of each sample was added to each lane.*

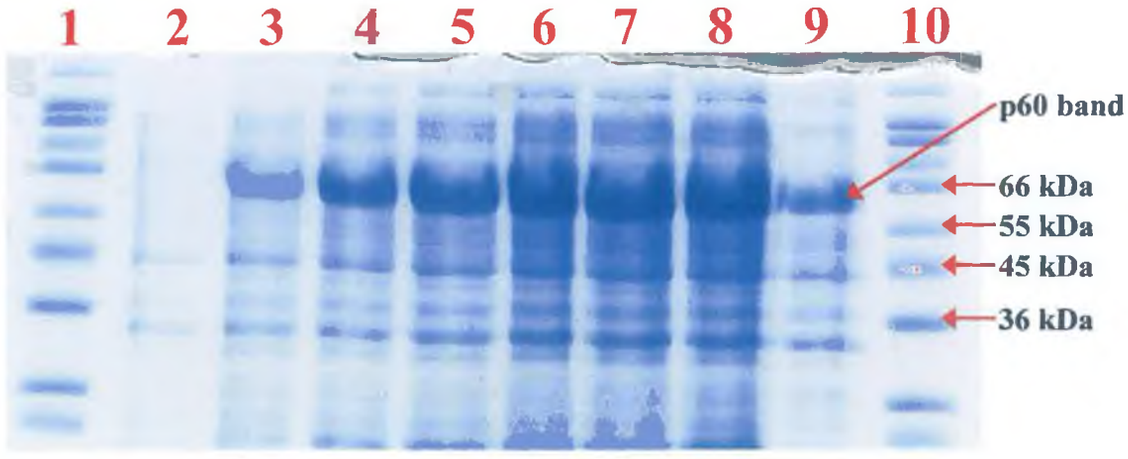


Figure 4.7: *Time course experiment for the expression of the p60 gene product. Lanes 1 and 10 represent a Sigma wide range molecular weight marker and lanes 2-9 represent E. coli cell lysates taken after induction with IPTG at 0, 1, 2, 3, 4, 5, 6 and 18 hours, respectively. 1 ml samples from the growing induced culture were taken at 1 hour intervals, centrifuged and the pellet resuspended in urea buffer as described in section 2.3.7. 20 µl of each sample was added to each lane.*

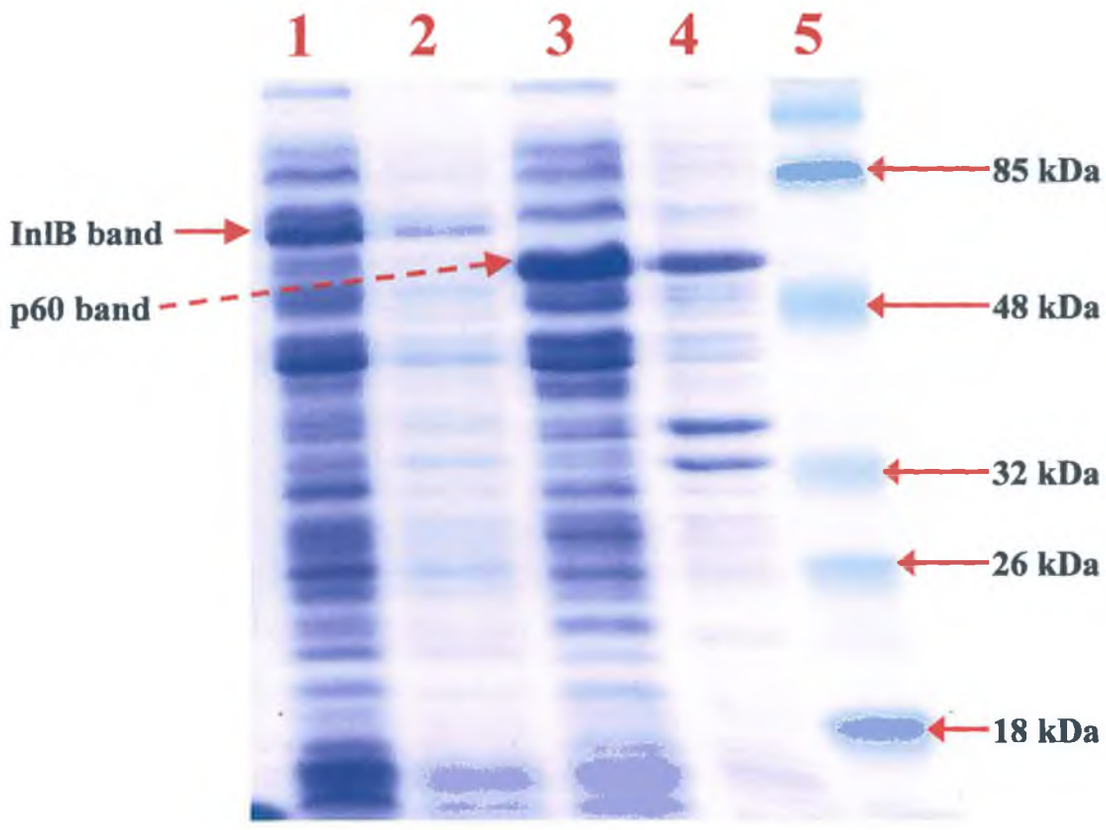


Figure 4.8: *Determination of protein solubility. E. coli cells harbouring each protein were induced for 5 hours and the cells recovered by centrifugation. Pellets were resuspended in PBS and sonicated (soluble fraction). The remaining insoluble matter was separated by centrifugation and resuspended in 8 M urea buffer (insoluble fraction) to solublise the remaining protein aggregates. Lanes 1 and 2 represent E. coli InlB soluble and insoluble lysate fractions, lanes 3 and 4 represent E. coli p60 soluble and insoluble lysate fractions and lane 5 represents a Pierce Blue Ranger prestained marker with weights indicated. From the gel it can be clearly seen that the majority of each protein is expressed as soluble material.*

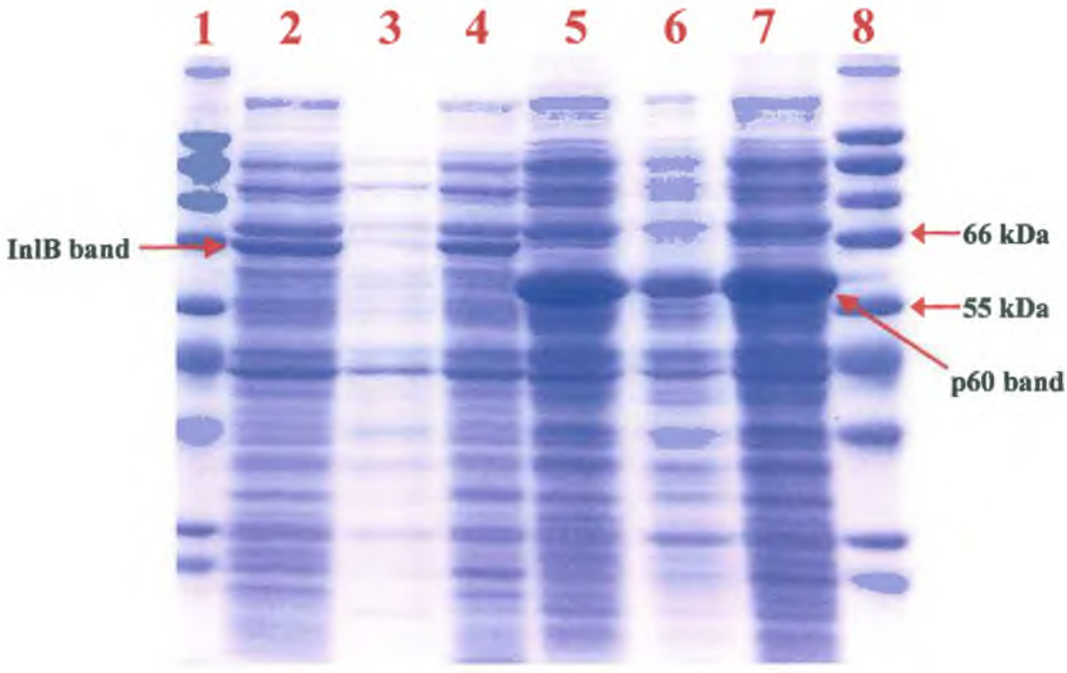


Figure 4.9: *Determination of the cellular location of each protein. E. coli cells harbouring each protein were induced with IPTG of 5 hours and 2 x 5 ml of each cell culture harvested. The cell pellets were resuspended in either PBS and sonicated (native sonicated cytoplasmic extract) or TES buffer (section 2.3.8) for 1 hour at 4°C (native periplasmic extract). The remaining pellet from the periplasmic extract was also resuspended in PBS and sonicated (native cytoplasmic extract minus periplasmic extract). Lanes 1 and 9 represent a Sigma wide range marker, lanes 2-4 represent E. coli InlB native cytoplasmic extract, periplasmic extract and native cytoplasmic extract minus the periplasmic fraction, respectively. Lanes 5-7 represent E. coli p60 native cytoplasmic extract, periplasmic extract and native cytoplasmic extract minus the periplasmic fraction, respectively. From the gel it can be seen that both the majority of the InlB and p60 gene products are expressed in the cytoplasm with little or no protein being expressed in the periplasm (lanes 3 and 6).*

4.2.6 *Sequencing of the cloned InlB and p60 gene sequences*

Plasmid DNA containing either the InlB or p60 gene sequence was sent to MWG-Biotech (UK) Ltd, for sequencing. Sequencing results, received as linear nucleotide sequences, were subsequently translated into amino acid sequences and aligned with the known amino acid sequence of each protein using the ClustalW multiple alignment program (see section 2.3.13). Aligned sequences were imported into Genedoc, a program for the manipulation of aligned sequences, and the similarities compared. Figures 4.10 and 4.11 show the alignments of both cloned gene sequences with their known protein sequence. Both the p60 and InlB cloned gene sequences show 100% identity with their respective sequences provided by Genedoc (X52268 and AJ012346 for the p60 and InlB genes, respectively).

4.2.7 *Determination of the optimum IPTG concentration*

To determine the optimum IPTG concentration for the expression of each gene product, a series of time course experiments (section 2.3.7) were performed with various IPTG concentrations. Figure 4.12 shows the SDS-PAGE time course gels of the expression of both gene products, induced with various IPTG concentrations. Time course experiments were performed with IPTG concentrations of 1 mM (gels labelled A), 0.5 mM (gels labelled B), 0.1 mM (gels labelled C) and 0.5 mM (gels labelled D). A time course experiment was also performed with no induction (0 mM IPTG, gel labelled E) as a negative control. No expressed p60 nor InlB proteins bands were observed with out the induction of IPTG which indicate that *E. coli* strains, such as XL10 Gold, harbouring the *laqI*^{q} gene, produce sufficient quantities of the *lac* repressor protein to prevent undesired basal expression of recombinant proteins. Reducing the IPTG concentration from 1 mM to 0.05 mM had no significant effect on the expression of the p60 gene product (Figure 4.12, gels A-D). Slight variations in the sonication of each sample can result in the observation of unevenly expressed bands on the same gel. This is evident for example in the p60 gel C where the p60 band in lane 4 (expression after 3 hours of induction) is more concentrated than in lane 5 of the same gel (expression after 4 hours of induction). Decreasing the IPTG concentration from 1 mM to 0.5 mM seemed to reduce the expression of the InlB gene product (gels A-D) with an IPTG concentration of between 1 and 0.5 mM producing the highest level of expression. Again, it is evident that variation in the InlB protein concentration between samples has occurred due to the uneven sonication of the *E. coli* cell samples (gel B, lanes 5 and 6). Due to the type of sonicator used, exact replication (exact position and dept of the probe) of sonication conditions were difficult.

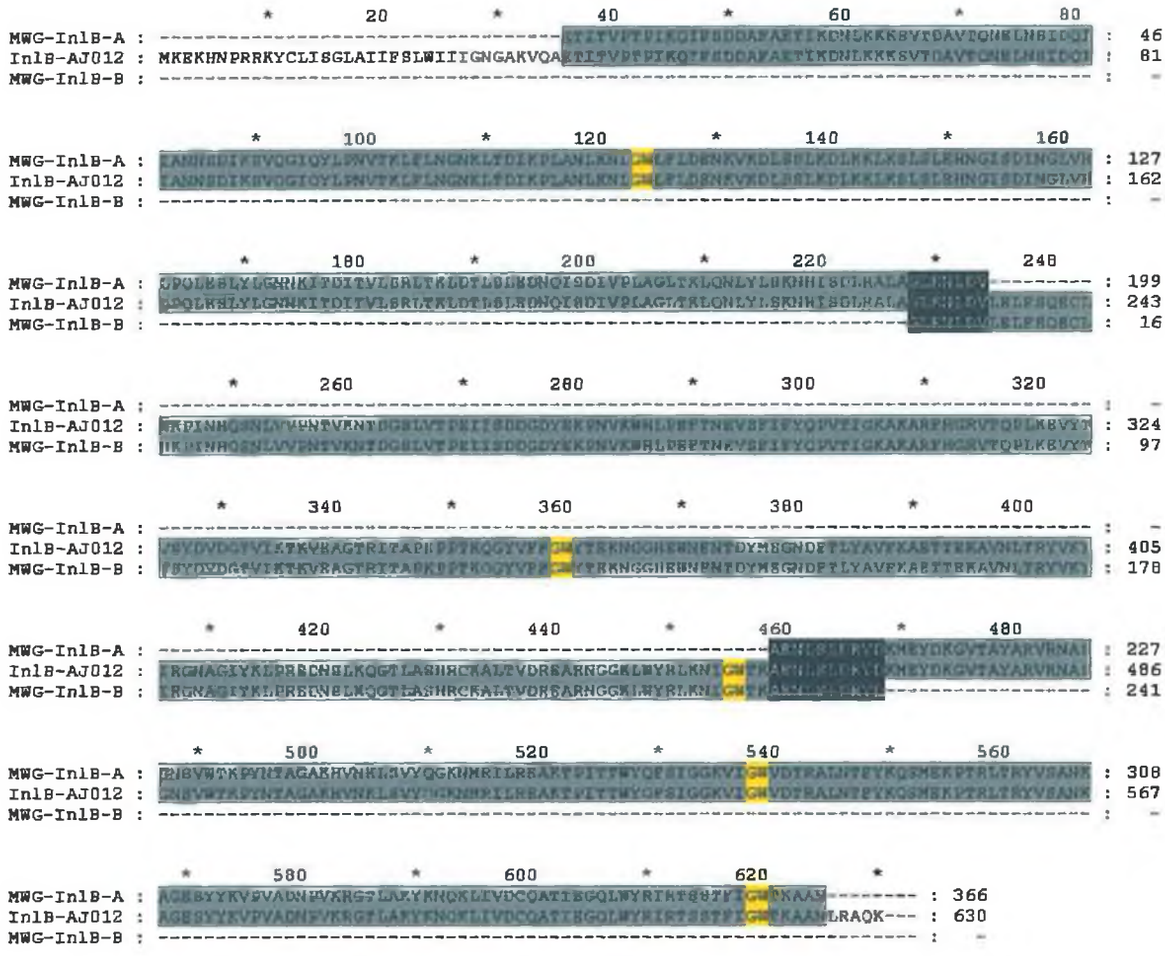


Figure 4.10: *Alignment of the cloned InlB gene sequence with the* ***InlB*** *gene sequence obtained from Genebank (accession number AJ012346). As the* ***InlB*** *gene is approximately 1900 bp (630 amino acids) in length the cloned gene sequence had to be sequenced in both directions (MWG-InlB-A) and upon receiving the sequencing results a primer designed for the sequencing of the inner region (MWG-InlB-B). The nucleotide sequences received from MWG-Biotech Ltd, UK, were translated into amino acid sequences and aligned using the ClustalW program. Aligned sequences were then imported into Genedoc for further sequence manipulation. The amino acid sequences highlighted in black show 100% identity in all three sequences (overlap of MWG-InlB-A and MWG-InlB-B sequencing results of the InlB gene with the AJ012346 InlB sequence) and the amino acid sequences highlighted in grey show 100% identity between the sequenced cloned DNA and the InlB gene sequence provided by Genebank. The GW repeats involved in the anchoring of InlB to the cell membrane are highlighted in yellow. The signal peptide in InlB accounts for the first 35 amino acids (not highlighted) and the sequence encoding this peptide was not cloned into E. coli. No sequencing results were obtained for the last 5 amino acids of the InlB sequences (not highlighted) possibly due to the nature of the primers used for sequencing but these amino acids were cloned into E. coli and should be present. Overall the sequencing results obtained from MWG-Biotech shows 100% identity with the InlB gene sequence obtained from Genebank.*

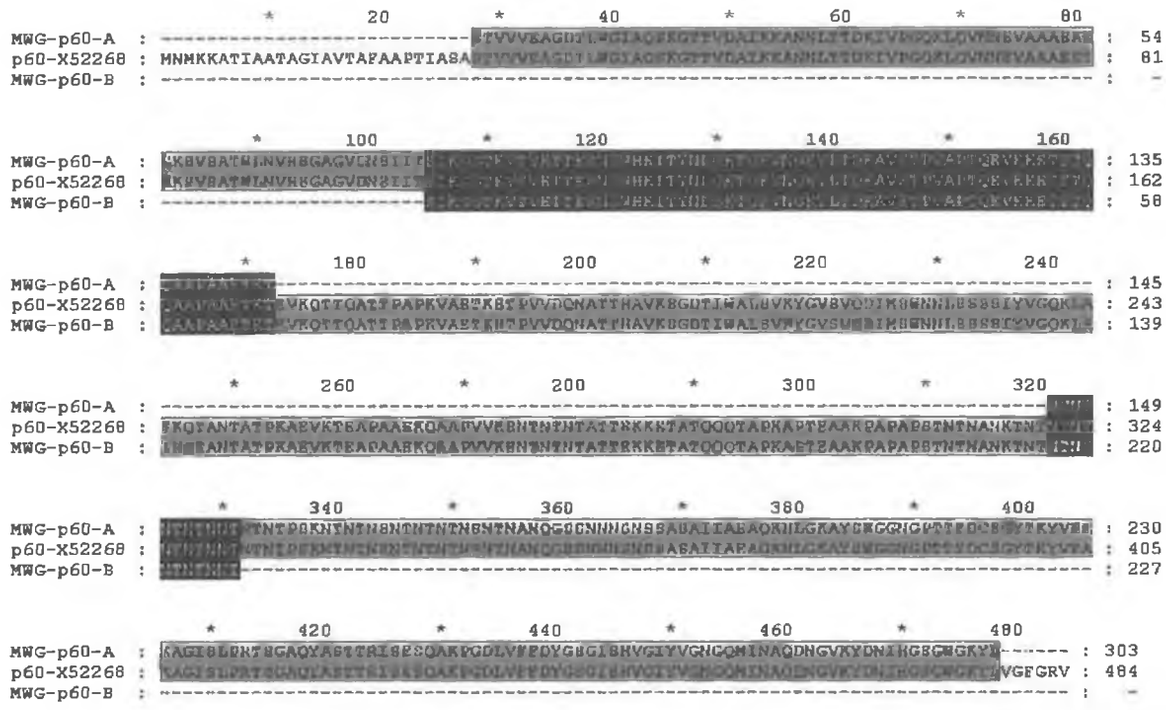


Figure 4.11: *Alignment of the cloned p60 gene sequence with the p60 gene sequence obtained from Genebank (accession number X52268). As the p60 gene is approximately 1450 bp (484 amino acids) in length the cloned gene sequence had to be sequenced in both directions (MWG-p60-A) and upon receiving the sequencing results a primer designed for the sequencing of the inner region (MWG-p60-B). The nucleotide sequences received from MWG-Biotech Ltd, UK, were translated into amino acid sequences and aligned using the ClustalW program. Aligned sequences were then imported into Genedoc for further sequence manipulation. The amino acid sequences highlighted in black show 100% identity in all three sequences (overlap of MWG-p60-A and MWG-p60-B sequencing results of the InlB gene with the X52268 p60 sequence) and the amino acid sequences highlighted in grey show 100% identity between the sequenced cloned DNA and the InlB gene sequence provided by Genebank. The signal peptide in p60 accounts for the first 27 amino acids (not highlighted) and the sequence encoding this peptide was not cloned into E. coli. No sequencing results were obtained for the last 6 amino acids of the p60 sequences (not highlighted) possibly due to the nature of the primers used for sequencing. Overall the sequencing results obtained from MWG-Biotech shows 100% identity with the p60 gene sequence obtained from Genebank. It should be noted that the only the amino acid sequences are aligned and some silent mutations or PCR errors may have occurred at the nucleotide level, which are not seen here.*

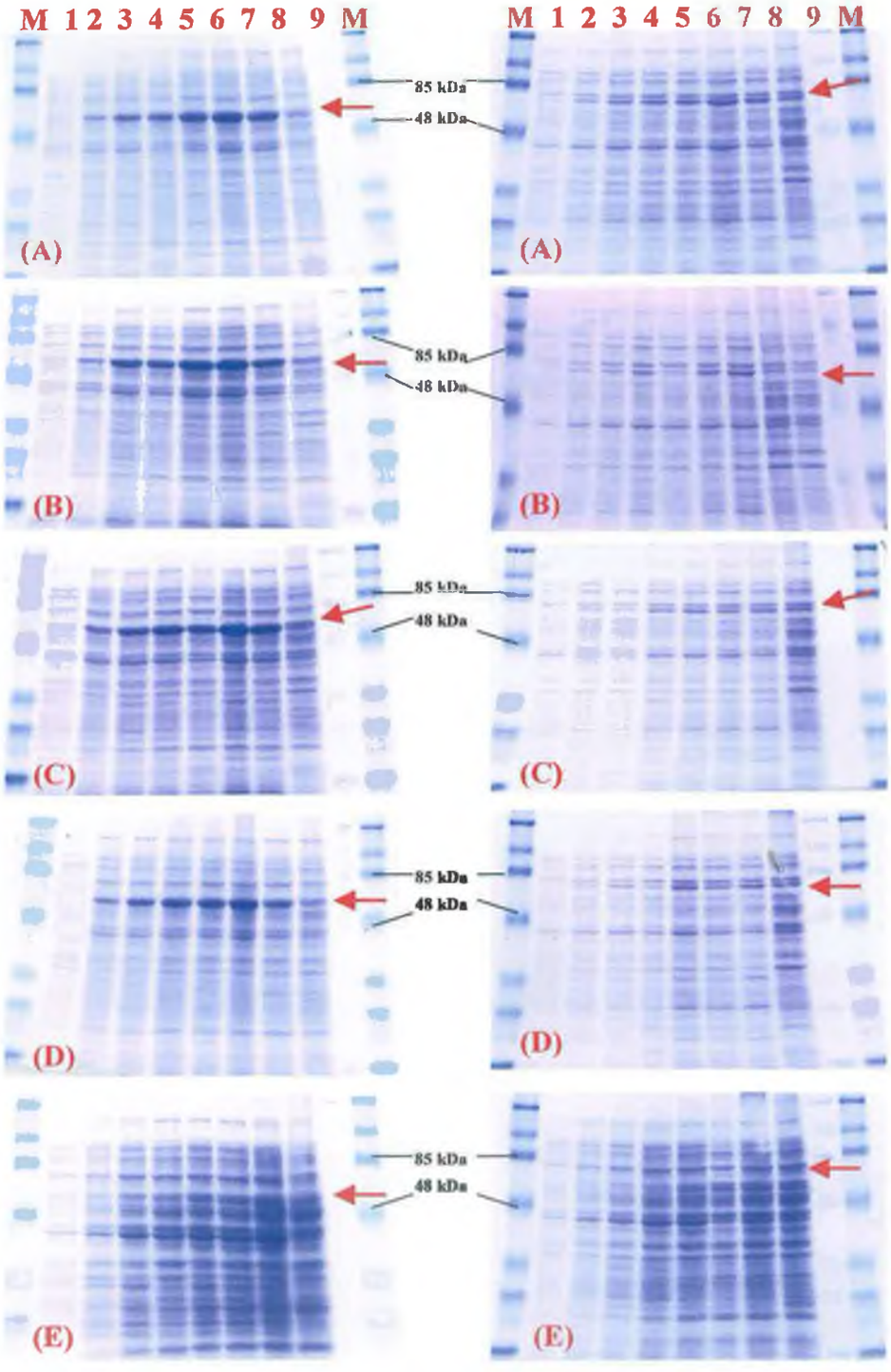


Figure 4.12: *Effect of IPTG concentration on the expression of the InlB and p60 gene sequences. Gels A-E represent p60 (left) and InlB (right) gene products induced with 1 mM, 0.5 mM, 0.1 mM, 0.05 mM and 0 mM IPTG, respectively. Lanes M represent a Pierce Blue Ranger marker, lanes 1-8 represent 0, 1, 2, 3, 4, 5, 6 and 22 hours of induction, respectively, while lane 9 was left blank (some overflow from the marker may have contaminated this lane). The expected InlB or p60 bands are indicated by a red arrow. No p60 nor InlB band was observed with no induction indicating that the expression of both proteins is tightly controlled (E. coli XL10 Gold contain the lacI^q gene).*

4.2.8 *Purification of the InlB and p60 gene products by IMAC*

Proteins containing a 6xHis affinity tag, encoded at either the N or C terminus, can easily be purified by binding to Ni-NTA. The pQE-60 vector used to clone both the InlB and p60 gene sequences encodes a 6xHis tag at the C terminus of the recombinant protein. The 6xHis tag is poorly immunogenic, and at pH 8.0 the tag is small, uncharged, and therefore does not generally affect secretion, compartmentalisation, or folding of the fusion protein in the cell (QIAgen Ltd, 2000). Nitrilotriacetic acid (NTA) is a tetradentate chelating adsorbent that occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag (Ueda *et al.*, 2003). The high surface concentration of the NTA ligand is sufficient for the binding of approximately 5-10 mg of 6xHis tagged protein per millilitre of resin (QIAgen Ltd, 2000). In general, histidine residues in proteins are relatively rare, amounting to approximately only 2% of the amino acid content of globular proteins; in addition, only half of them are exposed on the protein surface (Ueda *et al.*, 2003). However, certain proteins from *E. coli*, namely superoxide dismutase (8 histidines, 195 amino acids), chloramphenicol acetyltransferase (12 histidines, 219 amino acids) and heat-shock protein (14 histidines, 624 amino acids), have been identified, which might bind to IMAC columns depending on the conditions used (Müller *et al.*, 1998a). Increasing the ionic strength of buffers can lead to suppression of secondary, electrostatically undesirable interactions while augmenting protein binding to chelate complexes. Therefore, for the specific purification of recombinant proteins from *E. coli* by immobilised metal affinity chromatography, stringent binding and washing conditions should be used. The running buffer used for the purification of the p60 and InlB proteins consisted of PBS with 1% (v/v) Tween 20, 500 mM NaCl and 10 mM β-mercaptoethanol. The addition of Tween 20 to the buffer reduces non-specific hydrophobic interactions, while the addition of 500 mM NaCl reduces non-specific ionic interactions and the repulsion of the positively charged recombinant p60 or InlB proteins with the positively charged Ni-NTA resin. The addition of 10 mM β-mercaptoethanol reduces the co-purification of host proteins that may have formed disulfide bonds with the protein of interest during cell lysis.

E. coli cells harbouring a gene insert were induced with 1mM IPTG for 5 hours and the recombinant gene products purified as described in section 2.3.9. Figure 4.13 shows a typical purification of both recombinant proteins. Lanes 2-5 show the purification of the recombinant p60 protein while lanes 6-9 show the purification of the recombinant InlB protein. From lane 2 the rp60 protein is observed to be over expressed in the *E. coli* lysate and binds to the Ni-NTA column resulting in a decrease in the 60 kDa band observed in

the flow through (lane 3). Limiting the amount of resin used to purify a recombinant protein reduces the purification of non-specific or weakly bound proteins by introducing competition for binding to the resin. Therefore, to improve the quality of the purified p60 product, the amount of resin used for its purification was kept low which resulted in some of the recombinant protein remaining in the flow through fraction (lane 2). The recombinant p60-Ni-NTA complex was washed (lane 4) with running buffer (section 2.3.9) and the protein eluted with sodium acetate buffer, pH 4.5 (lane 5). The histidines residues in the 6xHis tag have a pK_a of approximately 6.0 and will become protonated if the pH is reduced (pH 4.5-5.3). Under these conditions the 6xHis tagged protein can no longer bind to the nickel ions and will dissociate from the Ni-NTA resin. Figure 4.13, lanes 5 and 9 show the elution of the recombinant p60 and InlB protein with sodium acetate buffer pH 4.5. The elution of each protein resulted in the presence of a concentrated protein band at the expected molecular weight. As the concentration of expressed InlB (lane 6) is lower than that of expressed p60 protein (lane 2), all the expressed InlB protein can bind to the Ni-NTA resin as observed with the loss of the InlB protein in the flow through (lane 7). However, as can be seen in lane 3, the concentration of p60 protein in the cytoplasmic extract is higher than the binding capacity of the 1 ml of Ni-NTA resin used, and therefore a small amount of p60 protein is present in the flow through. The same volume of resin was used to purify both proteins, so the eluted p60 and InlB protein bands are observed to be similar in concentration. The presence of faint unknown contaminating protein bands (which are barely visible on the gel) are probably the result of co-purification of host protein with the InlB or p60 proteins or the slight non-specific binding of host proteins to the resin.

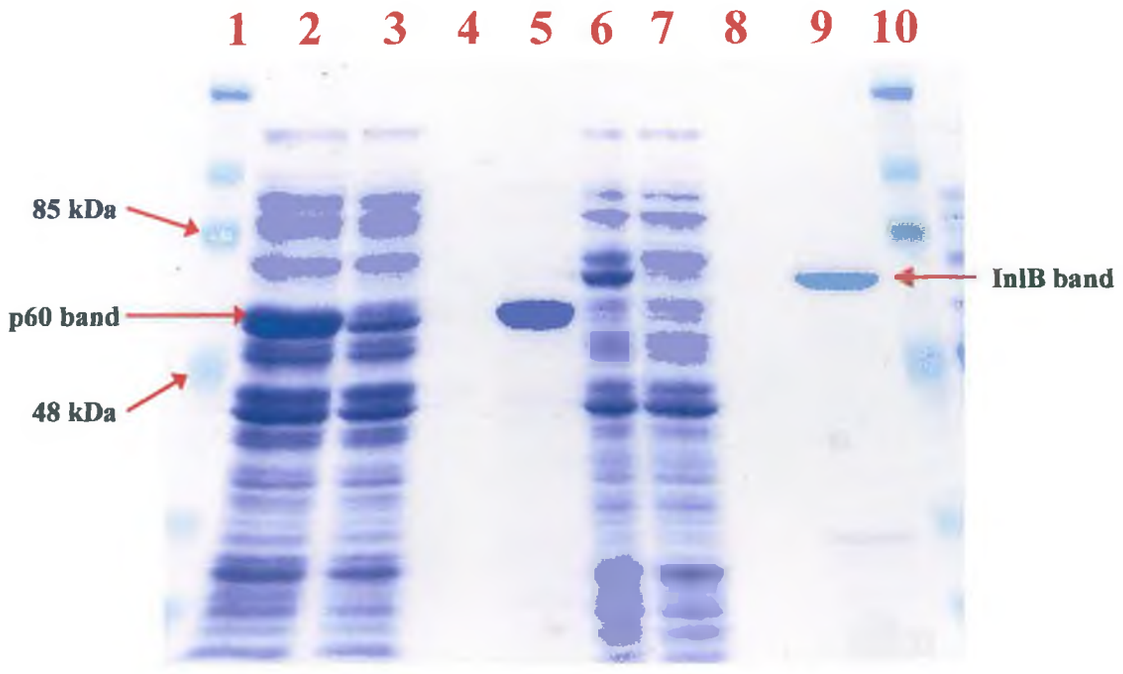


Figure 4.13: *Purification of the InlB and p60 gene products by immobilised metal affinity chromatography (IMAC). E. coli cells harbouring a recombinant gene product were induced with 0.5 mM IPTG for 5 hours at 30°C. After 5 hours the cells were centrifuged for 20 minutes at 4000 rpm and the pellets resuspended in PBS containing 0.5 M salt to a volume of 1/10 the original culture volume and sonicated (section 2.3.9). Cell lysates were incubated with a Ni-NTA resin (1/3 the lysate volume) for 1 hour at room temperature with constant mixing. The mixture was then centrifuged at 4000 rpm for 5 minutes and the supernatant decanted into a fresh tube (flow through). The Ni-NTA pellet with bound His tagged protein was washed 3 times with PBST containing 0.5 M salt and 10 mM β-mercaptoethanol as described in section 2.3.9. Bound His tagged proteins were eluted by incubation with sodium acetate buffer, pH 4.5 and neutralised with 2 M Tris. Purified proteins were analysed by SDS-PAGE to determine the purity of the protein and stored at –20°C until required. Lanes 1 and 10 represent a Pierce Blue Ranger pre-stained molecular weight marker with the 48 and 85 kDa bands indicated. Lanes 2-5 represent E. coli pQE-p60 (pQE-60 plasmid with p60 gene insert) cell lysate after 5 hours of induction with IPTG, E. coli pQE-p60 flow through after incubation for 1 hour with a Ni-NTA resin, bound p60-Ni-NTA resin washed with PBST and p60 protein eluted with pH 4.5, respectively. Lanes 6-9 represent E. coli pQE-InlB (pQE-60 plasmid with InlB gene insert) cell lysate after 5 hours of induction with IPTG, E. coli pQE-InlB flow through after incubation for 1 hour with a Ni-NTA resin, bound InlB-Ni-NTA resin washed with PBST and recombinant InlB protein eluted with pH 4.5, respectively.*

4.2.9 *Immunoreactivity of the recombinant p60 and InlB proteins*

To determine the potential use of the rp60 and rInlB proteins in an immunoassay for the detection of *Listeria monocytogenes* or for the production of anti-p60/InlB protein-specific antibodies, the immunoreactivity of each protein with anti-*Listeria monocytogenes* derived antibodies was evaluated. Anti-p60 and anti-InlB monoclonal antibodies were used to detect the rp60 and rInlB proteins by Western blotting, while anti-InlB extract and anti-*Listeria monocytogenes* polyclonal antibodies were used to detect the recombinant proteins by ELISA.

Figure 4.14, shows the detection of the rp60 and rInlB proteins by Western blotting. *E. coli* lysates harbouring the recombinant proteins were probed with a protein-specific monoclonal antibody (lanes 1 and 3) or probed with a monoclonal antibody specific for the 6xHis affinity tag (lanes 6 and 7) found at the C terminus of each protein. Both the rp60 and rInlB proteins could be specifically detected with monoclonal antibodies raised against the native form of the protein indicating that both proteins can be used for the generation of protein-specific antibodies. The 6xHis tag of each protein was easily detected with an anti-His tag-specific monoclonal antibody indicating the tag is not hidden in the protein structure and no proteolytic activity has occurred at the C-terminus of the protein.

Detection of the recombinant proteins under native conditions was performed by ELISA using polyclonal antibodies raised against InlB extract and *Listeria monocytogenes* cells (described in chapter 3). The recombinant p60 protein was successfully detected with the anti-*Listeria monocytogenes* polyclonal antibody in an inhibition ELISA format (Figure 4.15). Decreasing concentrations of recombinant p60 protein were incubated with the anti-*Listeria monocytogenes* antibody and added to recombinant p60-coated ELISA plates. While the anti-*Listeria monocytogenes* antibody did recognise the rp60 protein, the sensitivity of the curve was not good (limit of detection of approximately 3 μg/ml of purified protein) and a specific monoclonal or recombinant antibody should be used in development of an immunoassay with the rp60 protein. The recombinant InlB protein was detected by sandwich ELISA (Figure 4.16) as described in section 2.3.12. The results presented in Figure 4.15 and 4.16 show the immunoreactivity of the recombinant proteins in native conditions and indicate that both proteins can be used in the development of a protein-specific immunoassay or for the generation of protein-specific antibodies by immunisation or by bio-panning a combinatorial antibody library.

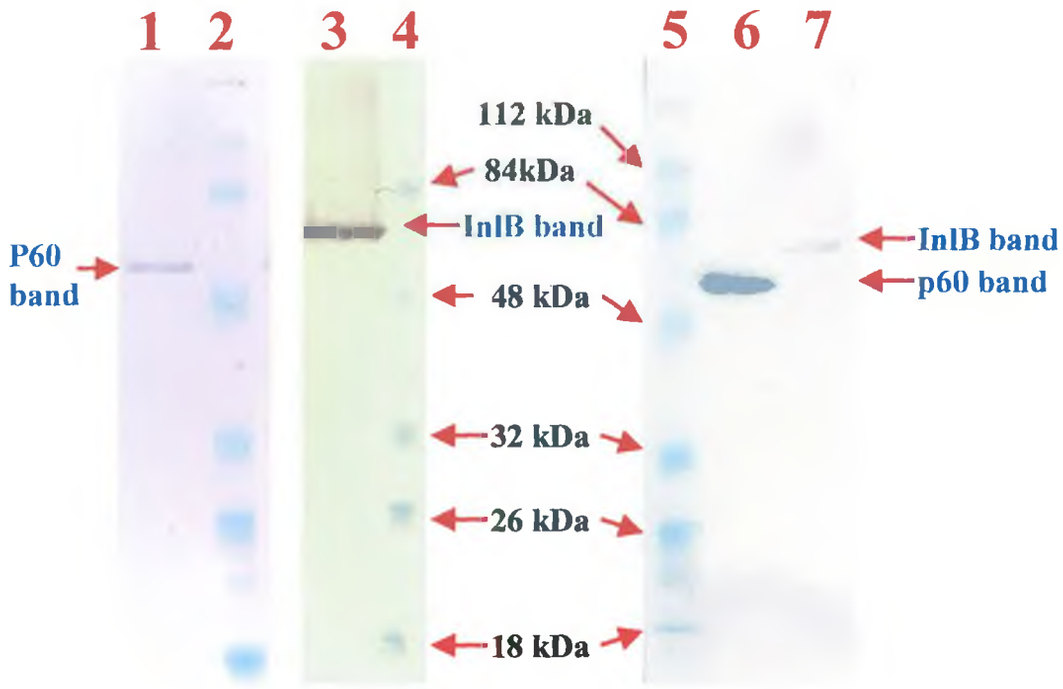


Figure 4.14: *Detection of the recombinant p60 and InlB proteins by Western blotting. E. coli lysates containing the recombinant p60 or recombinant InlB proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane as described in section 2.2.7. Lanes 2, 4 and 5 represent a Pierce Blue Ranger pre-stained marker with molecular weights indicated. Lane 1 represents the detection of recombinant p60 protein with an anti-p60-specific monoclonal antibody, lane 3 represents the detection of InlB with an anti-InlB-specific monoclonal antibody and lanes 6 and 7 represent the detection of p60 and InlB with an anti-His tag monoclonal antibody (Sigma), respectively. The detection of both proteins with specific monoclonal antibodies confirms the immunoreactivity of the recombinant proteins with antibodies specific for the native form of each protein. The detection of the His tag of each protein confirms that the His tag is accessible and no cleavage of the tag has occurred. The anti-p60 and anti-InlB monoclonal antibodies used in this blot were donated by Stephen Hearty, Dublin City University.*

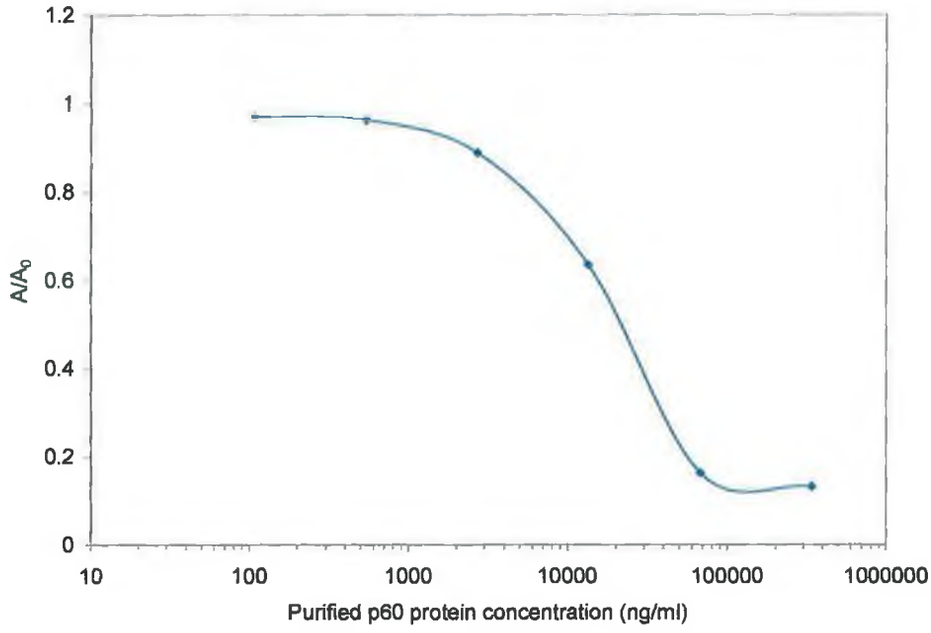


Figure 4.15: *Inhibition ELISA for the detection of recombinant p60 (rp60) with an anti-Listeria monocytogenes polyclonal antibody. To determine the immunoreactivity of the rp60 under native conditions and to evaluate its use in ELISA, an inhibition ELISA was developed with the anti-Listeria monocytogenes polyclonal antibody discussed in chapter 3. The ELISA plate was coated with 30 μg/ml of purified rp60 and blocked with 5% (w/v) milk marvel. Decreasing concentrations of purified rp60 were incubated with a 1/5000 dilution of the anti-Listeria monocytogenes antibody for 30 minutes before being added to the plate in triplicate. The inhibition curve shows a large decrease in binding with increasing competing rp60 concentrations.*

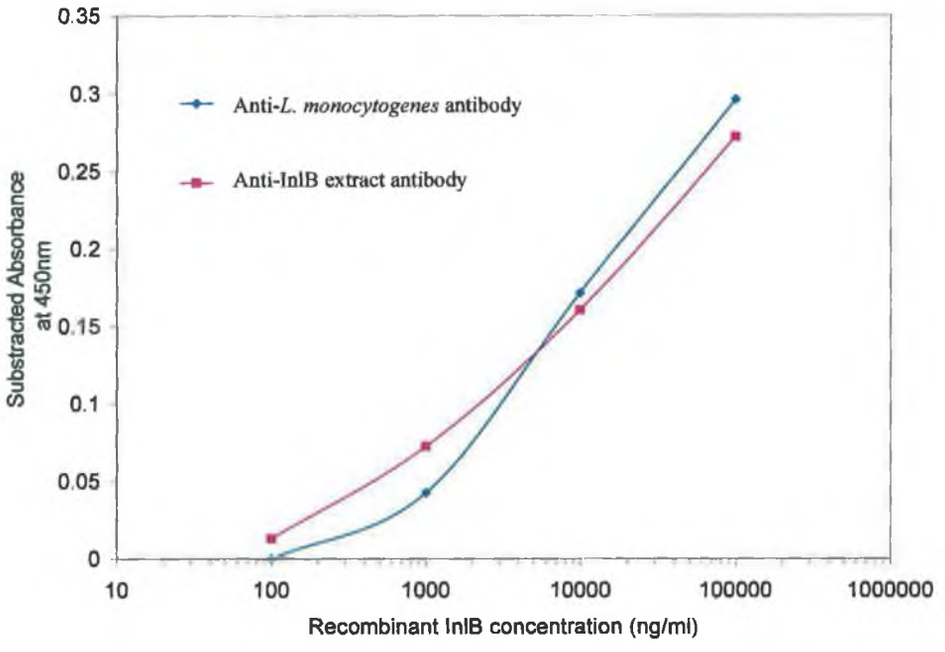


Figure 4.16: *Sandwich ELISA for the detection of recombinant InlB (rInlB) with an anti-InlB Extract-HRP conjugated polyclonal antibody. To determine the immunoreactivity of the rInlB under native conditions and to evaluate its use in ELISA, a sandwich ELISA was developed with the anti-InlB extract and anti-Listeria monocytogenes polyclonal antibodies discussed in chapter 3. An ELISA plate was coated with 30 µg/ml of purified anti-InlB extract or anti-Listeria monocytogenes antibody and blocked with 5% (w/v) milk Marvel. Decreasing concentrations of purified rInlB protein were added to the ELISA plate in triplicate and incubated for 1 hour as described in section 2.3.12. Bound protein was detected with a 1/100 dilution of an anti-InlB extract-HRP-labelled polyclonal antibody (section 2.3.11) and the average of triplicate results plotted. The curves shown represent the capture of the rInlB protein with either the anti-InlB extract or anti-Listeria monocytogenes antibody and the detection of bound rInlB with the HRP-conjugated anti-InlB extract polyclonal antibody. The absorbance values shown are the absorbance values obtained minus the background values. The result shows the immunoreactivity of the rInlB protein in native conditions and indicates that either antibody can be used for the capture of the rInlB protein on the surface of the ELISA plate.*

4.3 *Discussion*

The aim of this chapter was to generate sufficient quantities of recombinant InlB and p60 proteins for use in the development of an immunoassay for the detection of *Listeria monocytogenes* cells and for the selection of protein-specific recombinant antibodies (described later in chapter 5). The InlB and p60 gene sequences were successfully amplified from genomic DNA extracted from *Listeria monocytogenes* cells and the optimum annealing temperature determined as shown in Figure 4.2. Fresh amplified PCR products were cloned into the TA cloning vector, pCR 2.1 and transformed into *E. coli* INV αF' cells. Transformants containing amplified PCR products were plated on 2xTY agar plates containing X-Gal providing blue-white screening for the identification of positive clones (Figure 4.3). Plasmid DNA was extracted from selected clones, digested with *NcoI* and *BamHI* restriction enzymes and run on an agarose gel (Figure 4.5). The InlB and p60 gene sequences were then purified from the gel and ligated into the pQE-60 vector for the over expression and purification of the recombinant InlB and p60 proteins. As described in section 4.2.3, the pQE-60 vector contains an optimised promoter-operator element consisting of a phage T5 promoter (recognised by *E. coli* RNA polymerase) and two *lac* operator sequences which increase *lac* repressor binding and ensure efficient repression of the powerful T5 promoter. Each vector also contains a synthetic ribosomal binding site for high translation rates, a 6xHis tag (located 3' to the multiple cloning site) for convenient detection and purification, strong transcriptional terminators to prevent read-through transcription and ensure stability of the expression product and a β-lactamase gene (bla) conferring resistance to ampicillin.

A series of time course experiments were performed to determine the expression of the InlB and p60 gene products. Figures 4.6 and 4.7 show the expression of the InlB and p60 proteins upon induction with 1 mM IPTG. Both proteins were easily expressed in *E. coli* XL10 Gold cells, with the *Listeria monocytogenes* extracellular protein, p60 being expressed at a significantly higher level. Both proteins were mainly expressed in the soluble form with little or no protein forming inclusion bodies (Figure 4.8). By comparing cytoplasmic lysates and periplasmic lysates from cells harbouring each protein sequence, it was determined that both the InlB and p60 proteins were expressed mainly in cytoplasm (Figure 4.9). The cloned InlB and p60 gene sequences were sequenced (section 4.2.6) and their amino acid sequences compared with the protein amino acid sequences provided by Genebank. Both the InlB and p60 cloned amino acid sequences showed 100% identity with the protein sequences provided by Genebank (accession numbers AJ012346 and X52268). To evaluate the optimum IPTG concentration for the expression of the InlB and

p60 gene products, a series of time course experiments were performed with various IPTG concentrations. Decreasing the IPTG concentration from 1 mM to 0.05 mM had no significant effect on the expression of the p60 and InlB gene products. Without the induction of IPTG, no p60 nor InlB expression was evident, which indicates that *E. coli* strains, such as XL10 Gold, harbouring the *laqI* ^{q} gene, produce sufficient quantities of the *lac* repressor protein to prevent undesired basal expression of recombinant proteins.

Stringent binding and washing conditions should be used for the specific purification of recombinant proteins from *E. coli* by immobilised metal affinity chromatography (discussed in section 4.2.8). The running buffer used to purify the p60 and InlB proteins (section 2.3.9) was designed to reduce non-specific hydrophobic interactions by the addition of 1% (v/v) Tween 20. Non-specific ionic interactions and the repulsion of the positively charged recombinant p60 or InlB proteins with the positively charged Ni-NTA resin were reduced by the addition of 500 mM NaCl. The co-purification of host proteins that may have formed disulfide bonds with the protein of interest during cell lysis was also reduced by the addition of 10 mM β-mercaptoethanol. Figure 4.13 shows the purification of the recombinant p60 and InlB proteins as described in section 2.3.9. Ni-NTA bound His-tagged proteins were eluted by reducing the pH to 4.5 and analysed by SDS-PAGE. Figure 4.13, lanes 5 and 9 shows the elution of the recombinant p60 and InlB proteins resulting in the presence of concentrated protein bands (representing the rp60 and rInlB proteins) at the expected molecular weights. Overall, the purification of the recombinant proteins by IMAC allowed the one step purification of each protein to good levels of homogeneity without the need for subsequent purification steps.

To evaluate the potential use of the rp60 and rInlB proteins in an immunoassay for the detection of *Listeria monocytogenes* or for the production of anti-p60/InlB protein-specific antibodies, the immunoreactivity of each protein with anti-*Listeria monocytogenes*-derived antibodies was determined. Both the rp60 and rInlB proteins could be specifically detected with monoclonal antibodies raised against the native form of the protein and against the 6xHis tag (Figure 4.14), indicating that both proteins can be used for the generation of protein specific antibodies and that the 6xHis tag is not hidden in the protein structure or no proteolytic activity has occurred at the C-terminus of the protein. The detection of both recombinant proteins by ELISA (Figure 4.15 and 4.16) shows the immunoreactivity of the recombinant proteins in native conditions and indicates that both proteins can be used in the development of a protein-specific immunoassay or for the generation of protein specific antibodies by immunisation or by bio-panning a combinatorial antibody library.

Overall, cloning, expression and purification of the *Listeria monocytogenes* p60 and InlB gene sequences in *E. coli* using the strategy described in this chapter provides a safe (no need to handle live *Listeria monocytogenes* cells), reliable (correct DNA sequences are cloned in *E. coli*) and convenient (bacterial cells can easily be induce to express recombinant proteins with IPTG) method for the production of high levels of recombinant protein (18 and 7mg/l for p60 and InlB, respectively (see section 4.2.5)) and allows the simple one step purification (IMAC) of desired protein. To my knowledge this is the first time that both InlB and p60 have been expressed in *E. coli*.

Chapter 5

Selection of recombinant scFv antibodies to *Listeria monocytogenes*

5.1. *Introduction*

As discussed in chapter 1, the emergence of recombinant antibody phage display technology has transformed the way in which we generate antibodies for the specific detection of a chosen analyte (e.g. *Listeria monocytogenes*). Engineering antibodies and antibody fragments in *E. coli,* allows modifications to be introduced in the primary antibody sequence leading to affinity maturations, generation of fusion proteins and the addition of detection and purification tags. The aim of the work described in this chapter was to produce a recombinant single chain Fv fragment antibody specific for *Listeria monocytogenes* cells. The selected antibody would be characterised and used to develop an immunoassay to detect *Listeria monocytogenes.*

The results presented in this chapter involve the generation of two antibody libraries developed from mice immunised with heat-treated (HT) *Listeria monocytogenes* cells and InlB protein extract. The selection of phage-scFv antibodies from these libraries and a naïve human antibody library donated by Cambridge Antibody Technology Ltd, Cambridge, UK, is described and selected antibodies characterised. The chapter is set out in four sections describing the production of murine antibody libraries, bio-panning of the murine antibody libraries against *Listeria monocytogenes* cells, bio-panning of a naïve human antibody library against *Listeria monocytogenes* cells and finally, bio-panning of a naïve human library antibody against *Listeria monocytogenes* invasion-associated proteins, Internalin B and p60.

Antibody variable domain genes were amplified from RNA extracted from the spleens of immunised mice according to the protocols described by Krebber *et al.* (1997a). Amplified antibody variable heavy and light chain genes were assembled by splice overlap extension PCR and ligated into a pAK100 vector for phage display of the scFv antibodies. Two murine antibody libraries, produced from RNA taken from mice immunised with *Listeria monocytogenes* cells and from mice immunised with InlB extract, were screened against HT-*Listeria monocytogenes* cells using a process known as bio-panning. Six phage-scFv antibodies were selected from the anti-InlB extract library and three phage-scFv antibodies selected from the anti-*Listeria monocytogenes* antibody library. The scFv genes from each of the selected antibodies were sequenced and the genes aligned using a multiple alignment program. Antibodies were further evaluated for soluble expression and reactivity with other non-*Listeria monocytogenes* strains of bacteria. Each of the nine selected phage scFv-antibodies failed to express scFv antibody in soluble form. Also each of the phage-scFv antibodies was observed to cross-react with

bacteria from the genus *Listeria* and therefore were not further characterised for the development of an immunoassay for the specific detection of *Listeria monocytogenes* cells.

In search of a species specific recombinant antibody, a naïve human antibody library, first described by Vaughan *et al.* (1996), with a diversity of 1.4×10^{10} clones, was used to select for scFv antibodies with the ability to bind *Listeria monocytogenes* cells. The antibody library was bio-panned with HT-*Listeria monocytogenes* cells and the scFv genes of each of the selected antibodies sequenced. The antibodies were also further evaluated for soluble expression and reactivity with other non-*Listeria monocytogenes* strains of bacteria. Four rounds of bio-panning were performed and two phage-scFv antibodies selected. Alignment of the antibody gene sequences revealed that both antibodies were identical. However, this antibody was observed to cross react with non-*Listeria monocytogenes* bacterial cells and did not express as soluble scFv antibody.

In order to refine antibody specificity, the naïve human antibody library was also bio-panned with purified recombinant Internalin B and p60 invasion-associated proteins. A number of antibodies were selected from the library that recognised the *Listeria monocytogenes* invasion-associated protein, InlB. Further examination of these antibodies, revealed that even though the antibodies recognised InlB, none of the selected antibodies reacted with *Listeria monocytogenes* cells. Internalin B is a membrane associated protein (see chapter 1 for a description of InlB) which is anchored to the cell surface via a glycine-tryptophan rich repeat region. Antibodies, binding to this region would react with soluble InlB protein but would not be able to bind *Listeria monocytogenes* cell-associated InlB.

A soluble single chain Fv fragment antibody, capable of binding both the recombinant and native forms of InlB was used to develop an inhibition ELISA-based immunoassay for the indirect detection of *Listeria monocytogenes* cells. InlB was extracted from *Listeria monocytogenes* cells and reacted with *E. coli* anti-InlB scFv antibody culture supernatant. Antibody and InlB extract were subsequently added to recombinant InlB-coated ELISA plates, with an increase in binding observed with decreasing InlB extract concentrations. Intra- and interday assay variability studies were carried out to determine the reproducibility of the immunoassay developed.

5.2. *Production of murine antibody libraries for the selection of anti-Listeria monocytogenes single chain Fv antibodies*

The Krebber system (Krebber *et al.*, 1997a) described in section 1.13, is designed for the reliable cloning of functional antibody variable domains. This system was used to develop scFv antibody libraries from spleen cells of mice immunised with heat-treated *Listeria monocytogenes* cells and InlB extract.

5.2.1 *Immunisation of mice with heat-treated Listeria monocytogenes cells and InlB extract*

Antibody libraries developed from immunised hosts have a distinct advantage over naïve antibody libraries in that the hosts immune system is primed towards the antigen of interest before the antibody variable genes are extracted. To increase the possibility of selecting high affinity antibodies Balb/c mice were immunised with *Listeria monocytogenes* cells or InlB extract as described in section 2.4.1.1. Serum from the tail of each mouse was analysed by ELISA (section 2.2.3) and the antibody titres observed (Figures 5.1 and 5.2). Serial dilutions of mice antiserum were added to ELISA wells pre-coated with either heat-treated (HT) *Listeria monocytogenes* cells or InlB extract and detected with a rabbit anti-mouse HRP labelled antibody. Mice A and B immunised with InlB extract titred out at approximately 1/50,000 and 1/100,000, respectively, when assayed over InlB extract-coated wells (Figure 5.1). Mice A and B immunised with HT-*Listeria monocytogenes* cells both titred out between approximately 1/50,000 and 1/100,000 (Figure 5.2) when assayed over HT-*Listeria monocytogenes*-coated wells. These serum titrations indicated a sufficient response from the host to isolate splenomic mRNA as described in section 2.4.1.2.

5.2.2 *Selection of primers for the amplification of murine heavy and light chain genes*

The primers listed in section 2.4.2.1 were based on the primer set described by Krebber *et al.* (1997a), for assembling mouse scFv fragments in the orientation V_L-$(G_4S)_4$-V_H, where V_L and V_H are the variable light and heavy chain domains, respectively, and $(G_4S)_4$ is the 20 amino acid glycine-serine repeat linker. Assembled mouse scFv fragments are compatible with all the pAK vector constructs presented in Figure 1.12. The primer set incorporates all mouse V_H and V_λ and V_κ sequences collected in the Kabat data base (Kabat *et al.*, 1991) and combines some extended primer sets (Kettleborough *et al.*, 1993; Ørum *et al.*, 1993 and Zhou *et al.*, 1994).

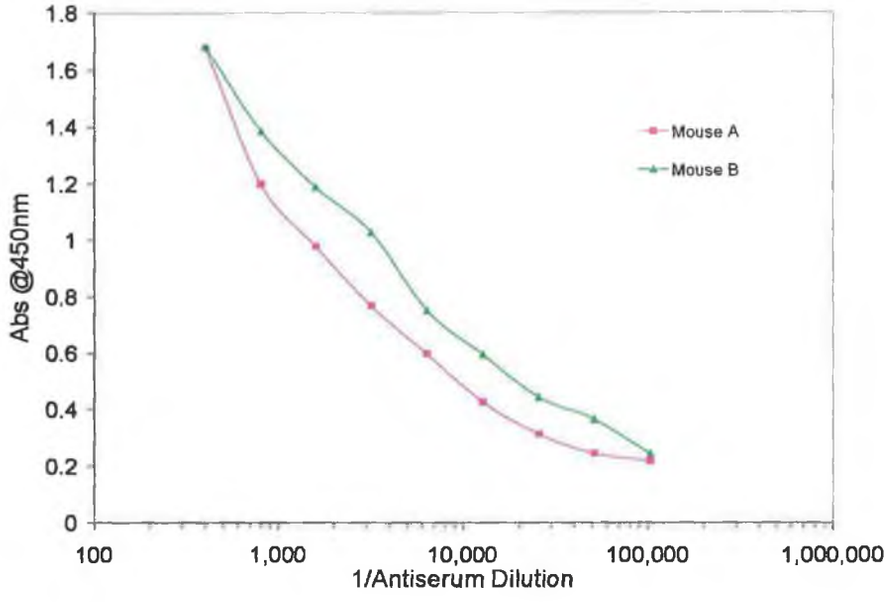


Figure 5.1: *Titre of tail bleed serum taken from mice immunised with InlB extract. The serum was diluted in PBS and added to wells coated with InlB extract. Bound mouse antibodies were detected using a rabbit anti-mouse HRP labelled antibody followed by the addition of OPD substrate. Absorbance values were read at 450 nm.*

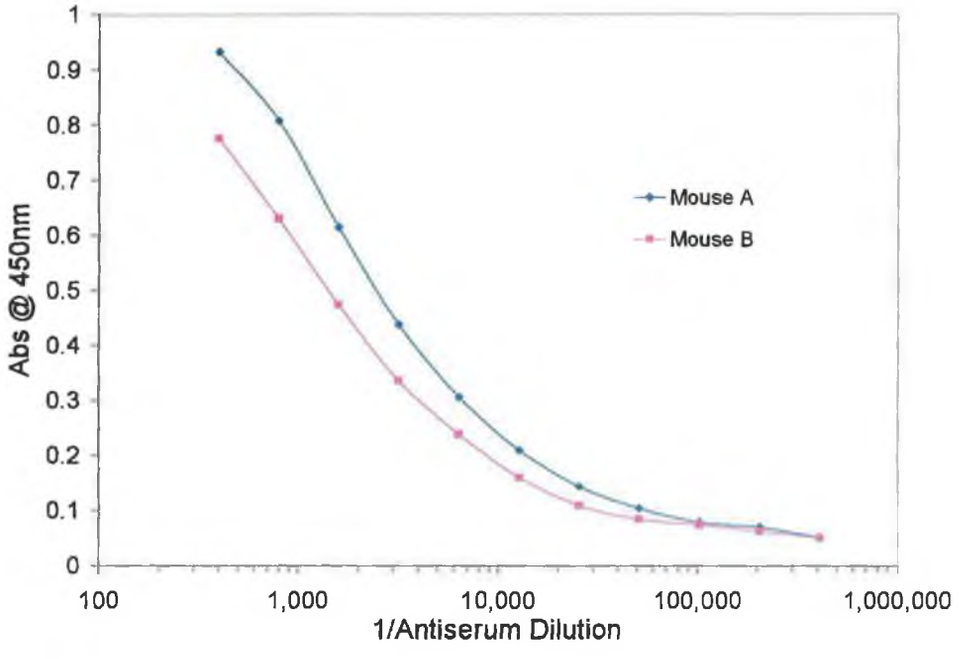


Figure 5.2: *Titre of tail bleed serum taken from mice immunised with heat-treated (HT) Listeria monocytogenes cells. The serum was diluted in PBS and added to wells coated with HT-Listeria monocytogenes cells. Bound mouse antibodies were detected using an anti-mouse HRP-labelled antibody followed by the addition of OPD substrate. Absorbance values were read at 450 nm.*

5.2.3 *Amplification of variable heavy (V_H) and variable Light (V_L) antibody domains*

Reverse transcription of mouse mRNA to cDNA was carried out as described in section 2.4.1.3 and used as a template for the amplification of mouse variable heavy and light chain genes. The amplified PCR products were electrophoresed on a 1% (w/v) agarose gel and the expected bands of 375-402 bp (Figure 5.3) for V_L or 386-440 bp (Figure 5.4) for V_H observed. The variable heavy and light chain fragments were purified from the gel as described in section 2.4.2.4.

5.2.4 *Assembly of V_H and V_L genes by splice by overlap extension (SOE) PCR*

Purified V_H and V_L domains were ligated together by SOE PCR as described in section 2.4.2.6 producing a 800 bp band (Figures 5.5 and 5.6). PCR amplification with different templates and primers has a wide range of optimal reaction conditions. Proper PCR amplification usually requires the determination of optimal conditions for the amplification of a desired product. A PCR Optimizer™ kit from Invitrogen was used to determine the optimal buffer composition for the SOE PCR. A buffer composition of 7.5 mM $MgCl_2$, pH 9.0 (buffer E) was optimal for both SOE PCR reactions (lane 6 in Figures 5.5 and 5.6).

5.2.5 *Digestion of pAK100 vector and SOE-PCR products formed by linking antibody light and heavy chain genes*

Gel purified pAK100 vector and SOE PCR products (section 2.4.2.4) were digested with *SfiI* restriction enzyme as described in section 2.4.2.8 overnight at 50°C. Restricted products were again purified from an agarose gel and ligated in the presence of DNA ligase (section 2.4.2.9) at 15°C overnight.

5.2.6 *Transformation of E. coli XL1-Blue competent cells with pAK100 vector containing light and heavy chain genes*

High efficiency competent *E. coli* XL1-Blue cells (section 2.4.2.10), were used for the production of a recombinant antibody libraries to *L. monocytogenes* and InlB extract using the procedure described in section 2.4.2.11. Ligation reactions of pAK100 vector and SOE-PCR products were transformed resulting in the production of recombinant libraries consisting of approximately 5-6 x 10^3 transformants each.

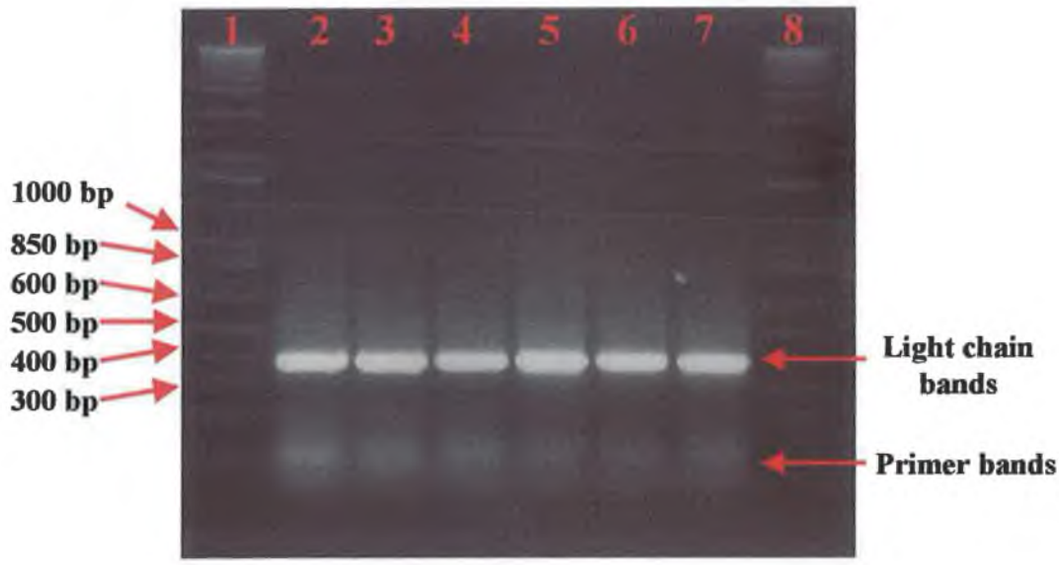


Figure 5.3: *Light chain PCR amplification. Rows 1 and 8 are 1kb DNA ladder. Lanes 2-4 are light chain DNA bands from mice immunised with HT-L. monocytogenes and lanes 5-7 are light chain DNA bands from mice immunised with InlB extract.*

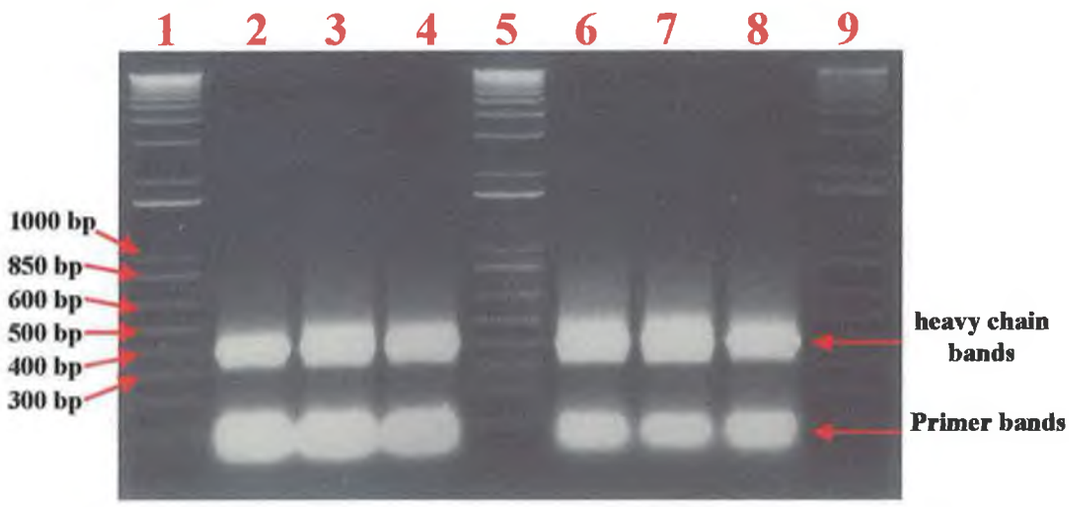


Figure 5.4: *Heavy chain PCR amplification. Rows 1 and 9 are 1kb DNA ladder. Lanes 2-4 are heavy chain DNA bands from mice immunised with HT-L. monocytogenes* cells *and lanes 6-8 are heavy chain DNA bands from mice immunised with InlB extract.*

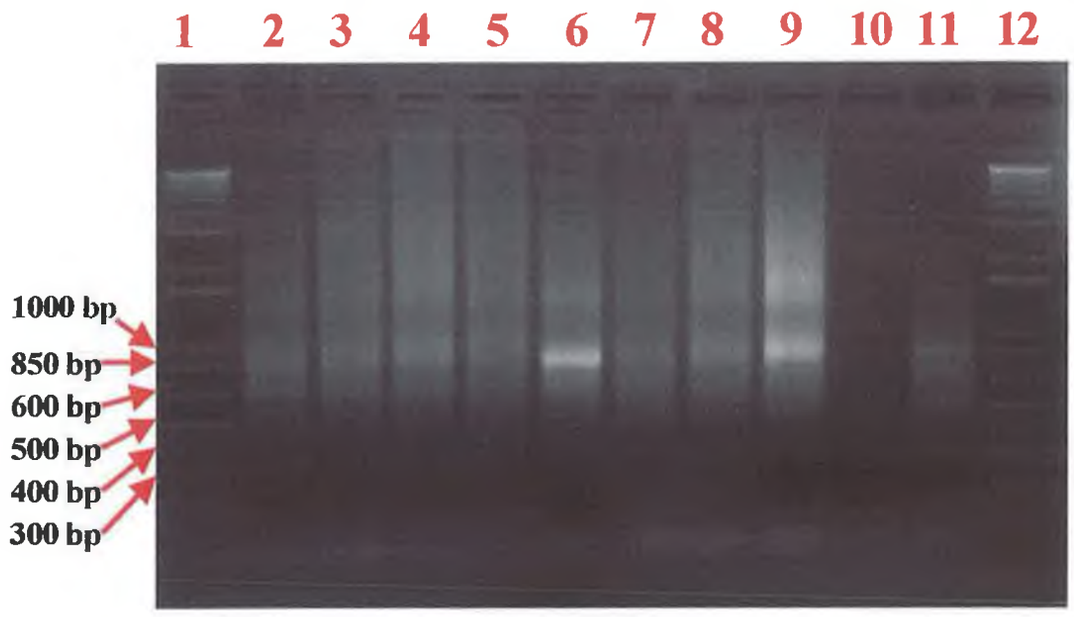


Figure 5.5: *SOE PCR optimisation of variable domain DNA from mice immunised with HT-Listeria monocytogenes. Lanes 1 and 12 represent a Gibco 1kb plus DNA ladder and lanes 2-11 represent optimisation buffers A-J (see section 2.4.2.6 for buffer compositions). Buffer E (lane 6, 7.5 mM $MgCl_2$, pH 9.0) produced the best product (clear band at 800bp).*

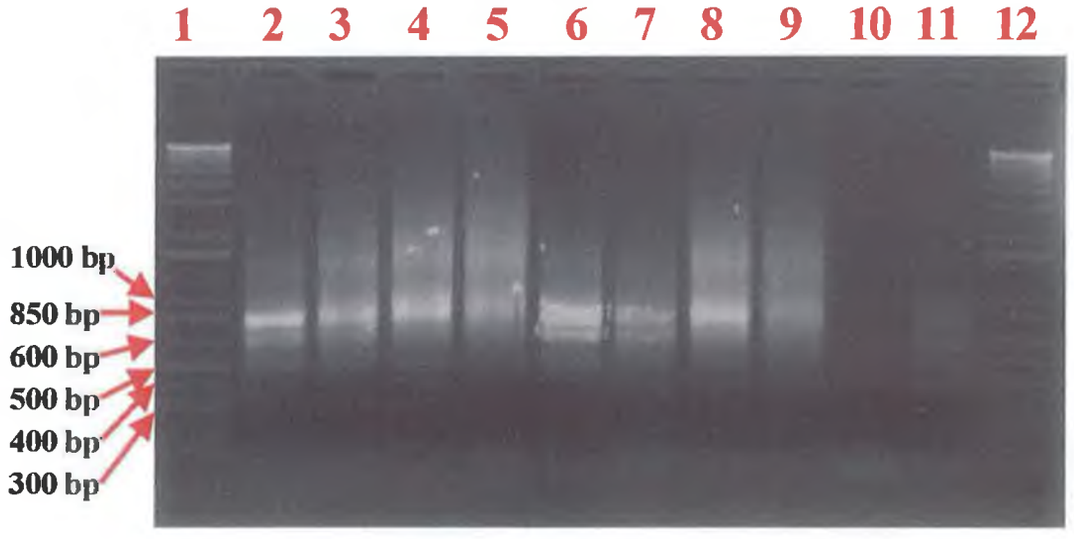


Figure 5.6: *SOE PCR optimisation of variable domain DNA from mice immunised with InlB extract. Lanes 1 and 12 represent a Gibco 1kb plus DNA ladder and lanes 2-11 represent optimisation buffers A-J. Buffer E (lane 6, 7.5 mM $MgCl_2$, pH 9.0) produced the best product while buffer A (lane 1, 7.5nmM $MgCl_2$, pH 8.5) also produced a good 800 bp band.*

5.3. *Production of single chain Fv antibodies to Listeria monocytogenes cells*

The selection of scFv antibodies to a given target usually requires the enrichment of specific antigen-binding clones by bio-panning and the characterisation of binders by ELISA. This section describes the selection of phage scFv antibodies to HT-*Listeria monocytogenes* cells, the alignment of antibody genes and the specificity of the antibodies for their target, HT-*Listeria monocytogenes* cells.

5.3.1 *Pre-panning selection and screening of functional scFv sequences to Listeria monocytogenes cells and characterisation by ELISA*

Ninety five colonies from each library were picked from the library stock plates before phage rescue and bio-panning. A phage ELISA (section 2.4.3.6) was carried out with each clone chosen and the results recorded (Figure 5.7). Three clones from the anti-InlB extract antibody library (clones B1, G9 and B12, Figure 5.7, panel A) and three clones from the HT-*Listeria monocytogenes* antibody library (clones H1, H3 and H11, Figure 5.7, panel B) showed binding to the immobilised HT-*Listeria monocytogenes* cells. Positive clones were used to inoculate 5 ml 2xTY media and phage produced overnight at 26°C (section 2.4.3.1). Phage scFv supernatant from each of the chosen clones were further characterised by ELISA using an inhibition format (section 2.2.5). Phage scFv supernatant was incubated with decreasing concentrations of HT-*Listeria monocytogenes*-cells for 30 minutes at 37°C and then added to HT-*Listeria monocytogenes* coated ELISA plate wells. Bound phage scFv antibodies were detected with a rabbit anti-bacteriophage Fd antibody and the results plotted in Figure 5.8. All three clones selected from the anti-InlB extract library (clones B1, G9 and B12) recognised HT-*Listeria monocytogenes* cells in solution (Figure 5.8, panel A) showing a decrease in normalised absorbance with increasing HT-*Listeria monocytogenes* cell concentrations. The three clones selected from the anti HT-*Listeria monocytogenes* library did not recognise HT-*Listeria monocytogenes* cells in solution and showed no decrease in binding with increasing HT-*Listeria monocytogenes* cell concentrations. Glycerol stocks were made of each positive clone as described in section 2.4.3.7 and stored at –80°C.

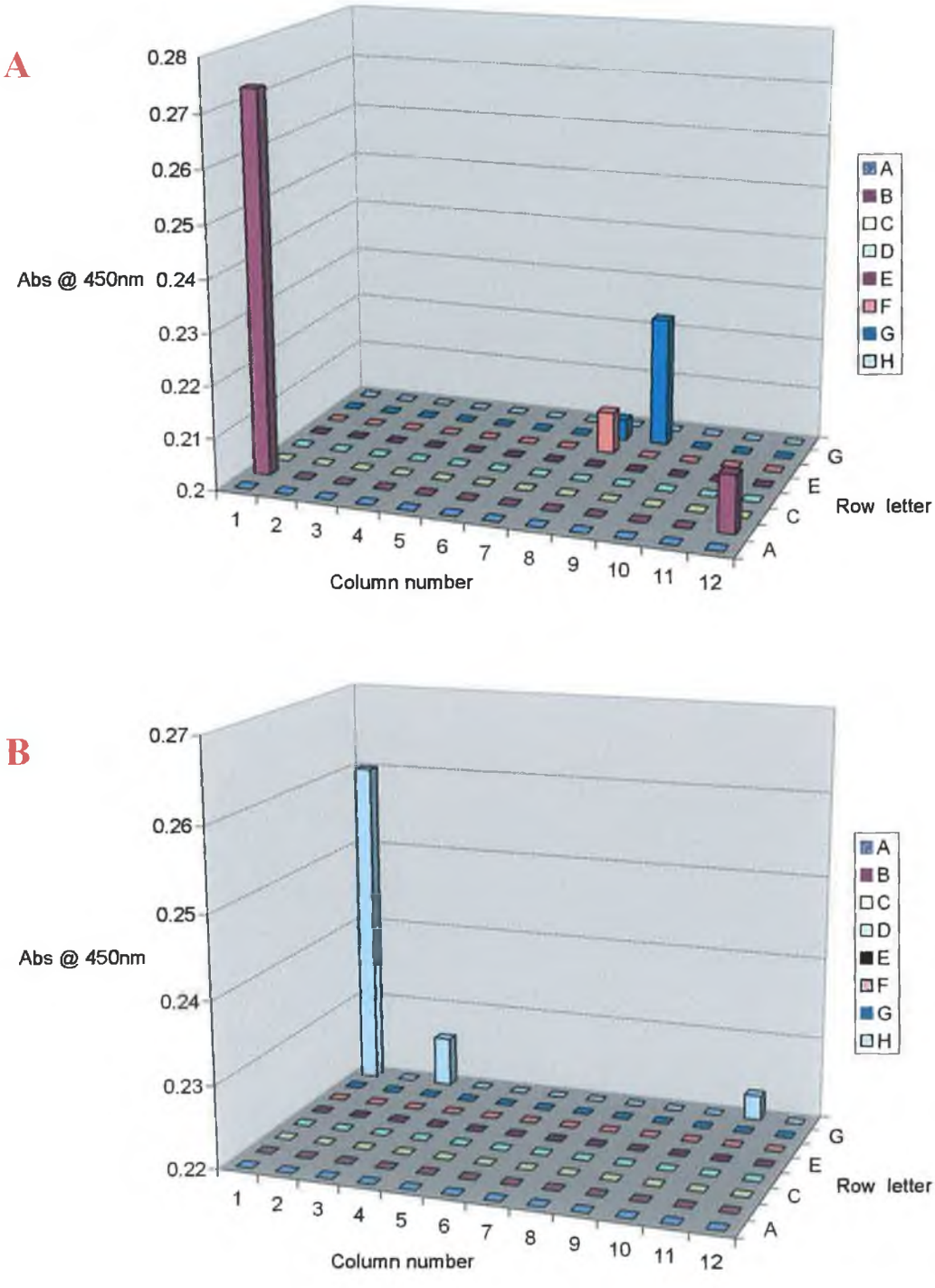


Figure 5.7: *Phage ELISA results from the selection of 95 clones from both the InlB and Listeria monocytogenes libraries with immobilised L. monocytogenes cells. Panel (A) represents the results for the InlB extract antibody library and panel (B) the results for the Listeria monocytogenes antibody library.*

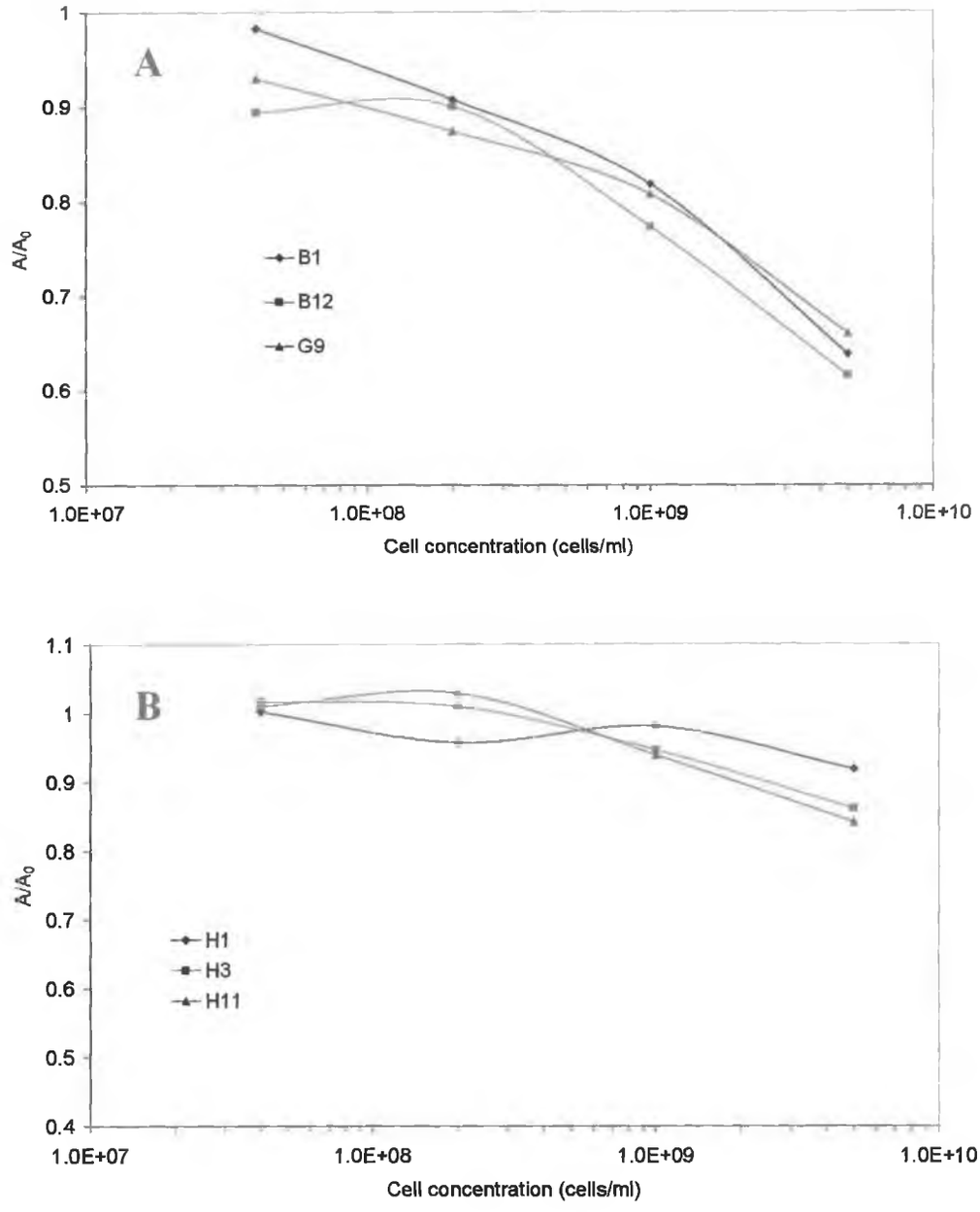


Figure 5.8: *Inhibition ELISA curves from phage-scFv clones selected from both libraries. Clones were expressed in 5 ml of 2xTY media overnight at 26°C and incubated with decreasing concentrations of heat-treated Listeria monocytogenes cells for 30 minutes. Antibody and cells were then added to ELISA plate wells that had being coated with $1x10^8$ cells/ml heat-treated Listeria monocytogenes cells and blocked with 5% (w/v) milk marvel. Three clones form the InlB extract library (labelled A) and three clones from the Listeria monocytogenes library (labelled B) were assayed. Clones B1, B12 and G9 from the InlB library showed competition with free Listeria monocytogenes cells while clones H1, H3 and H11 from the Listeria monocytogenes library showed no competition.*

5.3.2 *Selection and screening of functional scFv sequences to Listeria monocytogenes cells by bio-panning*

Prior to bio-panning both antibody libraries against HT-*Listeria monocytogenes* cells, a phage rescue was performed as described in section 2.4.3.1 and phage displaying scFv antibodies precipitated using PEG/NaCl (section 2.4.3.2) and resuspended in sterile filtered PBS. Rescued phage scFv antibodies were pre-blocked with 2% (w/v) milk marvel for 20 minutes before being added to immunotubes coated with $1x10^8$ cells/ml HT-*Listeria monocytogenes* cells and blocked with 5% (w/v) milk marvel. Phage scFv antibodies were incubated in the immunotubes for 2 hours at room temperature (section 2.4.3.4) and eluted phage scFv antibodies re-infected into *E. coli* XL-1 blue cells as described in section 2.4.3.5. A phage titre was carried out with the re-infected phage scFv clones and a titre of $1x10^4$ and $5x10^3$ colony forming units (cfu) observed for the InlB extract and HT-*Listeria monocytogenes* antibody libraries, respectively. Ninety five clones from each library were characterised by phage ELISA (Figure 5.9) against immobilised HT-*Listeria monocytogenes* cell-coated plates. Five clones from the anti-InlB extract library (clones F1, D3, G7, B10 and H1, Figure 5.9, panel A) and five clones from the anti- HT-*Listeria monocytogenes* cell library (clones D2, D6, D8, D11, C6, Figure 5.9, panel B) showed binding to immobilised HT-*Listeria monocytogenes* cells. Phage scFv antibodies were produced in 5 ml cultures over night at 26°C from all ten positive clones. Overnight phage scFv supernatant from each of the chosen clones were further characterised by an inhibition ELISA as described in section 2.2.5. Phage scFv supernatant was incubated with decreasing concentrations of HT-*Listeria monocytogenes* cells for 30 minutes at 37°C and then added to HT-*Listeria monocytogenes*-coated ELISA plate wells. Bound phage scFv antibodies were detected with a rabbit anti-bacteriophage Fd antibody and the results plotted in Figure 5.10. Three of the five clones selected from the anti-InlB antibody library (clones D3, G7 and B10, Figure 5.10, panel A) recognised HT-*Listeria monocytogenes* cells in solution showing a decrease in absorbance with increasing HT-*Listeria monocytogenes* cell concentrations. Clones D6, D8 and D11 from the anti-HT-*Listeria monocytogenes* antibody library also recognised HT-*Listeria monocytogenes* cells in solution showing a decrease in absorbance with increasing HT-*Listeria monocytogenes* cell concentrations (Figure 5.10, panel B). Figure 5.11 shows an inhibition ELISA curve of all nine phage scFv clones selected (3 from section 5.3.1 and 6 from section 5.3.2) form both antibody libraries.

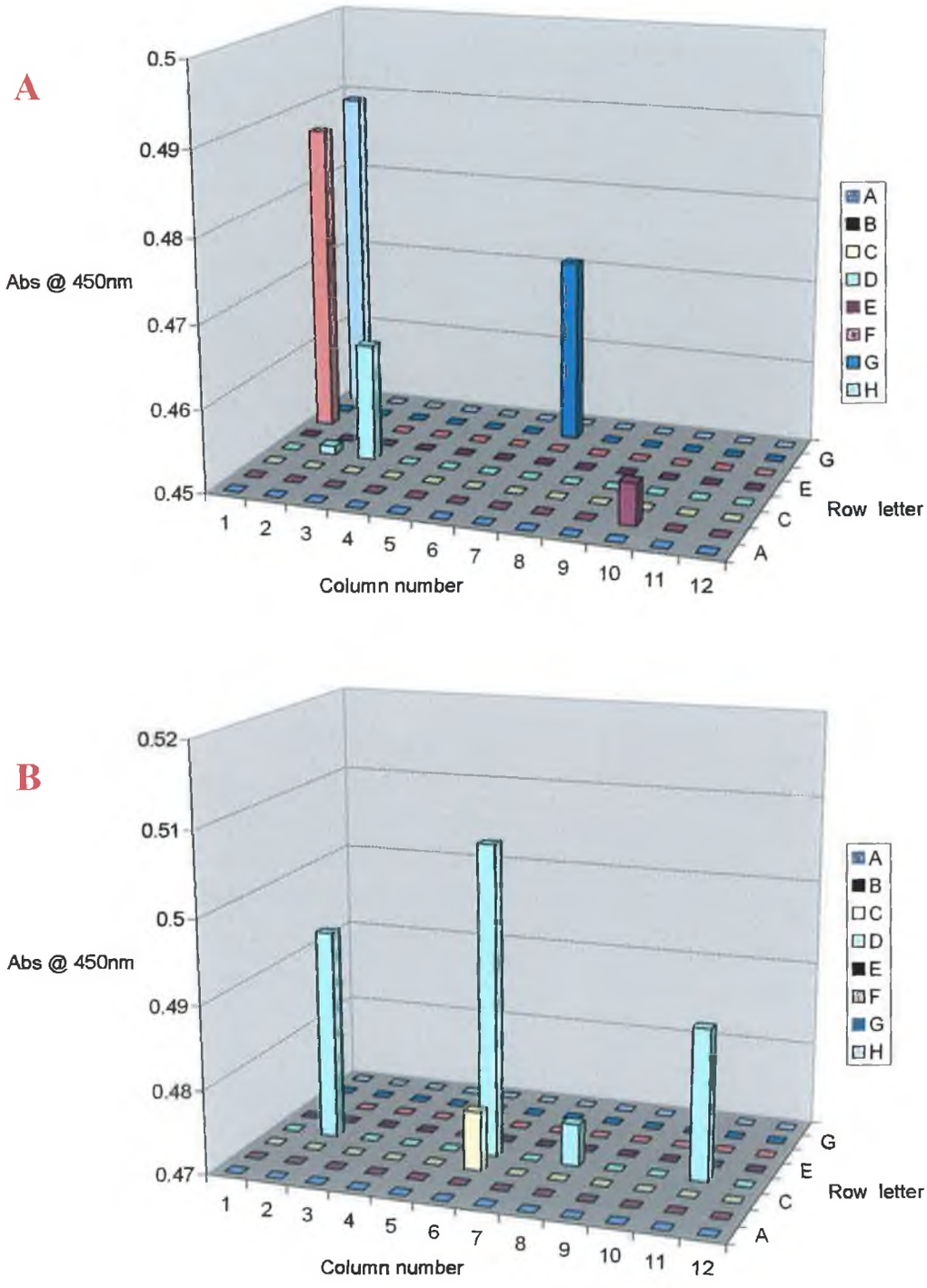
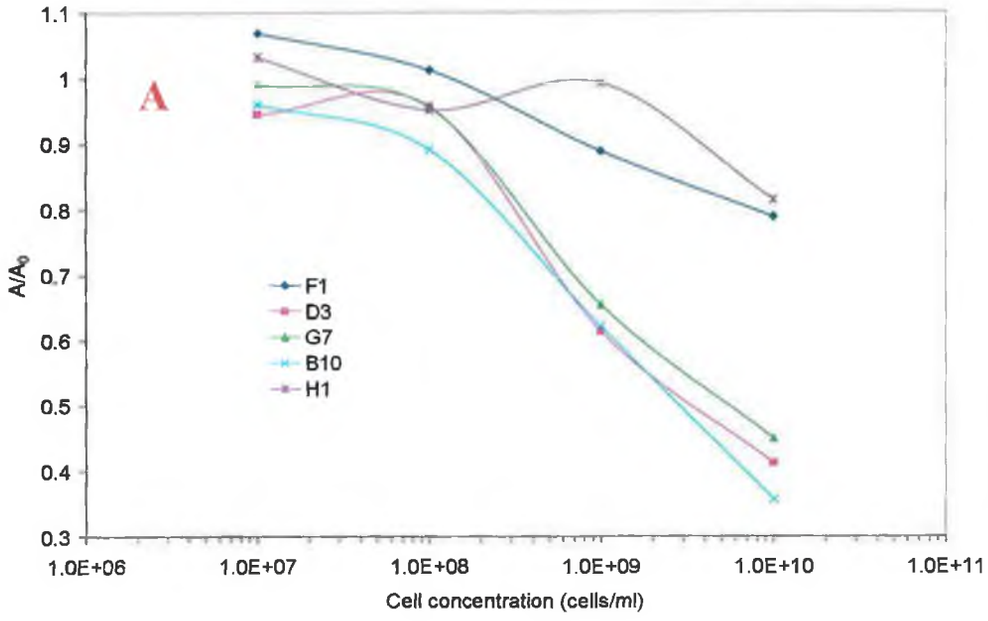


Figure 5.9: *Phage ELISA results after bio-panning both the InlB and HT-Listeria monocytogenes libraries with HT-Listeria monocytogenes cells. Panel (A) represents the results for the InlB extract library and panel (B) the results for the Listeria monocytogenes library.*



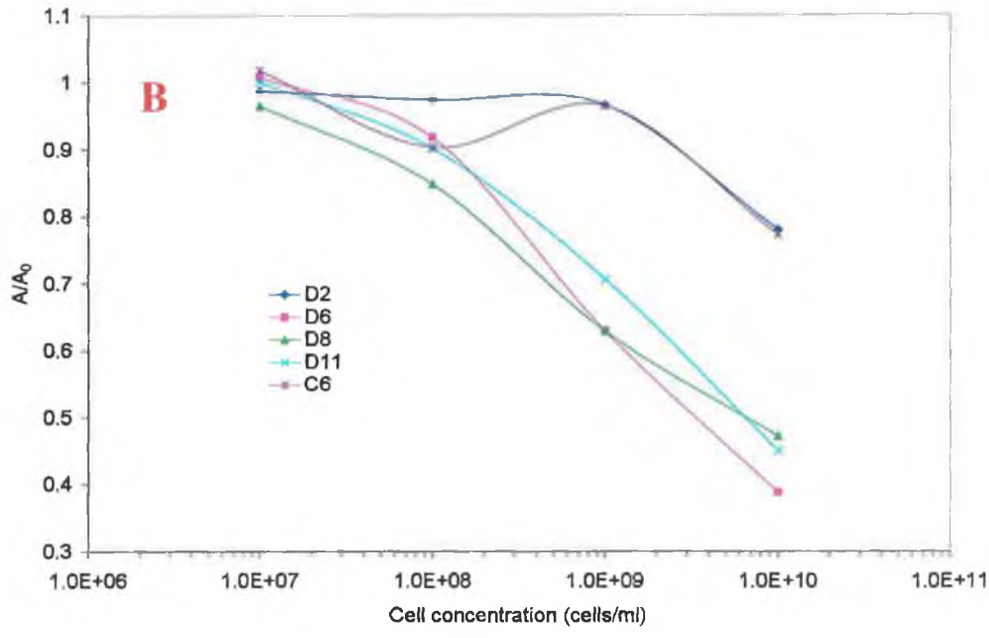


Figure 5.10: *Inhibition ELISA curves from phage-scFv clones selected from both libraries. Clones were expressed in 5 ml of 2xTY media overnight at 26°C and incubated with decreasing concentrations of heat-treated (HT) Listeria monocytogenes cells for 30 minutes. Antibody and cells were then added to ELISA plate wells that had been coated with $1x10^8$ cells/ml heat-treated Listeria monocytogenes cells and blocked with 5% (w/v) milk marvel. Five clones from the InlB library (labelled A) were assayed with three clones, D3, G7 and B10 showing competition with free HT-Listeria monocytogenes cells. Five clones from the Listeria monocytogenes library (labelled B) were also assayed with three clones, D6, D8 and D11 showing competition with free HT-Listeria monocytogenes cells. Slight competition was observed with the negative clones at high cell numbers possibly due to non-specific interference with binding due to the sheer high numbers of cells present.*

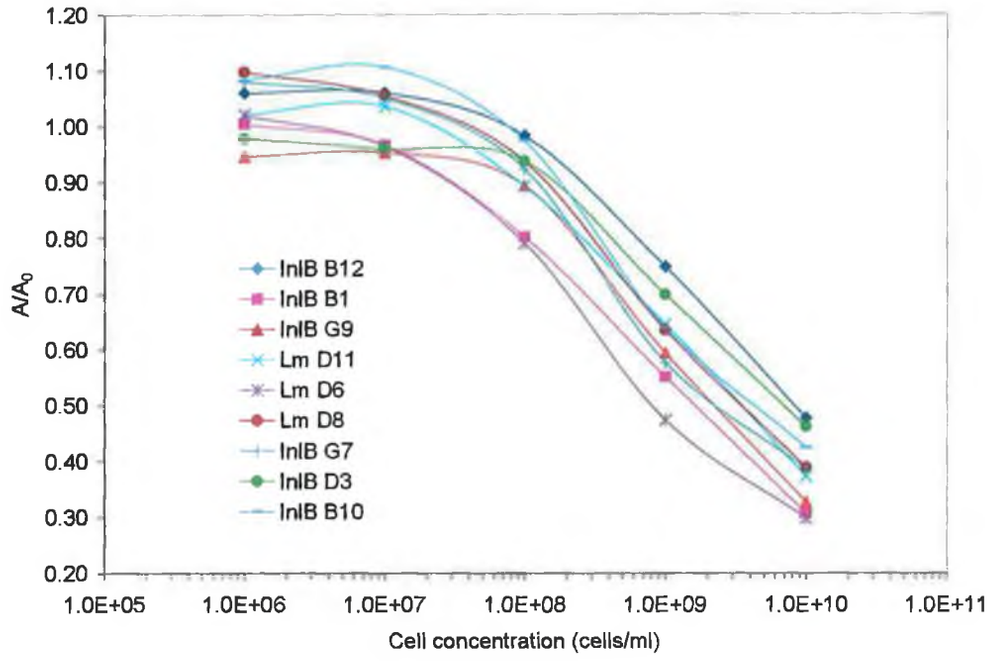


Figure 5.11: *Inhibition ELISA curves of all nine phage-scFv clones. Each clone was grown in 5 ml 2xTY media overnight at 26°C. Phage-scFv supernatant was incubated with decreasing concentrations of HT-Listeria monocytogenes cells for 30 minutes at 37°C. Antibody and cells were added to HT-Listeria monocytogenes-coated ELISA plates and bound phage-scFv antibodies detected with rabbit anti-bacteriophage Fd antibody. Clones InlB B1 (InlB library) and Lm D6 (Listeria monocytogenes library) showed the greatest levels of detection of approximately* $1x10^7$ *cells/ml.*

5.3.3 *Alignment of phage-scFv gene sequences and determination of antibody complementarity determining regions*

Bacterial stocks containing the pAK100-scFv gene sequences from the clones selected in sections 5.3.1 and 5.3.2 were grown overnight in 5 ml 2xTY containing 1% (w/v) glucose and 25 μg/ml chloramphenicol at 37°C. Plasmid DNA was purified according to section (2.4.2.7) and vacuum dried. DNA was sent to MWG-Biotech Ltd, UK, for sequencing. On receiving the DNA sequence results, the DNA sequences were translated to amino acid sequences using the Expasy translate tool (see section 2.4.3.8 for web addresses of programs used in this section) and the amino acid sequences pasted into the ClustalW program, a tool for multiple alignments. The aligned sequences from ClustalW were imported into the Genedoc program, which allows easy manipulation of sequence alignments. Using the Genedoc program identical residues were automatically identified and the CDR regions manually highlighted according to the Kabat scheme for the identification of antibody CDR regions (Figures 5.12 to 5.15).

5.3.3.1 *Alignment of the three clones selected from the InlB extract library prior to bio-panning*

Figure 5.12 shows the alignment of the three scFv clones selected from the anti-InlB library described in section 5.3.1. Light chain complementarity determining regions are highlighted in red while the heavy chain regions are highlighted in yellow. All three sequences show variation in amino acid compositions in both the framework and CDR regions. However, it can be clearly seen that there are many highly conserved residues (highlighted in black) which are present in all three clones and found throughout both the heavy and light chain domains. Each complementarity determining region is flanked on both sides by conserved residues (CDR L1 flanked by a C and W, CDR L3 flanked by a C and FGGG etc.). The complementarity determining regions of each clone show huge variations from the corresponding regions on the other clones, with the CDR L1 and H3 showing the greatest diversity with respect to identity and length. CDR L1 of clone B12 consists of 19 residues compared to 14 residues from that of clone G9. Likewise, the CDR H3 of B1 consists of 13 residues compared to 7 residues from that of clone B12. There appears to be a greater diversity in the light chain framework regions compared to that from the heavy chain framework regions as indicated by the lack of identical (highlighted in black if present in all three sequences and in grey if present in two sequences) residues. The 20 amino acid linker region (highlighted in black and labelled) allows enough

flexibility for both chains to associate to each other (see section 1.15.3 for effects of linker chain length on antibody domain association).

5.3.3.2 *Alignment of the three clones selected from the InlB extract library after bio-panning*

The three scFv clones selected from the anti-InlB library after bio-panning (section 5.3.2) are aligned in Figure 5.13. The number of conserved sequences (highlighted in black) is a lot higher than that observed in Figure 5.12 indicating a lesser degree of diversity between the sequences than that observed from the clones selected prior to bio-panning.

The gene encoding the heavy chain variable domain of an antibody is made up of three types of segments, V, D and J where V codes for approximately the first 90 residues, D mostly for the CDR 3 region and J for the remaining approximately 15 residues of the variable domain. These segments are randomly assembled (combinatorial joining) in an imprecise manner creating additional diversity (junctional diversity). In addition, extra nucleotides can be added during the joining procedure providing further diversity. The genetic mechanisms underlying the light-chain diversity are similar except that there are no D segments, and therefore the diversity of CDR 3 depends largely upon junctional diversity generated during V-J joining (Branden and Tooze, 1991). The light chain V genes from the antibodies described in this section were amplified by the light chain back primers listed in section 2.4.2.1. The light chain forward primers hybridise the J-element and encode three repeats of the Gly_4Ser linker. The heavy chain forward primers hybridise within the J region and the back primers encode for the V segments and overlap with the Gly-Ser linker. The resulting scFv formation is V_L-J_L-linker-V_H-D-J_H (Plückthun et al., 1996; Kebber et al., 1997). From the alignment in Figure 5.13, it seems that the light chains of the three antibodies have descended from the same original V and J segments as clones D3 and B10 show 100% light chain identity and clone G7 only varies in the first 3 amino acids (may vary more as no results were obtained for the rest of the chain), a substitution of isoleucine for leucine (possibly a base substitution of an adenine for a cytosine during amplification by PCR) and the presence of a stop codon (indicated by the dash highlighted in blue). The presence of the stop codon in the light chain of clone G7 may have occurred as the result of the substitution of a thymine for a cytosine in the codon sequence (the codons coding for glutamine (Q) are CAA and CAG while two of the stop codons are TAA and TAG) or simply may be a sequencing error. A base substitution of thymine for guanine could result in valine being translated instead of glycine as observed in the linker region of clone D3. This also may be an error in the PCR amplification or

sequencing. The heavy chain framework and CDR regions of clone G7 differ significantly from that of the other two clones. Clones B10 and D3, already observed to have identical light chains, also contain identical heavy chain framework regions 1 and 2 and CDR 1 and 2 regions. Considerable differences in identity are observed in the last 18 residues of the framework 3 region as well as the CDR 3 and Framework 4 regions. This observation indicates that the diversity between these clones is probably due to D and J_H segment rearrangements, which encode this region of the heavy chain domain.

5.3.3.3 *Alignment of the three clones selected from the HT-Listeria monocytogenes library after bio-panning*

The alignment of the 3 clones selected from the HT-*Listeria monocytogenes* antibody library is shown in Figure 5.14. From the number of conserved residues highlighted it is clear that there is very little diversity between the clones especially in the heavy chain region. On further investigation it is observed that clones D8 and D11 are actually the same clone and show 100% identity throughout both the light and heavy chain domains. Clone D6 has an identical heavy chain domain except for 4 amino acid substitutions in framework regions 1 (two amino acids), 2 and 3. The substitutions of a lysine (K) for a glutamine (Q) and a serine for a proline observed in framework regions one and two, could be the result of a single base substitution or PCR error, underlining the power of combinatorial library production in producing random diversity into a family of conserved residues during amplification of the variable genes. The remaining two substitutions observed would involve at least two base changes as the codons encoding for the substituted amino acids are quite different from that of the conserved amino acids at their corresponding positions. The heavy chain CDR regions of all three clones are identical which would lead us to believe that the heavy chain antigen binding potential of all three clones is the same. However, binding of antigen to the one or more of the CDR regions is also depended on the orientation (i.e. folding of the protein), which can cause a significant increase/decrease in binding. Substitution of a polar amino acid (serine) for a hydrophobic amino acid (proline) and a large amino acid (leucine) for a small amino acid (glycine) as observed in the framework regions of clone D6, can cause a significant change in CDR orientation. A glycine residue in the linker of clone D6 has been deleted (indicated by the dash and not highlighted) but this should have no significant effect on the association of the heavy and light chain domains as only 15 residues or more are needed for proper domain association. From the alignment and number of conserved residues, it is obvious that there is very little similarity between the light chain of clone D6 and the two other clones. According to the Kabat scheme for identifying CDR regions, the CDR 1 region is

always preceded by a cysteine, finishes at a tryptophan residue and is usually 10-17 residues in length. The light chain sequence of clone D6 does not contain a cysteine residue indicating the start of CDR1 making if very difficult to determine the exact starting point of the region. The length of the highlighted region (24 residues) is a lot longer than the expected 10-17 residues in the Kabat scheme. However, with alignment of an unsure sequence (clone D6) with a greater number of known sequences (Figure 5.15 or the Kabat database) the CDR regions can be determined more accurately (CDR 1 of clone D6 is actually 15 residues in length as observed in Figure 5.15).

5.3.3.4 *Alignment of all nine clones selected from both antibody libraries*

On examination of the aligned light chain sequences in Figure 5.15, it is evident that there is an extremely high level of similarity between the InlB extract library clones D3, B10, G7 and B12 and the HT-*Listeria monocytogenes* library clones D8 and D11 (actually same clone as discussed in section 5.3.3.3). Slight differences are observed between the six clones in both framework 1 and framework 3 regions. Multiple residue variations are observed the framework 1 region with the exception of the five amino acids preceding the CDR 1 region while slight amino acid substitutions are observed in the second framework region, most notably the substitution of an arginine for a cysteine in clones D8 and D11. The three CDR regions of all six clones show 100% identity except for two residues in the CDR 1 of clone B12 (indicated by white text) that seem to have been the result of an inversion of a cysteine-glutamine-tyrosine sequence. The apparent high level of similarity of the light chain genes examined would indicate that they originated from the same V-J gene segments. These finding may indicate that there may have been cross contamination of variable genes prior to domain linking by SOE PCR, which could have occurred from purifying both sets of light chains from the same agarose gel (Figure 5.3). Clone B1, D6 and G9 show significant variation in both the framework and CDR regions. All aligned clones show differing degrees of variability in both framework and CDR regions, especially in the regions encoded by the D-J_H segments (CDR H3 and Framework 4). Clones B1 and D6 possess near identical heavy chains which is also unusual as each clone was selected from a different antibody library. This may again indicate some level of heavy chain cross contamination and also highlights the ability of PCR to amplify very small concentrations of contaminating DNA. Slight differences in amino acid structures in the framework (1, 2 and 3) and especially CDR (CDR H1) regions of both clones could result in both heavy chains having varied antigen binding abilities.

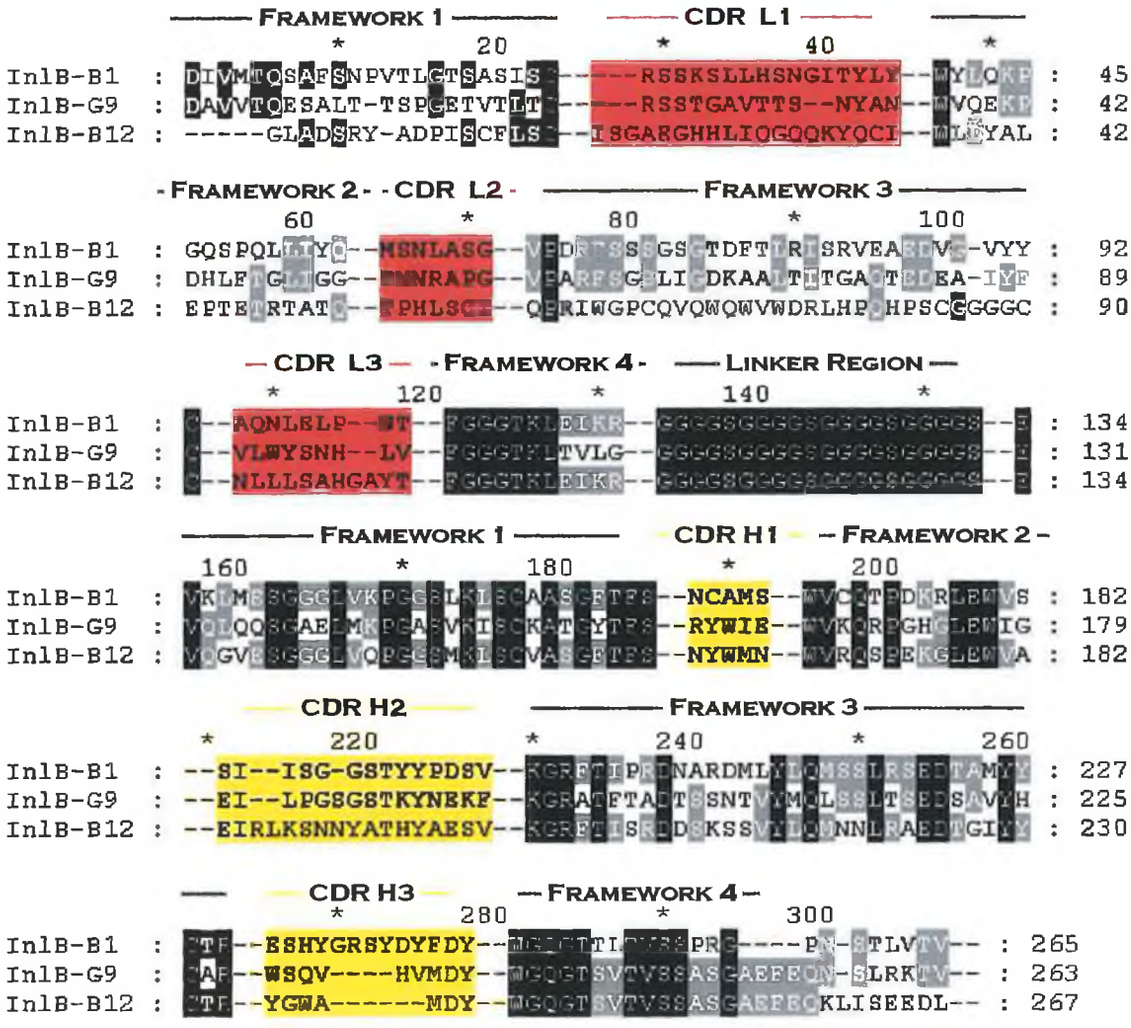


Figure 5.12: *Anti-InlB scFv gene sequences from the first round of selection (section 5.3.1) were translated into amino acid sequences using the Expasy Translate tool and aligned using the ClustalW multiple alignment program. Aligned sequences were imported into Genedoc for further manipulation and alignment. The sequences highlighted in black show 100% identity in all three gene sequences. The sequences highlighted in grey show identity in 2 out of the three sequences while sequences highlighted in red and yellow show the light and heavy chain complementarity determining regions (CDR), respectively.*

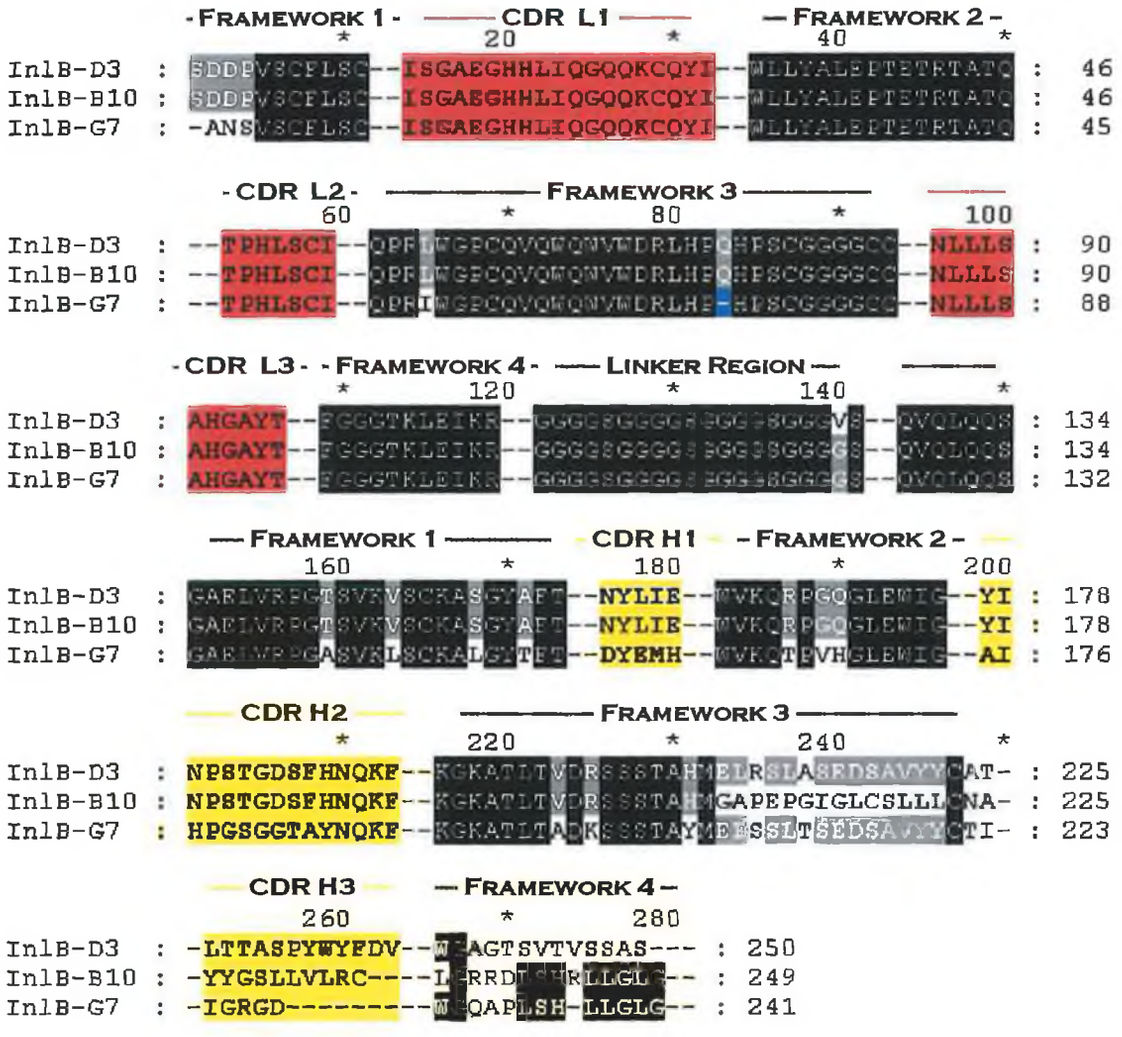


Figure 5.13: *Anti-InlB scFv gene sequences from the second round of selection (section 5.3.2) were translated into amino acid sequences using the Expasy Translate tool and aligned using the ClustalW multiple alignment program. Aligned sequences were imported into Genedoc for further manipulation and alignment. The sequences highlighted in black show 100% identity in all three gene sequences. The sequences highlighted in grey show identity in 2 out of the three sequences while sequences highlighted in red and yellow show the light and heavy chain complementarity determining regions (CDR), respectively.*

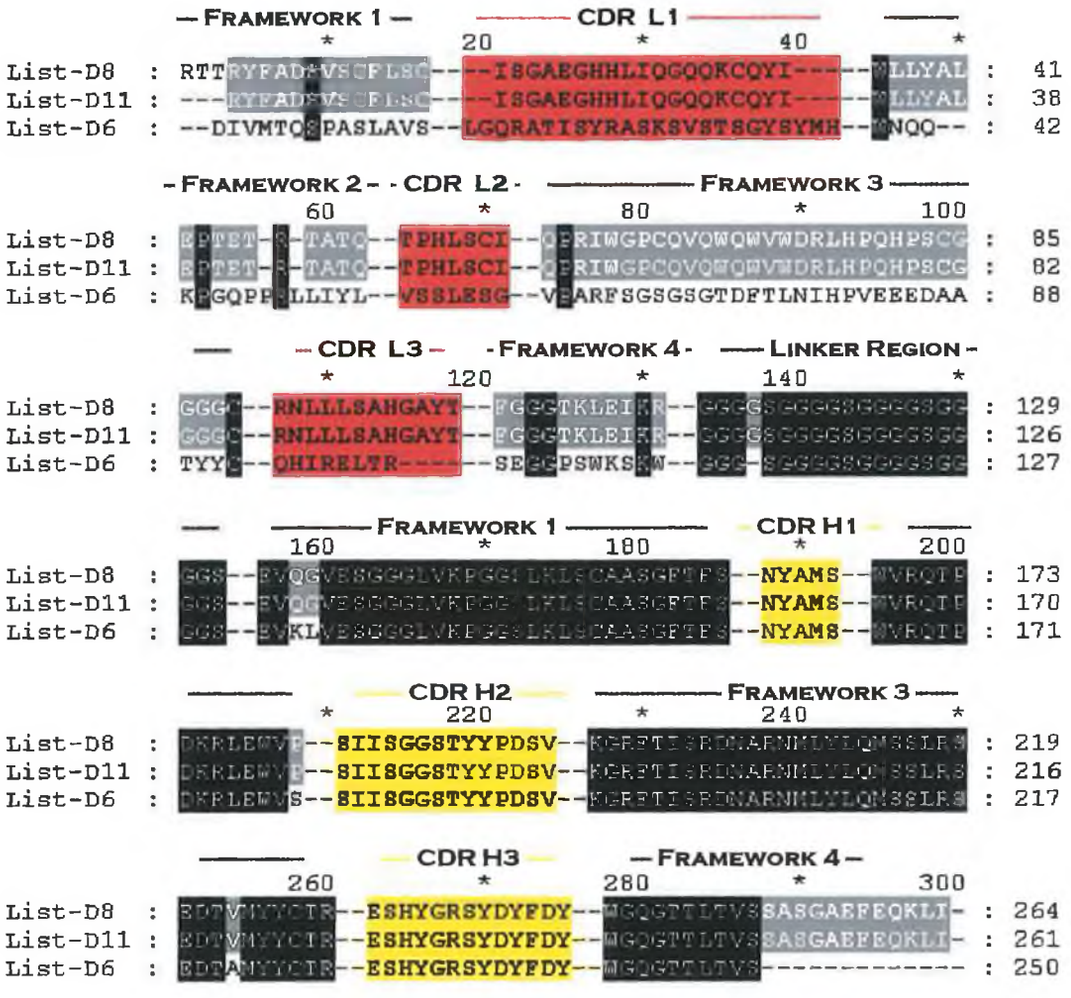


Figure 5.14: *Anti-Listeria monocytogenes scFv gene sequences from the second round of selection (section 5.3.2) were translated into amino acid sequences using the Expasy Translate tool and aligned using the ClustalW multiple alignment program. Aligned sequences were imported into Genedoc for further manipulation and alignment. The sequences highlighted in black show 100% identity in all three gene sequences. The sequences highlighted in grey show identity in 2 out of the three sequences while sequences highlighted in red and yellow show the light and heavy chain complementarity determining regions (CDR), respectively.*

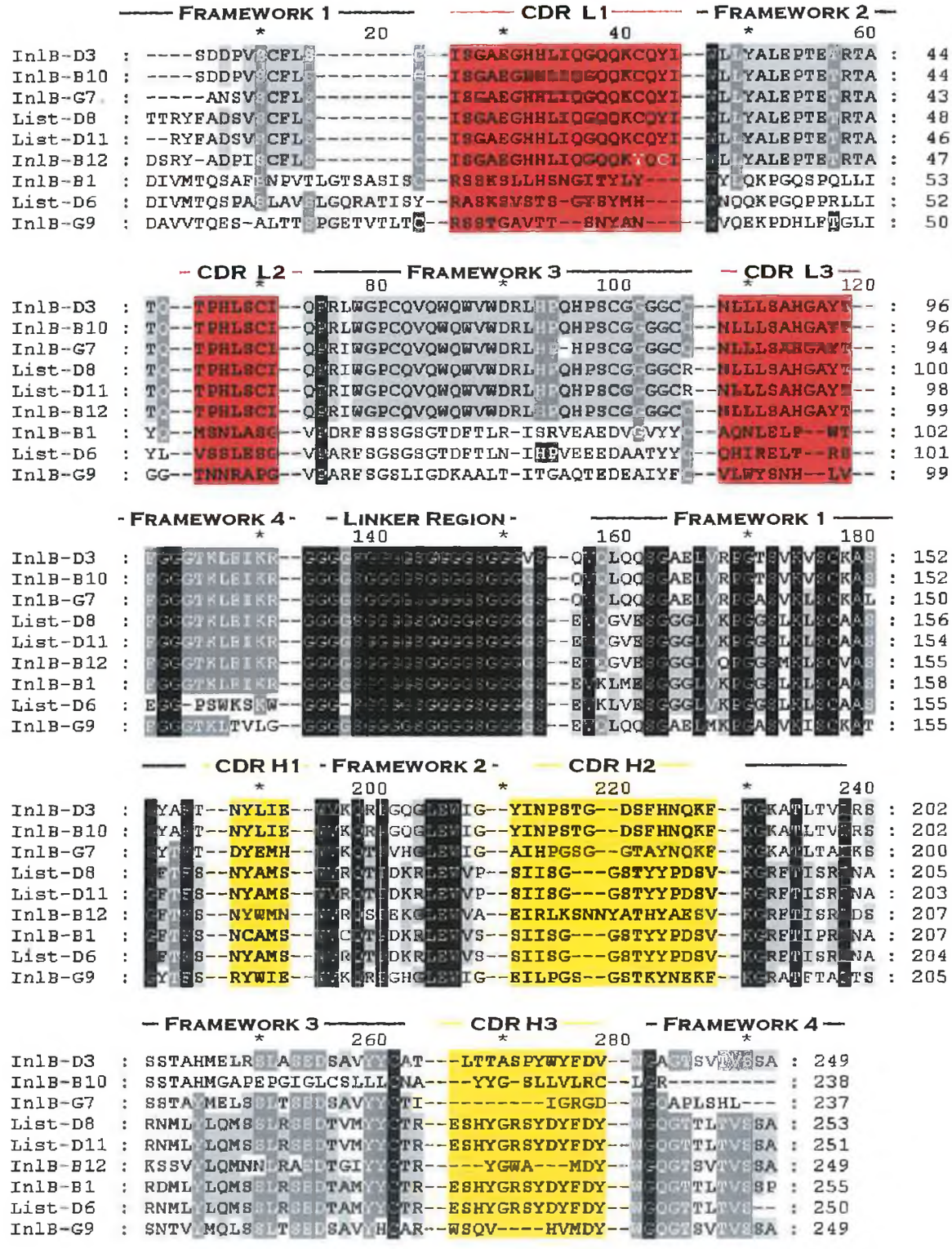


Figure 5.15: *All nine scFv gene sequences from both rounds of selection (section 5.3.1 and 5.3.2). The sequences highlighted in black show 100% identity in all three gene sequences. The sequences highlighted in dark grey show identity in 7 or more genes and the sequences highlighted in light grey show identity in 5 or more genes. Sequences highlighted in red and yellow show the light and heavy chain complementarity determining regions (CDR), respectively.*

5.3.4 *Homology modelling of scFv sequences using Swiss-Model and Swiss-PDB viewer*

The Protein Data Bank, established at Brookhaven National Laboratories (BNL) in 1971, is the single worldwide archive of structural data of biological macromolecules, which is now managed by the Research Collaboratory for Structural Bioinformatics (RCSB) (Berman *et al.*, 2000). The Protein Data Bank contains thousands (21,390 structures at the time printing) of protein structures deposited from research groups throughout the world. The accumulation of such a large collection of protein structural information can enable the 3D structure prediction of most linear polypeptide sequences. Many web-based protein-modelling programs (eg. SWISS-Model, 3D-JIGSAW etc.) are available without charge where the researcher can deposit their desired linear polypeptide sequence for modelling. The modelling program performs a blast search against all the sequences in the Protein Data Bank and models the linear sequence with the best blast result. The researcher receives the modelling result via e-mail as a PDB (Protein Data Bank) file, which can be then viewed using one of the web based PDB viewer programs (e.g. SWISS-PDB-Viewer, Rasmol, Protein Explorer etc.).

Figures 5.16 to 5.18 show illustrations of clone G9 (section 5.3.3.1), which has been modelled by SWISS-Model and viewed using the SWISS-PDB-Viewer. In order to illustrate the 3D nature of the antigen-binding domain of the antibody, several views have been presented. A side view of a ribbon representation of the scFv antibody is given in Figure 5.16, Panel A. Both the heavy and light chain variable domains can be clearly seen and are linked by the synthetic Gly_4Ser repeats introduced by the V_L-forward and V_H-back primers listed in section 2.4.2.1. Beta sheets are illustrated by flattened arrows with the arrow pointing towards the C terminus of the polypeptide chain. Both the linker and the loop structures are illustrated as solid lines. Each variable domain consists of nine Beta sheets and no helices (as described in section 1.8). The light chain CDR regions are highlighted in red and the heavy chain CDR regions in yellow. All CDR regions are labelled for easy identification. Figure 5.16, Panel B shows a front view (where the antigen would fit right down on the page, like an egg in an egg cup) of the antigen-binding region. From this view it can be clearly seen that the CDR L3 and H3 from the centre of the antigen-binding site and the other CDR regions form a cup shape around the two centre sites. Figure 5.17 Panel A shows an enlarged view of the antigen-binding site while Figure 5.17, Panel B illustrates a more realistic space fill view of the scFv with CDR regions coloured. The space fill model takes into account the amino acid side chain size and properties and estimates the Van der Waals interactions between the residues. A reverse view of the antigen-binding site (displayed in space fill) is presented in Figure

5.18. The antibody framework regions are illustrated as ribbon structures for easier chain differentiation and orientation. The framework regions act as foundations for the optimal orientation of the CDR regions for the binding of antigen.

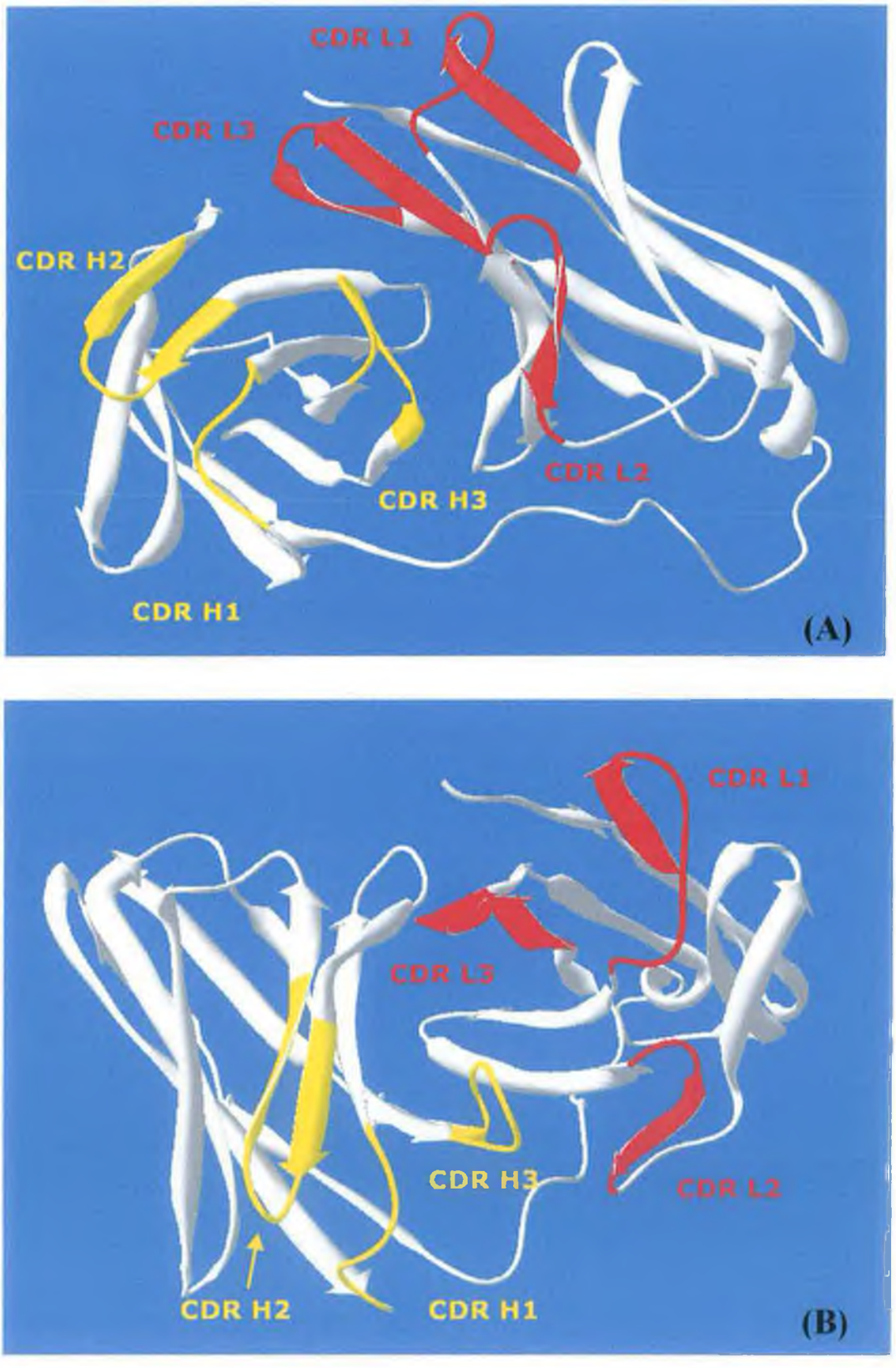


***Figure 5.16**: Homology model of the anti-InlB scFv antibody clone G9. (A) Side view illustration of the scFv showing the confirmation of both domains and (B) front view of the antigen-binding surface with labelled CDR regions.*

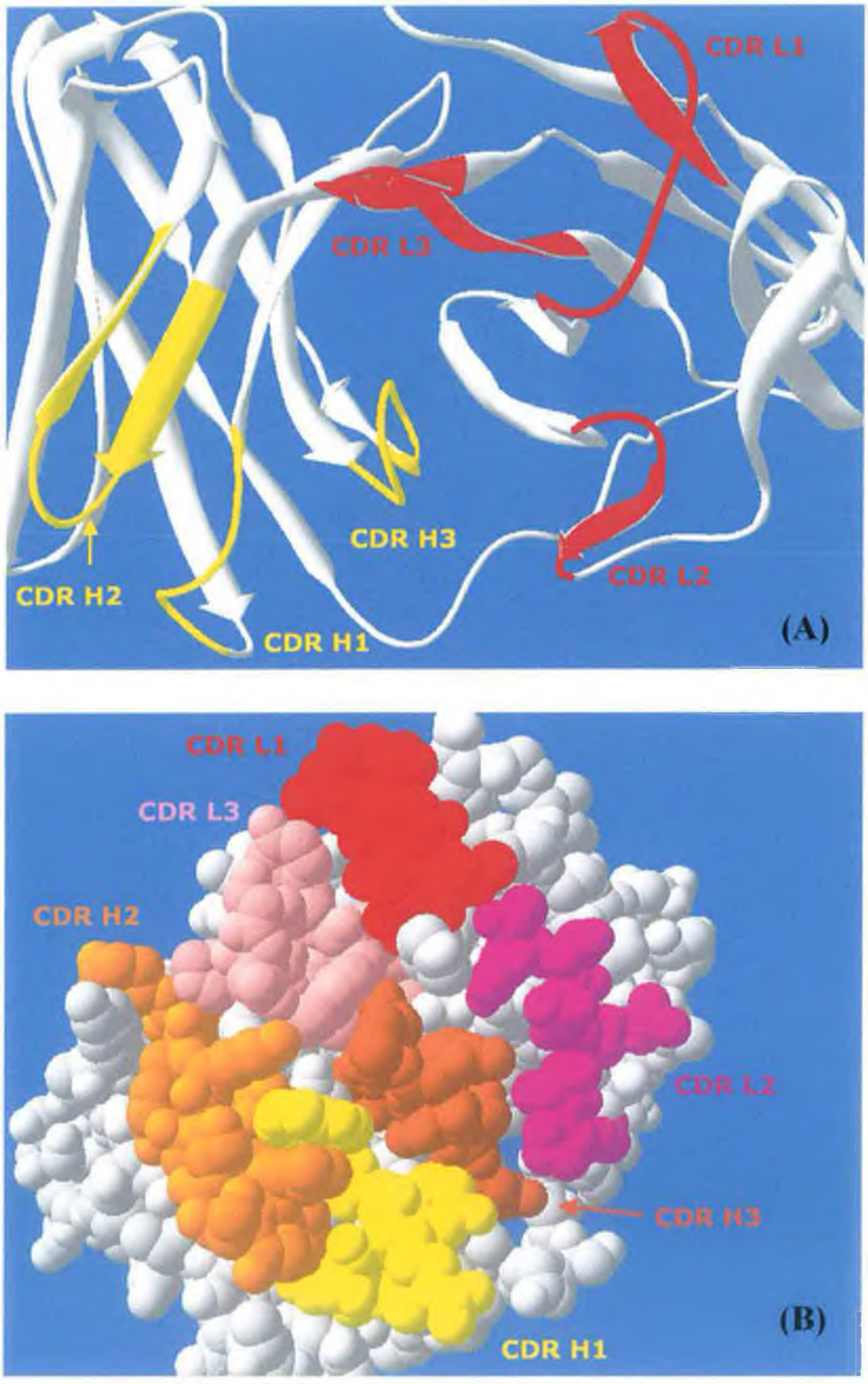


Figure 5.17: *Enlarged front view antigen-binding site of the scFv antibody. (A) Ribbon illustration of an enlarged view of the antigen-binding site and (B) a space fill model of the same view taking into account side chain orientation and Van der Waal's forces.*

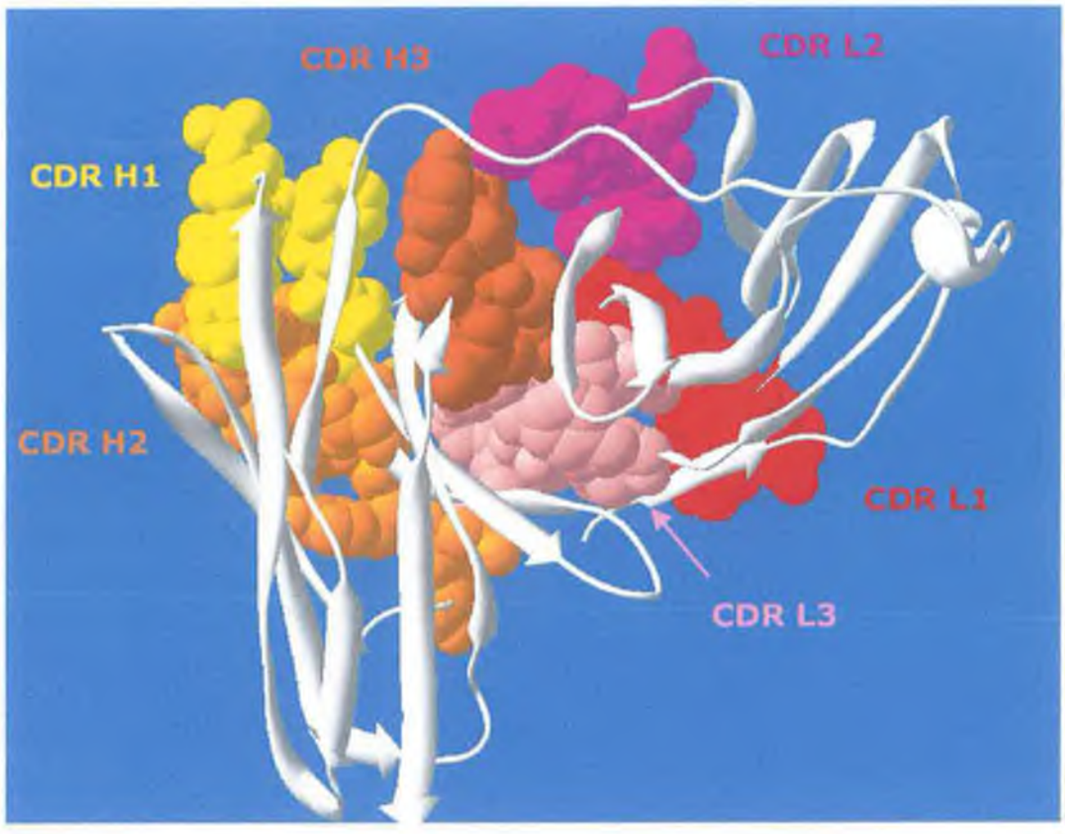


Figure 5.18: *Reverse view of the antigen-binding site presented in Figures 5.17 (A and B). The CDR regions are illustrated in space fill form while the framework regions are illustrated in ribbon structure. From this view, it can be seen how the framework regions orientate the CDR regions for antigen-binding. Mutations leading to amino acid substitutions in the framework regions can potentially alter the conformation of the antibody and increase/decrease the antibodies ability to bind its antigen. This view also illustrates that many of the residues found in the CDR regions are actually buried in the protein structure and not involved in antigen binding.*

5.3.5 *Expression of soluble scFv with the pAK400 vector*

Subcloning the scFv antibody genes into pAK400 (Figure 1.12) allows the enhanced expression of soluble scFv antibodies. The scFv gene sequences of the clones selected in sections 5.3.1 and 5.3.2, were subcloned into the pAK400 vector by *SfiI* restriction (section 2.4.2.8) of the pAK100 vectors carrying scFv genes (Figure 5.19) and transformed into the non-suppressor *E. coli* strain JM83 (section 2.4.2.11). Clones were grown in 2xTY media (section 2.4.3.9) and protein expression induced by 1 mM IPTG. The supernatant and cytoplasmic extracts of each clone were assayed by ELISA (section 2.4.3.10) for determination of antibody expression. However, subcloning the scFv genes into pAK400 did not result in the expression of soluble antibody. Varying the expression times and temperatures failed to have any effect on solublisation of the antibodies. A number strategies have been reported in the literature for the solublisation of scFv antibodies. Antibodies can be solublised using denaturing buffers such as those containing urea or guanidine hydrochloride and refolded by continuous dialysis (Park *et al.*, 2000a) or after affinity purification (Casalvilla *et al.*, 1999). Antibody solubility can also be increased by the co-expression of a gene encoding a molecular chaperone protein. Hayhurst and Harris, (1999) successfully increased the solubility of a single chain antibody (scAb) by co-expressing the *E. coli* skp chaperone gene. Hayhurst *et al.* (2003), also used the strategy of co-expressing the skp chaperone gene with scFv antibodies that they produced using the system designed by Krebber *et al.* (1997a). These authors also expressed the antibodies fused to human kappa light chain constant domain with and without co-expression skp chaperone gene. It was found that even though co-expressing the scFv antibody with the skp gene or expression of the antibody fused to a constant domain (scAb), increased the levels of soluble functional antibodies, expression levels were still very low. Due to the large signal amplification afforded by the anti-phage antibodies against the abundant g8p phage coat protein, phage-displaying scFvs or scAbs were still found to be superior ELISA reagents for immunoassays. The purpose of the production of scFv antibodies to *Listeria monocytogenes* in this project was for the generation of a species specific antibody, which could be used, for the detection of *Listeria monocytogenes* by ELISA. Therefore, the tendency of each clone to cross-react with other bacterial strains was evaluated (section 5.3.6) before further efforts to solublise the antibodies were pursued.

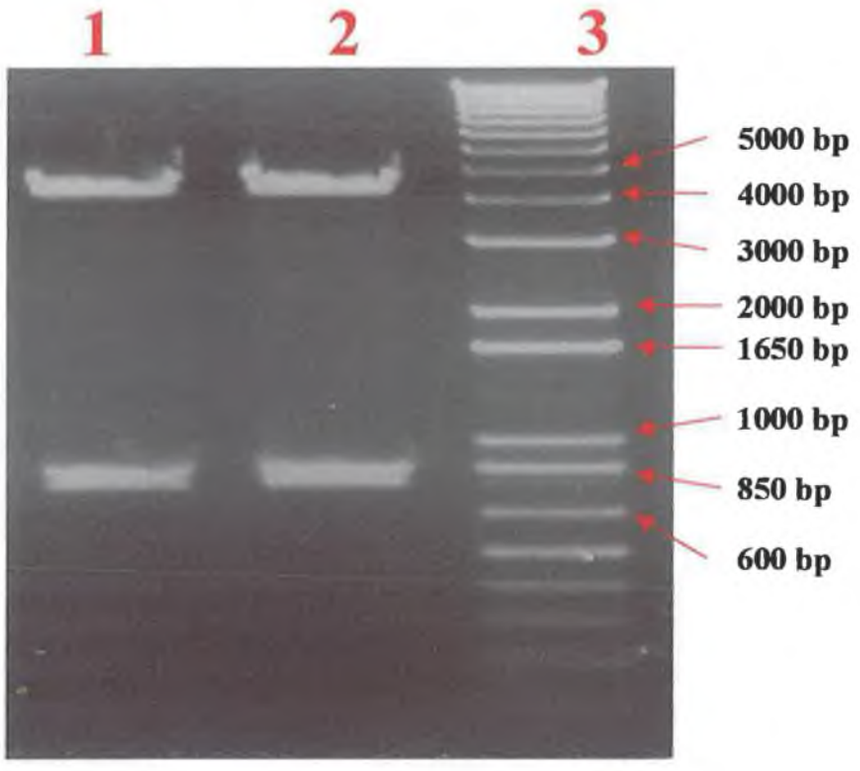


Figure 5.19: *Typical SfiI restriction of pAK100 plasmids with scFv gene inserts. The gel shows the restriction of the pAK100 vector (4425 bp) containing anti-InlB G9 (lane 1) and B1 (lane 2) scFv clones (800 bp). The marker (lane 3) is a Gibco 1kb plus DNA ladder with molecular weights indicated. The scFv gene sequences were purified from the gel and ligated into the pAK400 vector as described in sections 2.4.2.7 and 2.4.2.9.*

5.3.6 *Cross reactivity studies of phage-scFv clones*

As discussed in section 5.3.5, the purpose of the production of scFv antibodies to *Listeria monocytogenes* in this project was for the generation of a species-specific antibody, which could be used for the detection of *Listeria monocytogenes* by ELISA. To evaluate the tendency of each phage-scFv clone to cross-react, each antibody was tested for binding to intact *Listeria monocytogenes* cells, four non-pathogenic strains of the genus *Listeria* and the phylogenetically-related gram-positive rod, *Bacillus subtilis* (Vázquez-Boland *et al.*, 2001). Phage-scFv antibodies were added to ELISA plates coated with $1x10^8$ cells/ml of bacterial cells as described in section 2.4.3.11. The absorbance values observed for each antibody against the intact cells was recorded as a percentage of that observed against heat-killed *Listeria monocytogenes* cells as shown in Table 5.1. Each of the antibodies tested failed to recognise intact *Listeria monocytogenes* cells to the same degree as heat-killed cells. Only clones B1 and B12 showed binding of greater than 50% of that observed for heat-killed cells. Each phage-scFv clone recognised the four non-pathogenic strains of the genus *Listeria* and showed binding equal to or more than that observed to intact *Listeria monocytogenes* 4b. Clone B12 showed greater binding to the four non-pathogenic *Listeria* strains than that observed to heat-treated *Listeria monocytogenes*. Each phage-scFv clone also showed binding to intact *B. subtilis* cells, which was equal to or greater than that observed to intact *Listeria monocytogenes* cells. From the results presented in Table 5.1, solubilising the scFv antibodies will not yield an antibody specific for the detection of *Listeria monocytogenes*. Large naïve antibody libraries are available with antibody diversities of greater than 10^{10} clones that have the potential for generating high affinity antibodies that are specific for their target antigen (Vaughan *et al.*, 1996). In pursuit of a species-specific recombinant scFv antibody for the detection of *Listeria monocytogenes*, a naïve human antibody library (described in section 5.1), with an antibody diversity of 1.4×10^{10} clones (Vaughan *et al.*, 1996), was bio-panned against *Listeria monocytogenes* cells (section 5.4) and purified recombinant *Listeria monocytogenes* invasion-associated proteins, Internalin B (InlB) and p60 (section 5.5).

	HT-L.m 4b	*L. m 4b*	*L. in 6B*	*L. welsh.*	*L. ivan.*	*L. seel.*	*B. subtilis*
B1	++++	+++	+++	+++	+++	+++	++++
G9	++++	++	+++	+++	+++	++	++++
B12	++++	+++	+++++	+++++	+++++	+++++	+++
D3	++++	+	+++	++	+++	++	++
B10	++++	+	++	+	++	++	+
G7	++++	+	++	+	++	++	+
D11	++++	++	+++	+++	+++	+++	+++
D8	++++	++	+++	+++	+++	+++	+++
D6	++++	++	+++++	+++	++++	+++	++

Table 5.1: *Cross reactivity studies of phage-scFv antibodies by ELISA. The binding response minus background (wells blocked with milk) was expressed as a percentage of the binding response of each antibody over heat-killed Listeria monocytogenes 4b (HT-L.m 4b). Each antibody was tested against intact (not heat killed) Listeria monocytogenes 4b (L. m 4b), intact Listeria innocua 6B (L. in 6B), intact Listeria welshimeri (L. welsh.), Listeria ivanovii (L. ivan.), Listeria seeligeri (L. seel.) and Bacillus subtilis (S. subtilis).*

(+) represents 0-24% binding, (++) represents 25-49% binding, (+++) represents 50-74% binding, (++++) represents 75-100% binding, (+++++) represents >100% binding.

5.4 *Selection of scFv antibodies from a naïve human antibody library by bio-panning on Nunc immunotubes*

A naïve human antibody library with a diversity of 1.4 x 10^{10} clones (Vaughan *et al.*, 1996) was donated by Cambridge Antibody Technology Ltd, Cambridge, UK. This antibody library was used to select for scFv antibody sequences capable of binding to *Listeria monocytogenes* cells. Immunotubes were coated with HT-*Listeria monocytogenes* cells and blocked with 5% (w/v) milk marvel as described in section 2.4.4.3. Rescued phage displaying scFv particles were added to the immunotube and incubated for 2 hours at room temperature. Unbound phage-scFv particles were washed from the tube and phage binders eluted as described in section 2.4.4.3. Four rounds of bio-panning were performed against immobilised HT-*Listeria monocytogenes* cells and a phage ELISA carried out from clones selected from rounds 3 and 4 of bio-panning.

5.4.1 *Screening of scFv sequences to Listeria monocytogenes cells by phage ELISA*

Ninety-five clones from rounds 3 and 4 of bio-panning were selected and characterised by phage ELISA (Figure 5.20) against immobilised HT-*Listeria monocytogenes* cell-coated ELISA plates. Four clones from the third round of bio-panning (clones C11, F12, E10 and B6) and four clones from the fourth round of bio-panning (G4, F9, D1 and B5) were selected and further characterised by inhibition ELISA (Figure 5.21). Phage-scFv antibodies from all eight clones were produced in 5 ml cultures over night at 26°C. Phage scFv supernatant was incubated with decreasing concentrations of HT-*Listeria monocytogenes* cells for 30 minutes at 37°C and then added to HT-*Listeria monocytogenes* coated ELISA plate wells as described in section 2.4.4.5. Bound phage scFv antibodies were detected with a rabbit anti-bacteriophage Fd antibody and the results plotted in Figure 5.21. One of the four clones selected from the third round of bio-panning (clone F12, Figure 5.21, panel A) recognised HT-*Listeria monocytogenes* cells in solution showing a decrease in absorbance with increasing HT-*Listeria monocytogenes* cell concentrations. Clone B5 from the fourth round of bio-panning also recognised HT-*Listeria monocytogenes* cells in solution showing a decrease in absorbance with increasing HT-*Listeria monocytogenes* cell concentrations (Figure 5.21, panel B).

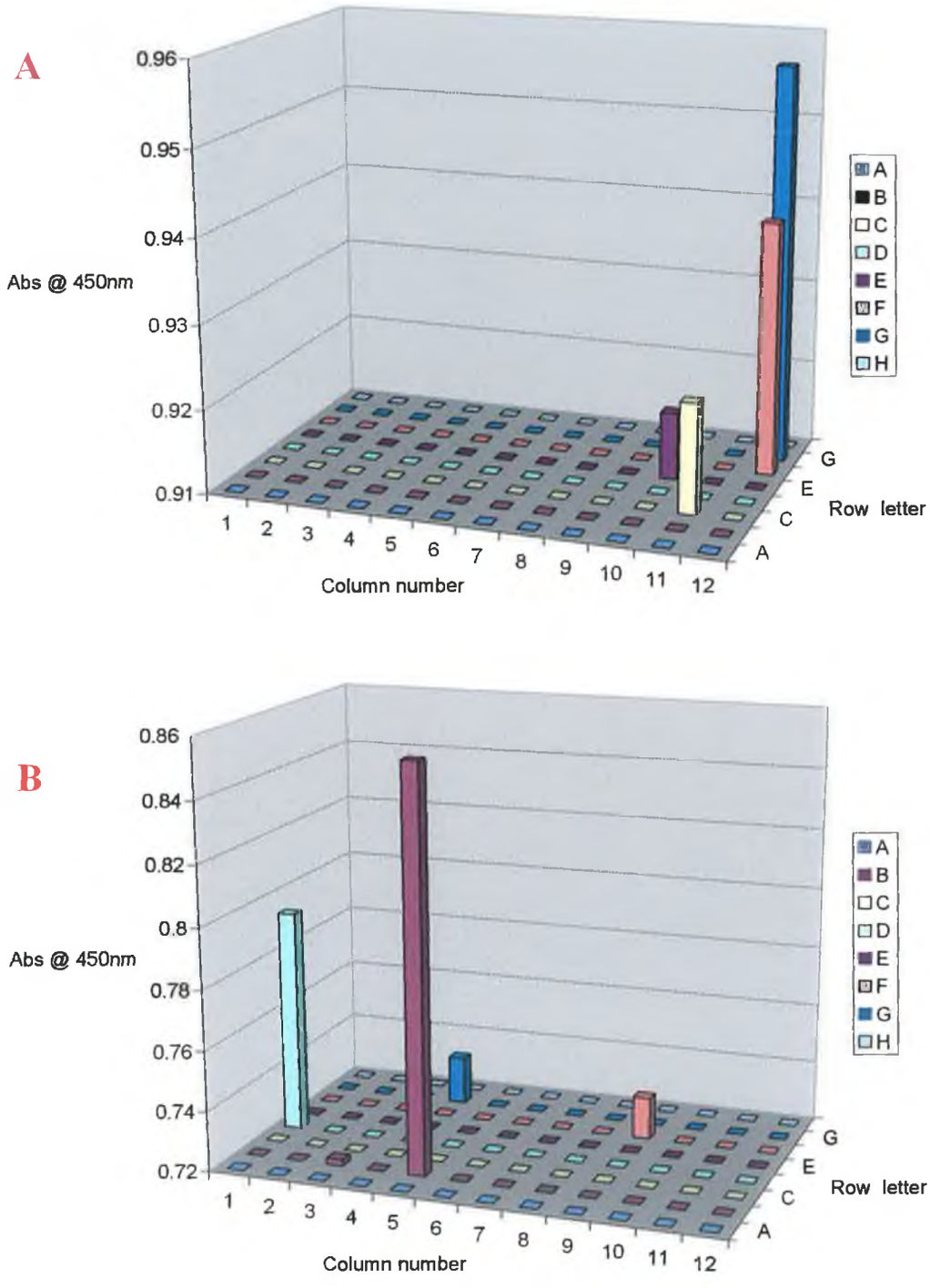


Figure 5.20: *Phage ELISA results after the third and fourth rounds of bio-panning the naïve human antibody library with HT-Listeria monocytogenes cells. Panel (A) represents the results for the third round of selection and panel (B) the results for the fourth round of selection.*

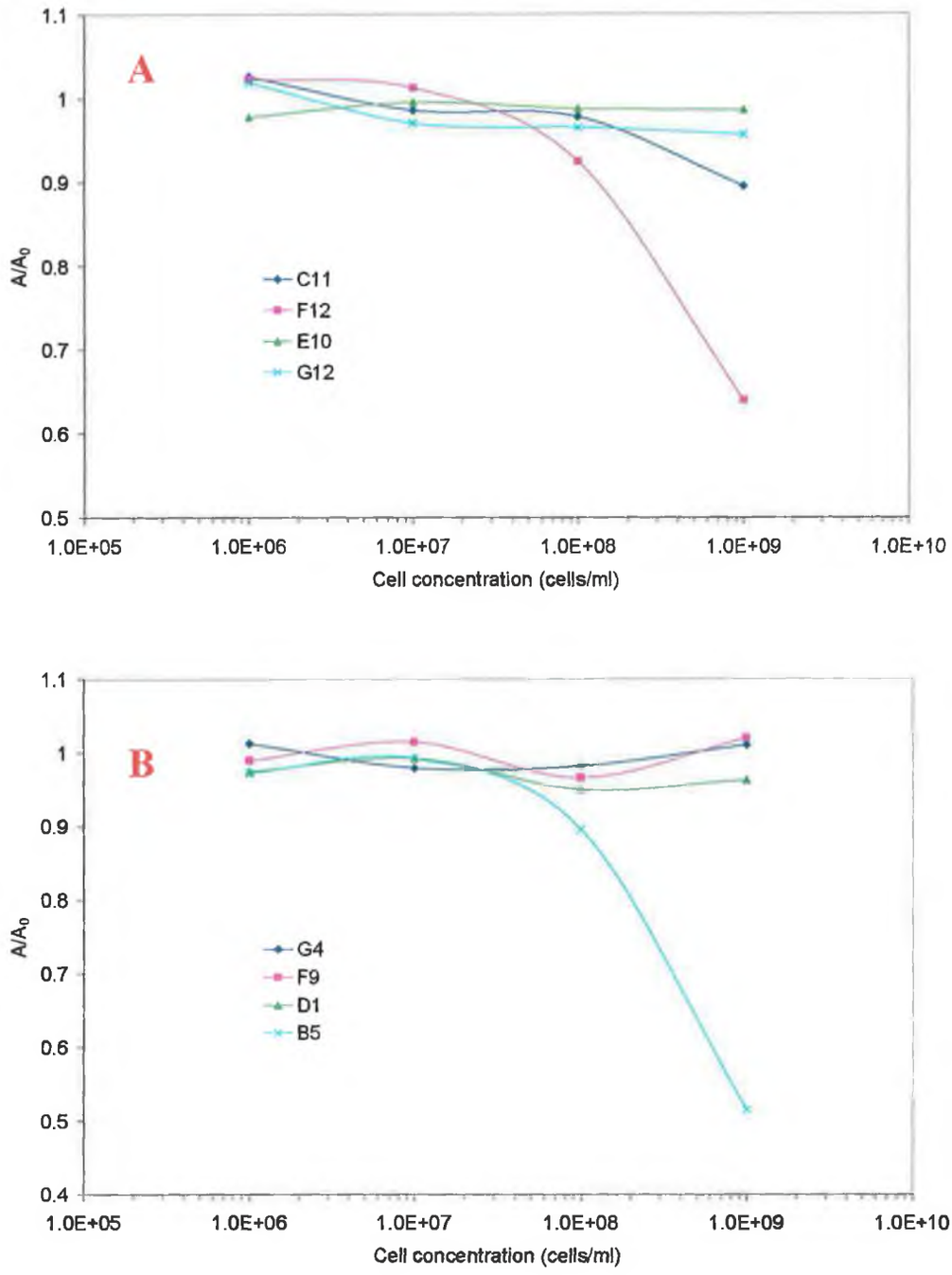


***Figure 5.21**: Inhibition ELISA curves from phage-scFv clones selected from both the third and fourth rounds of bio-panning. Clones were expressed in 5 ml of 2xTY media overnight at 26°C and incubated with decreasing concentrations of heat-treated (HT) Listeria monocytogenes cells for 30 minutes. Antibody and cells were then added to ELISA plate wells that had being coated with $1x10^8$ cells/ml heat-treated Listeria monocytogenes cells and blocked with 5% (w/v) milk marvel. Clone F12 from the third round of bio-panning (labelled A) and clone B5 from the fourth round of bio-panning showed competition with free HT-Listeria monocytogenes cells.*

5.4.2 *Determination of antigen specificity of clones F12 and B5 by Western blotting*

SDS protein extracts from *Listeria monocytogenes* 4b cells were prepared as described in section 2.4.4.6 and the extracted proteins separated by SDS-PAGE as outlined in section 2.2.6. The separated proteins were transferred to a nitrocellulose membrane and blocked with 5% (w/v) milk marvel solution overnight as discussed in section 2.2.7. The nitrocellulose membranes were later probed with the phage-scFv antibody clones F12 and B5 (Figure 5.22) and detected with a rabbit anti-bacteriophage Fd antibody. Both phage-scFv clones strongly recognised an unknown 38-43 kDa protein band and also produced a faint band at approximately 70-80 kDa (Figure 5.22). The identity of the 38-43 kDa band is currently unknown but it is probable that the faint band found at 70-80 kDa is dimeric form of the unknown protein.

5.4.3 *Sequencing and alignment of positive phage-scFv clones*

E. coli TG1 stocks containing the scFv gene sequences from the clones selected in section 5.4.1 were grown overnight in 5 ml 2xTY containing 1% (w/v) glucose and 100 µg/ml ampicillin at 37°C. Plasmid DNA was purified according to section (2.4.2.7) and vacuum dried. DNA was sent to Kate Duffy at Queens University Belfast, for sequencing. On receiving the DNA sequence results, the DNA sequences were translated to amino acid sequences using the Expasy translate tool (see section 2.4.3.8 for web addresses of programs used in this section) and the amino acid sequences were pasted into the ClustalW program, a tool for multiple alignments. The aligned sequences from ClustalW were imported into the Genedoc program, for the easy manipulation of sequence alignments. Using the Genedoc program identical residues were automatically identified and the CDR regions manually highlighted according to the Kabat scheme for the identification of antibody CDR regions (Figure 5.23). The heavy chain CDR regions are highlighted in yellow, the light chain CDR regions in red and the 15 amino acid (5 amino acid shorter than the Krebber antibodies shown in section 5.3.3) linker region highlighted in dark grey. The polyhistidine tag can be seen at the C terminus of the light chain region (highlighted in green). Upon alignment of both antibodies (clones B5 and F12) it was observed that both scFv gene sequences were identical. Therefore, further analysis was only carried out with one of the two antibodies, clone F12.

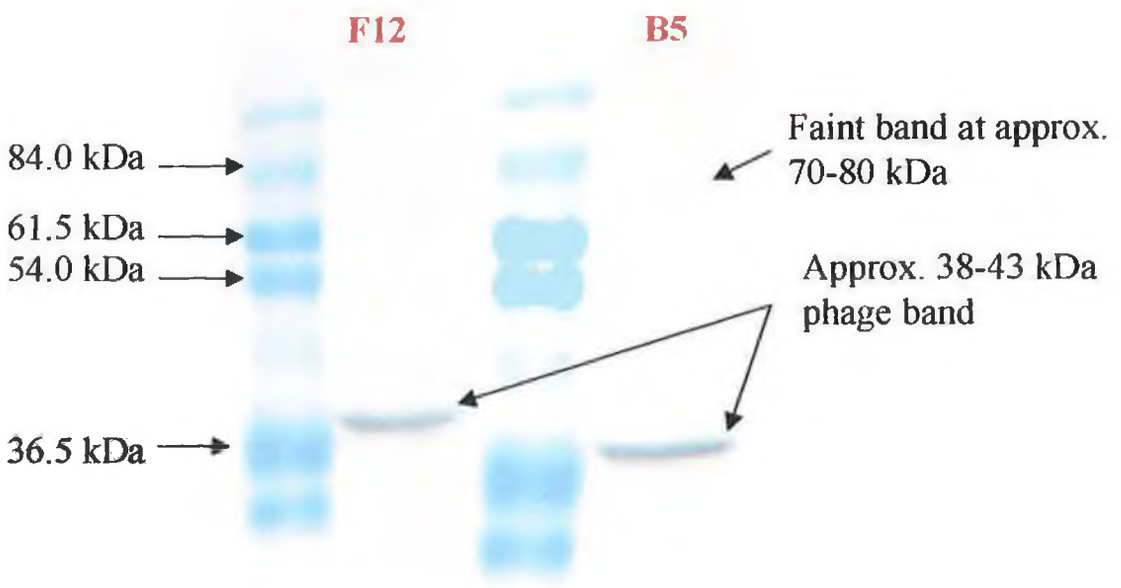


Figure 5.22: *Western blots of clone F12 and B5 phage supernatants against Listeria monocytogenes SDS-extract. 20 µl of L. monocytogenes SDS extract (section 2.4.4.6) was analysed by SDS-PAGE gel electrophoresis and probed with phage-scFv antibody supernatant by Western blotting. The marker used is a Sigma pre-stained marker with molecular weights indicated. Both phage-scFv clones were observed to react with two unknown protein bands, a strong 38-43 kDa band and a faint 70-80 kDa.*

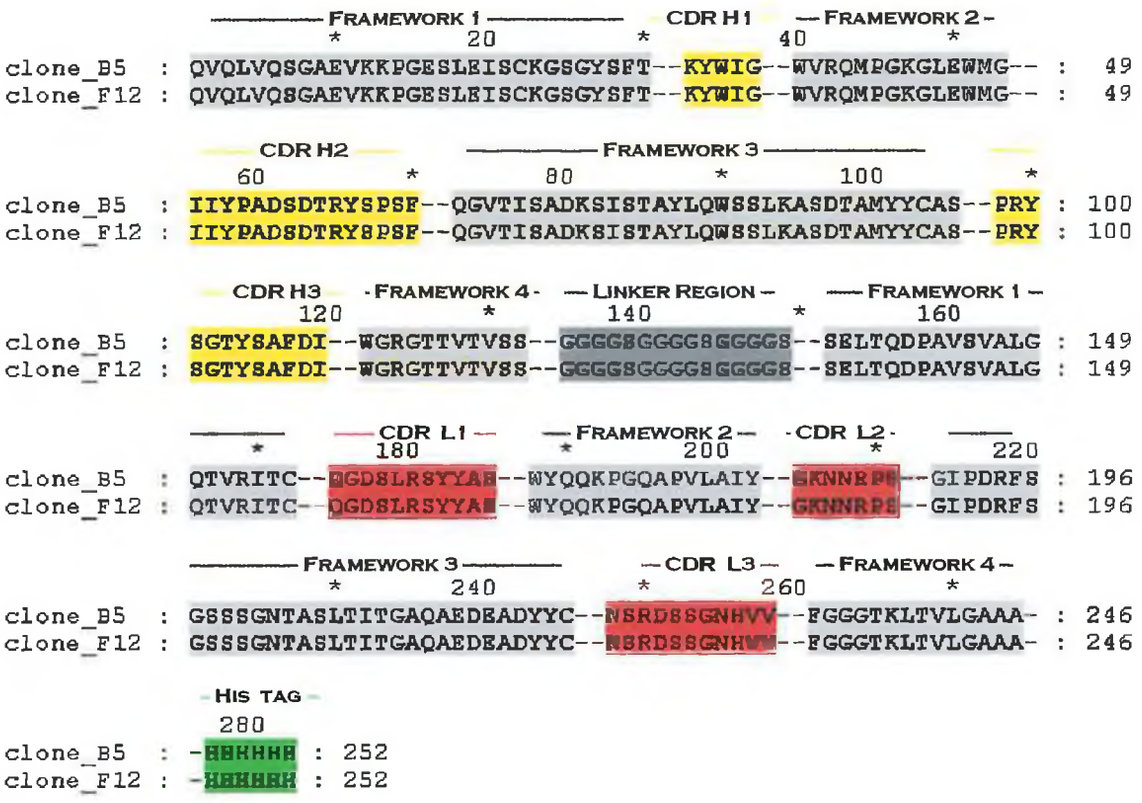


Figure 5.23: *Alignment of phage-scFv clones F12 and B5 selected from the naïve human library against Listeria monocytogenes cells. Both clones show 100% identity. Heavy chain CDR regions are highlighted in yellow and the light chain regions in red. The 15 amino acid linker region and 6 histidine tag are also highlighted.*

5.4.4 *Expression of soluble scFv antibodies and characterisation by ELISA and Western blotting*

The naïve human antibody library used to in this section allows the expression of soluble scFv antibodies without the need for subcloning into an expression vector (see section 1.14). Clone F12 was grown overnight in 2xTY media and soluble scFv antibody produced as described in section 2.4.4.9. An anti-fluorescein scFv (clone FITC-E2), first described by Vaughan *et al.* (1996), was donated by Cambridge Antibody Technology and used as a positive control in ELISA. A non-competitive ELISA was performed with the scFv supernatants and cytoplasmic extracts from both clones to determine the expression of soluble antibody as described in 2.4.3.10. Figure 5.24 shows the binding curves observed from the two clones against their respective antigens. The positive clone, clone FITC-E2, shows a good response to immobilised FITC-BSA from both antibody supernatant and cytoplasmic extract. However, clone F12 supernatant and cytoplasmic extract did not yield a measurable signal.

A time course experiment (described in section 2.4.4.11) was carried out with the cytoplasmic extract from clone F12 as shown in Figure 5.25. One millilitre aliquots from TG1 clone F12 cells induced with 1 mM IPTG were taken every hour and centrifuged at 14,000 rpm for 10 minutes. Cell pellets were resuspended in denaturing buffer (section 2.4.4.11) and sonicated. Cytoplasmic extracts were electrophoresed by SDS-PAGE and the protein bands stained with Coomassie blue as described in section 2.2.6. Clone F12 scFv bands were observed after 3, 4, and 5 hours of induction but was absent after 20 hours of induction indicating that the protein is degraded over time. Figure 5.26 shows a Western blot of clone F12 cytoplasmic extracts probed with an anti-c-myc-HRP-labelled monoclonal antibody. ScFv bands can be clearly seen in lanes 1 and 2 (denatured cytoplasmic extract at 4 and 5 hours after induction, respectively), corresponding to a size of approximately 29-30 kDa. No band is observed for the denatured extract after 20 hours of induction (lane 3), which corresponds with Figure 5.25. Low levels of scFv bands were observed for native cytoplasmic extracts after 4 and 5 hours of induction (lanes 7 and 8) indicating very low soluble expression of antibody.

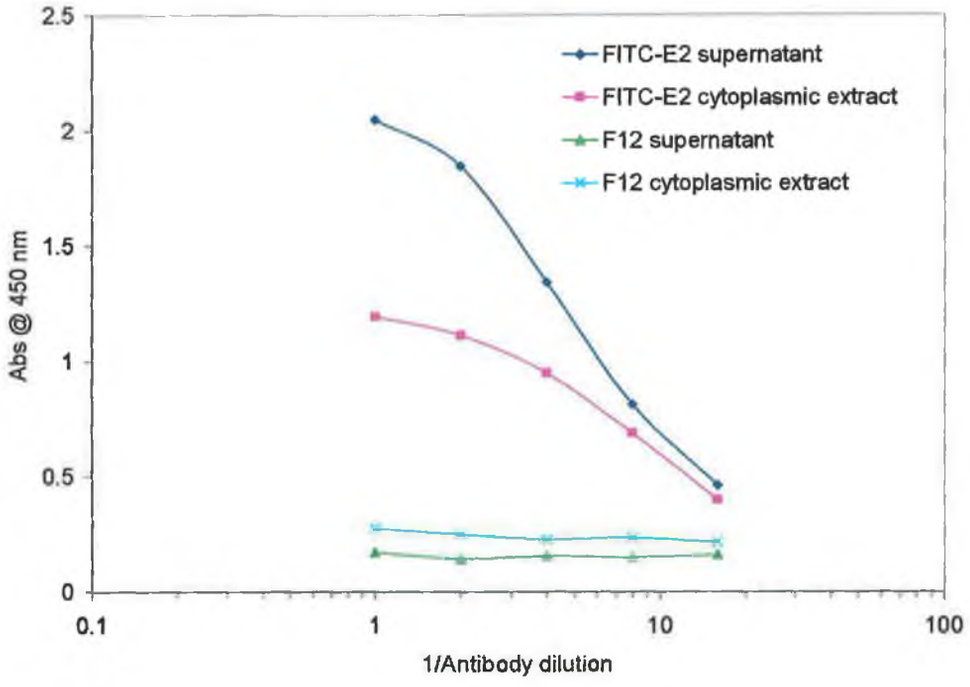


Figure 5.24: *Titre of F12 and FITC-E2 (positive control) supernatant and cytoplasmic extracts. Antibody supernatant and cytoplasmic extracts were diluted in PBS and added to ELISA plate wells coated with $1x10^8$ cells/ml HT-Listeria monocytogenes or 30 µg/ml FITC-BSA. Bound antibodies were detected with an anti-c-myc HRP-conjugated monoclonal antibody and absorbance read at 450 nm. The anti-fluorescein antibody (clone FITC-E2) produced a good response to immobilised fluorescein-conjugated BSA while clone F12 supernatant and cytoplasmic extract did not yield a response to immobilised HT-Listeria monocytogenes cells.*

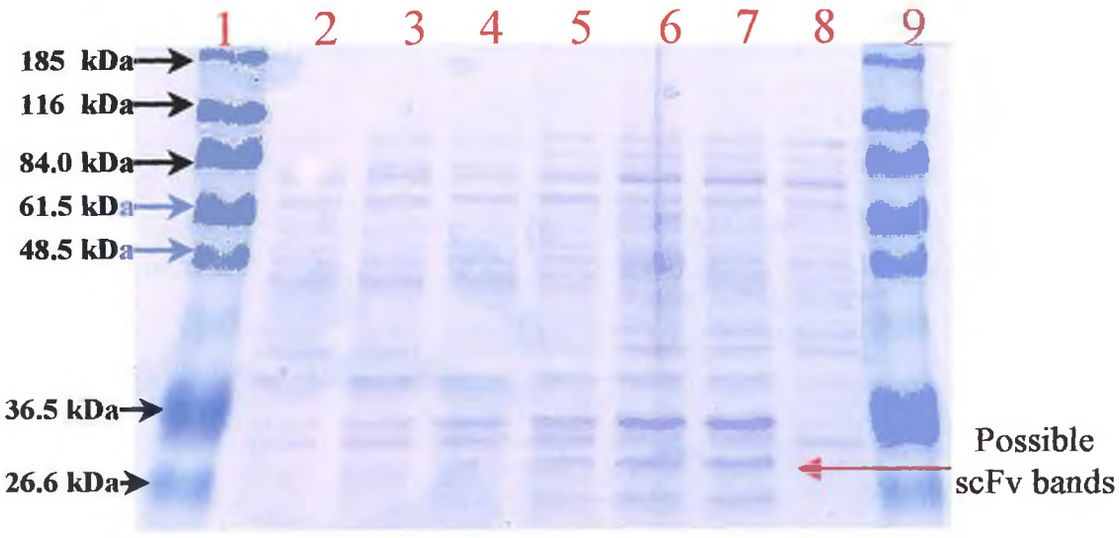


Figure 5.25: *Time course experiment of the expression of scFv antibody from clone F12. Lanes 2-8 represent denatured cytoplasmic extracts of clone F12 at times 0, 1, 2, 3, 4, 5 and 20 hours after induction with 1 mM IPTG as described in section 2.4.4.11. The red arrow indicates the possible scFv bands.*

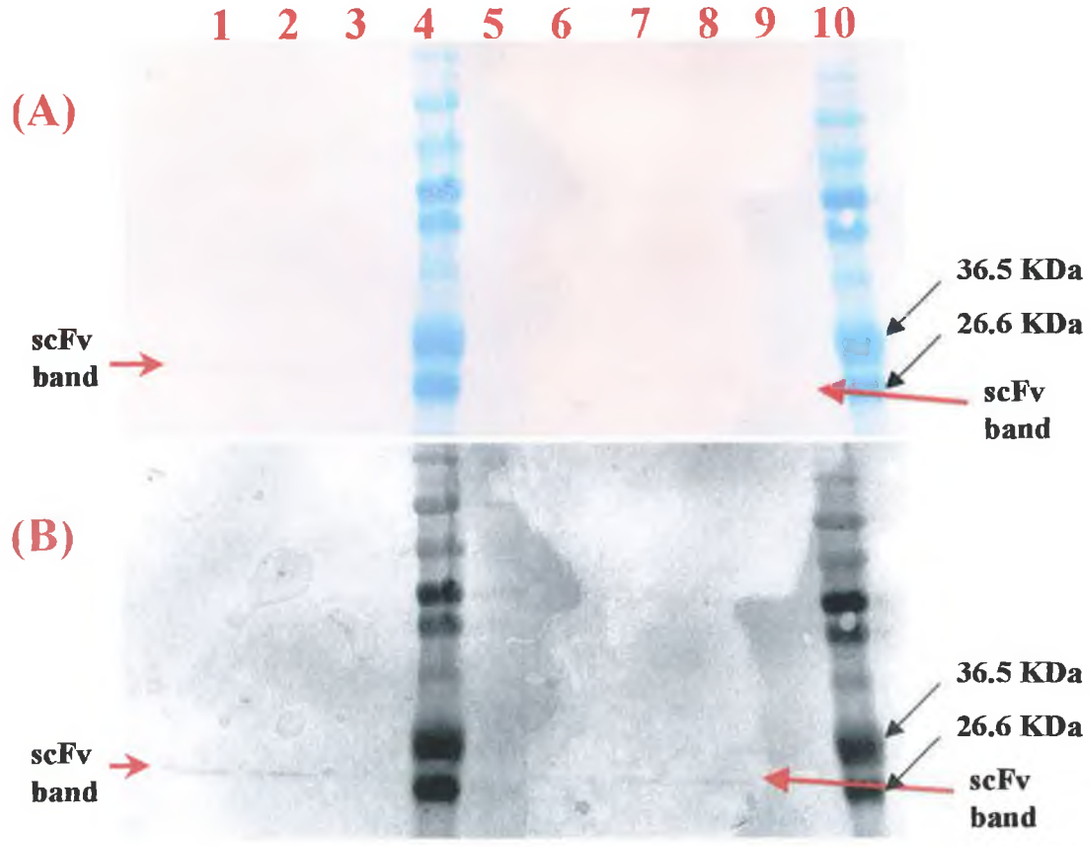


Figure 5.26: *Western Blot of clone F12 cytoplasmic extract at one-hour intervals. Lane 1, 2 and 3 are denatured cytoplasmic extract at time 4, 5 and 20 hours. Lanes 4 and 10 are a Sigma pre-stained marker and Lanes 5-9 are native cytoplasmic extracts at time 0, 3, 4, 5, and 20 hours. Panel B shows the blot from panel A in black and white for better visualisation of scFv bands (indicated by the red arrows).*

5.4.5 *Evaluation of clone F12 phage-scFv antibodies for use in an Inhibition ELISA*

As observed in section 5.4.4, induction of TG1 cells containing the F12 scFv gene sequence with IPTG, results in the majority of the antibodies forming inclusion bodies. As previously discussed in section 5.3.5, after attempts to increase the soluble expression of scFv antibodies, Hayhurst *et al.* (2003), found that the use of phage-scFv antibodies generated the best binding response in ELISA, probably due to the high copy number of the pVIII protein which was detected by the anti-bacteriophage antibody. Phage-scFv antibodies were also used to detect *Listeria monocytogenes* cells in an electrochemical immunosensor assay described by Benhar *et al.* (2001). An inhibition ELISA was developed using clone F12 phage-scFv antibodies incubated with decreasing HT-*Listeria monocytogenes* cell concentrations as described in section 2.4.4.5. Phage-scFv antibodies were incubated with cell concentrations from $2x10^{10}$ to $4x10^{4}$ cells/ml as presented in Figure 5.27. The inhibition ELISA was repeated using the same phage-scFv antibody supernatant over a four-day period. The sensitivity of the inhibition ELISA decreased dramatically over time (from approximately $5x10^{5}$ cells/ml at Day 1 to $5\text{-}6x10^{6}$ cells/ml at Day 4) with coefficients of variance between 12 and 20%. The loss of sensitivity of the inhibition ELISA is possibly due to the loss of the scFv antibodies from the surface of the phage. Johns *et al.* (2000), also reported a loss of reactivity in ELISA when storing phage antibodies for 4 days at 4°C. When analysing fresh phage antibody supernatant and 4 day old supernatant by Western blotting, the authors observed almost total cleavage of the scFv antibody from the phage coat protein pIII. From this observation and from the loss of sensitivity observed in ELISA with the phage-scFv clone F12, the use of phage-scFv antibodies in the development of a reproducible immunoassay is limited. As described in section 5.3.5, refolding of insoluble inclusion bodies or co-expression of a molecular chaperone protein can help increase the solubility of scFv antibodies. However, when assayed against intact cells as described in section 2.4.3.11, clone F12 phage-scFv antibodies generated a much decreased signal (over 70% decrease in signal when assayed against intact *Listeria monocytogenes* 4b) and showed no significant increase in reactivity to *Listeria monocytogenes* cells compared to the other bacterial strains tested (Table 5.2).

Table 5.2: *Cross reactivity studies of phage-scFv clone F12 antibodies by ELISA. The binding response minus background (wells blocked with milk) was expressed as a percentage of the binding response of the antibody over heat-killed Listeria monocytogenes 4b (HT-L.m 4b). The antibody was tested against intact (not heat killed) Listeria monocytogenes 4b (L. m 4b), Listeria innocua 6B (L. in 6B), Listeria welshimeri (L. welsh.), Listeria ivanovii (L. ivan.), Listeria seeligeri (L. seel.) and Bacillus subtilis (S. subtilis).*

	HT-L.m 4b	***L. m 4b***	***L. in 6B***	***L. welsh.***	***L. ivan.***	***L. seel.***	***B. subtilis***
F12	100%	29.0%	17.5%	18.4%	20.9%	18%	17.5%

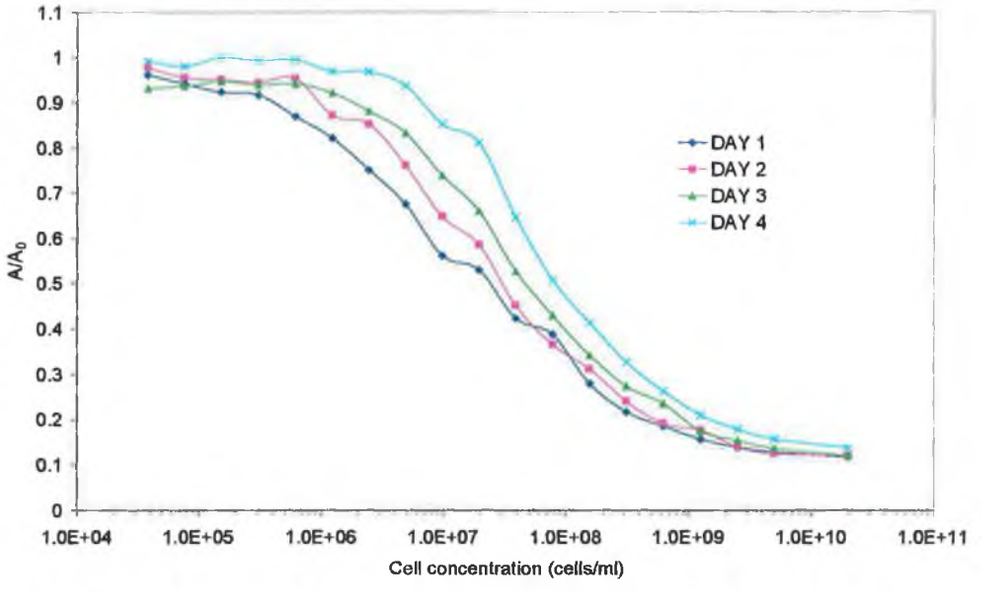


Figure 5.27: *Interday reproducibility study of the anti-HT-Listeria monocytogenes phage-scFv antibody clone F12. The antibody supernatant was incubated with decreasing concentrations of HT-Listeria monocytogenes cells as described in section 2.4.4.5. The sensitivity of the inhibition ELISA decreased dramatically over time (from approximately $5x10^5$ cells/ml at Day 1 to 5-$6x10^6$ cells/ml at Day 4) with coefficients of variance between 12 and 20%. The loss of sensitivity of the ELISA is possibly due to the loss of the scFv antibodies from the surface of the phage.*

5.5 *Selection of scFv sequences against purified recombinant InlB and p60 from a naïve human antibody library by bio-panning on Nunc immunotubes*

The naïve human antibody library donated by Cambridge Antibody Technology Ltd, Cambridge, UK, was used to select for scFv antibody sequences capable of binding to recombinant InlB (rInlB) and recombinant p60 (rp60). The recombinant proteins were purified from *E. coli* cytoplasmic extracts by immobilised metal affinity chromatography (IMAC) as described in section 2.3.9. Immunotubes were coated with 30 μg/ml of either rInlB or rp60 protein and blocked with 5% (w/v) milk marvel as described in section 2.4.4.3. Rescued phage-displaying scFv particles were added to the immunotube and incubated for 2 hours at room temperature. Unbound phage-scFv particles were washed from the tube and phage binders eluted as described in section 2.4.4.3. Four rounds of bio-panning were performed against immobilised recombinant protein and a phage ELISA carried out from clones selected from rounds 3 and 4 of bio-panning. Fifteen clones were found to react with rInlB (Table 5.3) and nine clones found to react with rp60 (Table 5.4).

5.5.1 *Screening of supernatant and periplasmic extracts of selected clones against recombinant protein and Listeria monocytogenes cells*

As discussed in section 5.4.5, phage-scFv antibodies can be unreliable reagents for the development of a reproducible immunoassay and the selection of a stable soluble antibody is an imperative. The 24 phage-scFv clones selected in Tables 5.3 and 5.4 were expressed as soluble scFv antibodies as described in section 2.4.4.9, and screened for reactivity against recombinant protein and *Listeria monocytogenes* cells. Table 5.5 shows the names and well positions of each of the clones analysed in Figure 5.28. Only two clones, clones G7 and A1 from round 4 of bio-panning against rInlB, expressed functional scFv antibodies in the supernatant. While both clones reacted well with rInlB, neither clone produced a measurable signal against intact *Listeria monocytogenes* cells (Figure 5.29, panel A). Cytoplasmic extracts from each of the selected clones were also analysed against recombinant protein and *Listeria monocytogenes* cells (Figure 5.29, panel B). Five clones (clone D3 from round 4 of bio-panning against rp60, clone H12 from round 3 of bio-panning against rInlB and clones G3, G7 and A1 form round 4 of bio-panning against rInlB) were observed to react with either rp60 or rInlB. Only one of the clones, clone D3 reacted with immobilised *Listeria monocytogenes* cells as well as protein. In fact, clone D3 seemed to react better with immobilised *Listeria monocytogenes* cells (absorbance of 1.294) than with immobilised rp60 (absorbance of 0.653), which was unexpected due to the high concentration of immobilised purified recombinant protein. However, with

further analysis of this clone, it was observed that the scFv was sticking to the blocking solution (milk marvel) and was not actually specific for either rp60 or *Listeria monocytogenes* cells. All four clones (clones G3, G7, H12 and A1) that recognised immobilised rInlB protein failed to generated a measurable signal against *Listeria monocytogenes* cells. Clone G7 was observed to react against purified rInlB protein in solution using an inhibition ELISA format (Figure 5.29) and showed good expression of scFv antibody in both the supernatant and cytoplasmic extract (Figure 5.28). This clone was therefore selected for the development of an immunoassay to detect InlB protein (and therefore indirectly *Listeria monocytogenes*) as described in section 5.5.2.

Table 5.3: *Number of clones selected for screening from rounds 3and 4 of bio-panning against recombinant InlB. Out of 95 clones selected from each round of panning 15 clones were positive (5 from round 3 and 10 from round 4) .*

Rounds of bio-panning	***No. of clones screened***	***No. of clones positive***
3	95	5
4	95	10

Table 5.4: *Number of clones selected for screening from rounds 3 and 4 of bio-panning against recombinant p60 protein. Out of 95 clones selected from each round of panning 9 clones were positive (all nine from round 4).*

Rounds of bio-panning	***No. of clones screened***	***No. of clones positive***
3	95	0
4	95	9

Table 5.5: *Table showing the well positions of the 24 clones selected from rounds 3 and 4 of panning. Each clone was assayed (Figure 5.28) against either rInlB or rp60 (rows B,C and D) and against Listeria monocytogenes cells rows (E, F and G). The clones highlighted in red were selected against rp60 in round 4 of panning, the clones highlighted in green and blue were selected against rInlB in rounds 3 and 4 of panning, respectively. The clone names represent the well positions from which they were selected on the phage ELISA plates.*

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
A	-	-	-	-	-	-	-	-	-	-	-	-
B	-	A3	E7	C9	G12	H12	G3	B10	C9	A3	-	-
C	-	E1	B9	C6	A10	H10	G7	A12	H12	-	-	-
D	-	C8	E2	D3	A12	-	E4	C7	A1	-	-	-
E	-	A3	E7	C9	G12	H12	G3	B10	C9	A3	-	-
F	-	E1	B9	C6	A10	H10	G7	A12	H12	-	-	-
G	-	C8	E2	D3	A12	-	E4	C7	A1	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

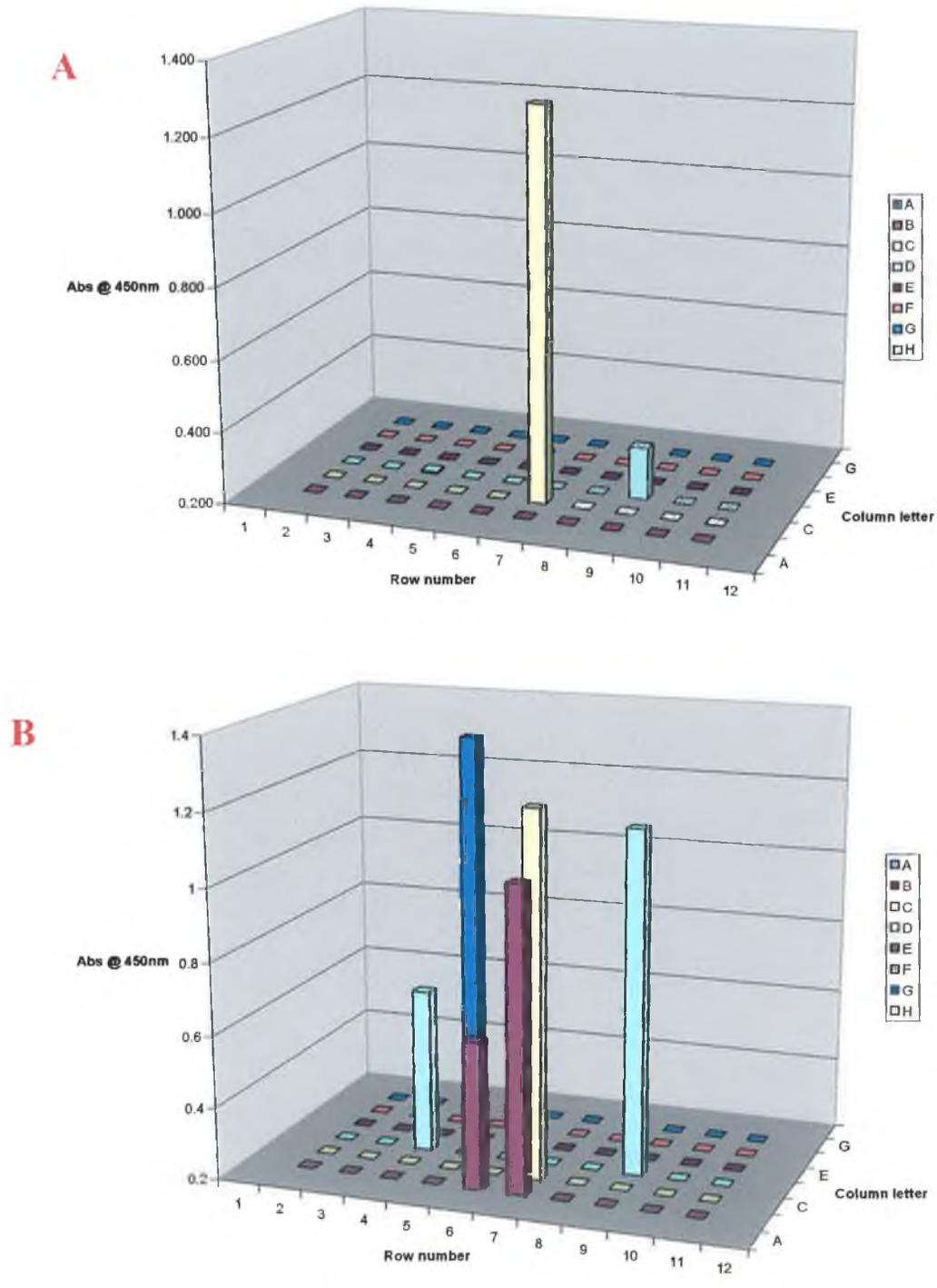


Figure 5.28: *Screening of selected clone supernatant and cytoplasmic extracts against recombinant protein and Listeria monocytogenes cells. Columns B, C and D were coated with either rInlB or rp60 while columns E, F and G were coated with 1x10^8 cells/ml intact Listeria monocytogenes cells. Table 5.5 (shown previously) shows the names and positions of each clone assayed in panels A and B..*

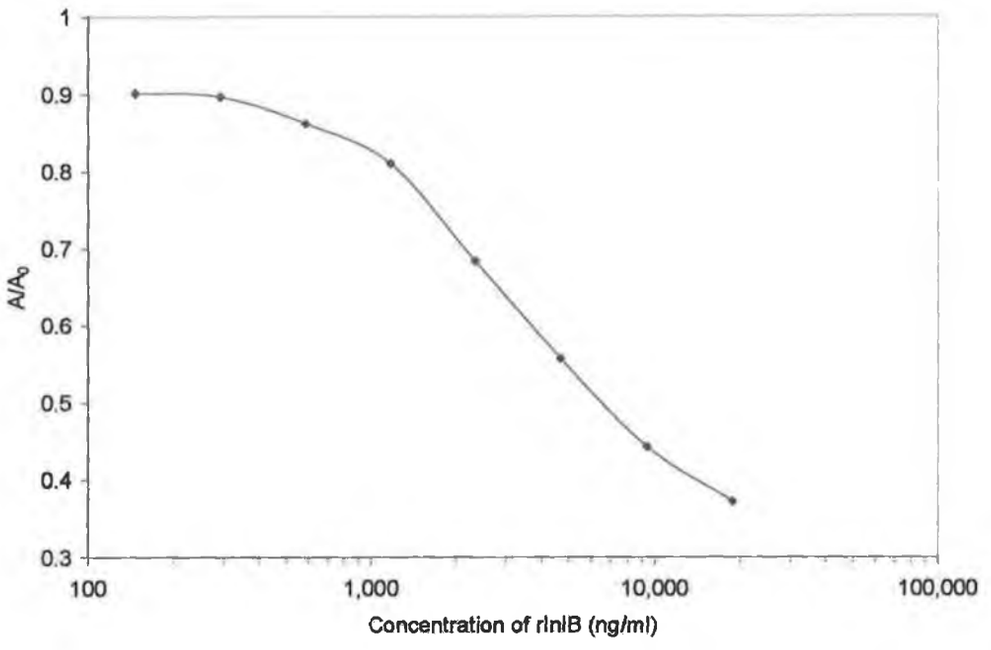


Figure 5.29: *An inhibition ELISA curve of clone G7 scFv supernatant against decreasing concentrations of rInlB over wells coated with 20 µg/ml rInlB. Antibody supernatant was incubated with purified protein for 30 minutes at 37°C and added to a rInlB protein coated ELISA in triplicate. The scFv antibody had a limit of detection of approximately 300 ng/ml of purified recombinant protein.*

5.5.2 *Development of an ELISA-based immunoassay for the indirect detection of Listeria monocytogenes cells using the anti-InlB clone G7 scFv antibody*

Soluble scFv antibodies were generated from the anti-InlB protein clone G7, which expressed well in both the culture supernatant and cytoplasmic extract (Figure 5.28). Slightly more antibody was secreted into the supernatant than found in the cytoplasmic extract, with max antibody production occurring 4-6 hours after induction (Figure 5.30). Antibody supernatant was subsequently used in all further analysis of the antibody due to its high concentration of antibody and ease of production. To determine the binding specificity of the scFv antibody, *E. coli* cell lysate containing the recombinant InlB (rInlB) protein and *Listeria monocytogenes* InlB enriched extract (see section 2.4.4.13) were probed with the scFv antibody supernatant by Western blotting. The clone G7 scFv supernatant recognised both the recombinant and native forms of Internalin B (Figure 5.31) and generates only a single band when probed against both *E. coli* cell lysate containing the recombinant InlB (rInlB) protein and *Listeria monocytogenes* InlB-enriched extract.

5.5.3 *Intra- and interday studies*

Clone G7 scFv antibody supernatant was used to develop an inhibition immunoassay for the detection of InlB protein from *Listeria monocytogenes* cell protein extracts as described in section 2.4.4.14. The assay was performed using eight InlB extract standards, assayed in triplicate and repeated over five days. Figure 5.32 shows the average interday inhibition ELISA standard curve plotted with a 4 parameter equation using BIAevaluation software. To assess the precision of the assay, the percentage coefficients of variance, standard deviation and percentage accuracy values are presented in Table 5.6 for both the inter- and intraday studies. The assay had a linear range of detection of between approximately $3x10^6$ and $1x10^8$ cells/ml with interday coefficients of variance of between 3.19 and 7.69%. The percentage accuracies of the curve ranged from 91.35% to 105.08% indicating the 4 parameter function provides an accurate representation of the sigmoidal relationship between the measured response and the logarithm of concentration observed for the immunoassay.

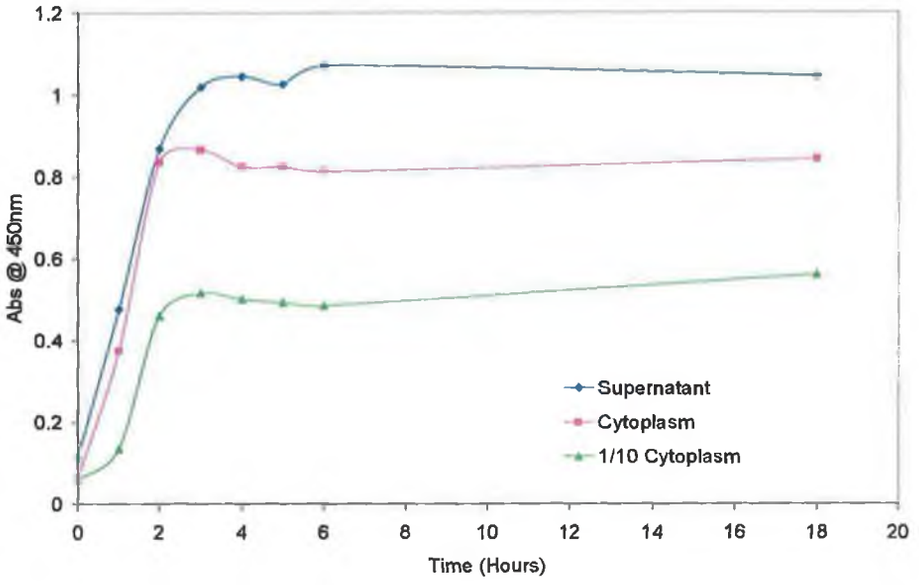


Figure 5.30: *Expression of ant-InlB scFv antibody (clone G7) in both the cytoplasm and supernatant over time. One millilitre samples were taken from the growing culture at one hour intervals and the supernatant and cytoplasmic extracts analysed for binding to rInlB coated ELISA wells. Bound antibody was detected using a mouse anti-c-myc-HRP labelled monoclonal antibody.*

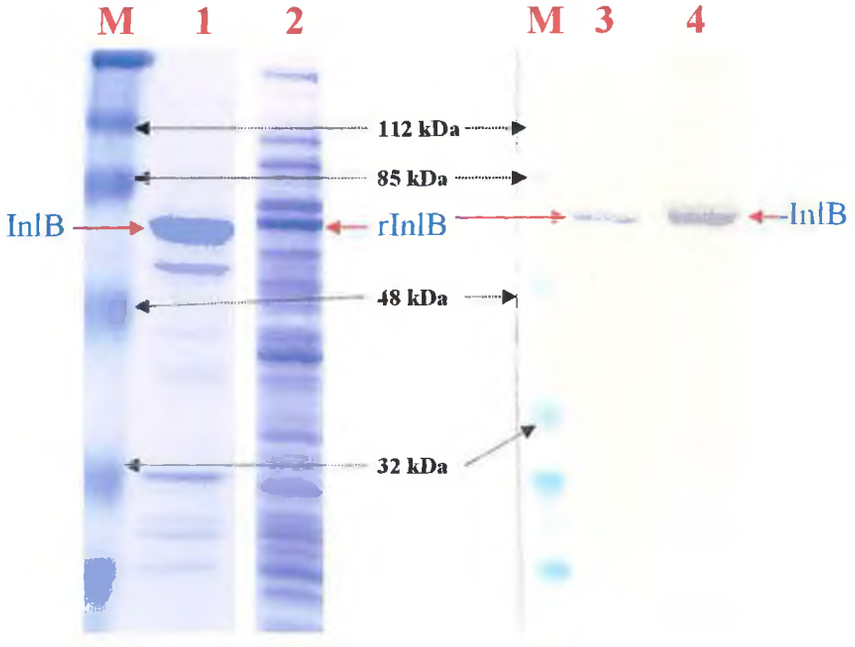


Figure 5.31: *SDS-PAGE and Western blot of InlB extract and recombinant InlB using the anti-InlB scFv clone G7. Lanes M represent a Pierce blue Ranger marker with weights indicated. Lanes 1 and 2 show InlB extract and rInlB lysate protein bands, respectively, separated by SDS-PAGE. Lanes 3 and 4 show western blots of rInlB lysate and InlB extract, respectively, probed with anti-InlB scFv G7 and detected with anti-c-myc-HRP monoclonal antibody.*

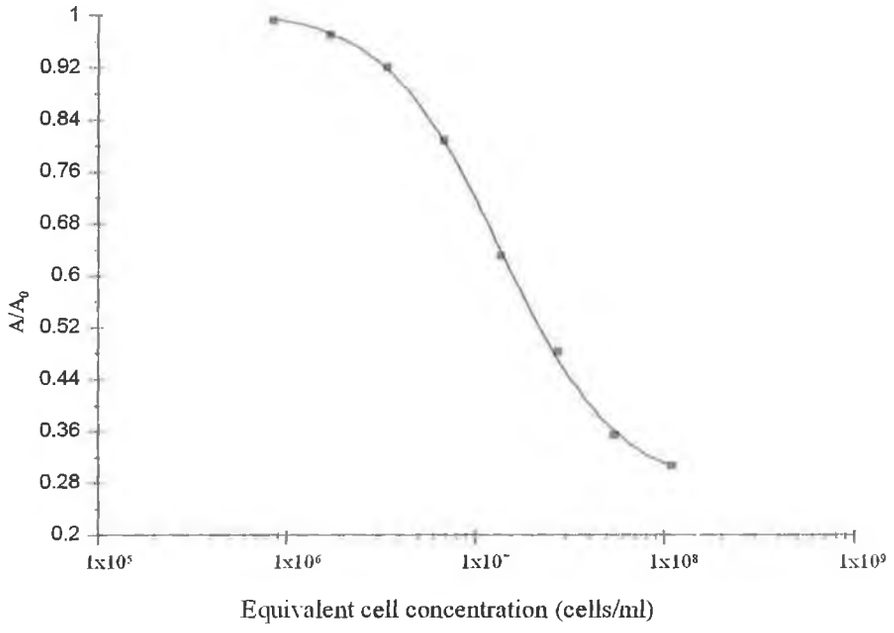


Figure 5.32: *Inhibition ELISA standard curve for the detection of InlB extract using the anti-InlB scFv clone G7. The curve here represents the average values of five assays with eight standards and each standard assayed in triplicate. The coefficients of variance, standard deviations and percentage accuracies are shown in Table 5.6. The equivalent Listeria monocytogenes cell concentrations for each InlB extract dilution was calculated and plotted against the observed absorbance values.*

Table 5.6: *Intra- and interday assay coefficients of variation (CV's) and percentage accuracies for the anti-InlB scFv antibody used in the inhibition assay described in section 5.5.3.*

	INTERDAY			**INTRADAY**		
Concentration (cells/ml)	**% CV**	**Back-calculated concentration.**	**% Accuracies**	**% CV**	**Back-calculated concentration.**	**% Accuracies**
1.09×10^8	6.08	1.06×10^8	102.75	4.05	8.94×10^7	118.23
5.47×10^7	7.69	5.80×10^7	93.90	3.37	5.84×10^7	93.24
2.73×10^7	4.84	2.60×10^7	105.08	5.33	2.40×10^7	112.39
1.37×10^7	5.47	1.42×10^7	96.46	5.46	1.46×10^7	93.06
6.84×10^6	5.81	6.78×10^6	100.88	7.28	6.57×10^6	103.96
3.42×10^6	4.05	3.30×10^6	103.45	5.19	3.40×10^6	100.53
1.71×10^6	3.19	1.74×10^6	98.47	6.19	1.77×10^6	96.31
8.54×10^5	3.56	9.28×10^5	91.35	2.06	9.06×10^5	94.02

5.5.4 Sequencing of the scFv clone G7 and determination of the antibody complementarity determining regions

Sequencing of the scFv gene was carried out as described in section 2.4.4.8. The CDR regions were determined according to the Kabat scheme for the identification of antibody CDR regions as previously described. Figure 5.33 shows the scFv gene sequence with the heavy chain CDR regions highlighted in yellow, the light chain CDR regions highlighted in red, the linker region in grey and both affinity tags highlighted in green.

5.5.5 *Cross-reactivity studies of the scFv*

To assess the binding reactivity of the scFv antibody clone G7 against non-pathogenic strains of bacteria, cross reactivity studies were performed as described in section 2.4.4.15. Figure 5.34 shows a SDS-PAGE gel of the Tris-Cl protein extracts from each bacterial strain examined (Panel A) and a Western blot of each extract probed with the clone G7 scFv antibody supernatant (Panel B). Only one distinct band was observed representing binding to the InlB protein, indicating that the scFv antibody supernatant does not react with Tris-Cl extracts from the non-*Listeria monocytogenes* strains tested. The clone G7 scFv antibody supernatant was also examined for binding to each extract in an inhibition ELISA format as described in section 2.4.4.15. As presented in Table 5.7, the scFv antibody supernatant from clone G7 did not react with any of the strains tested with less than four percent cross-reactivity observed from each strain as determined by ELISA.

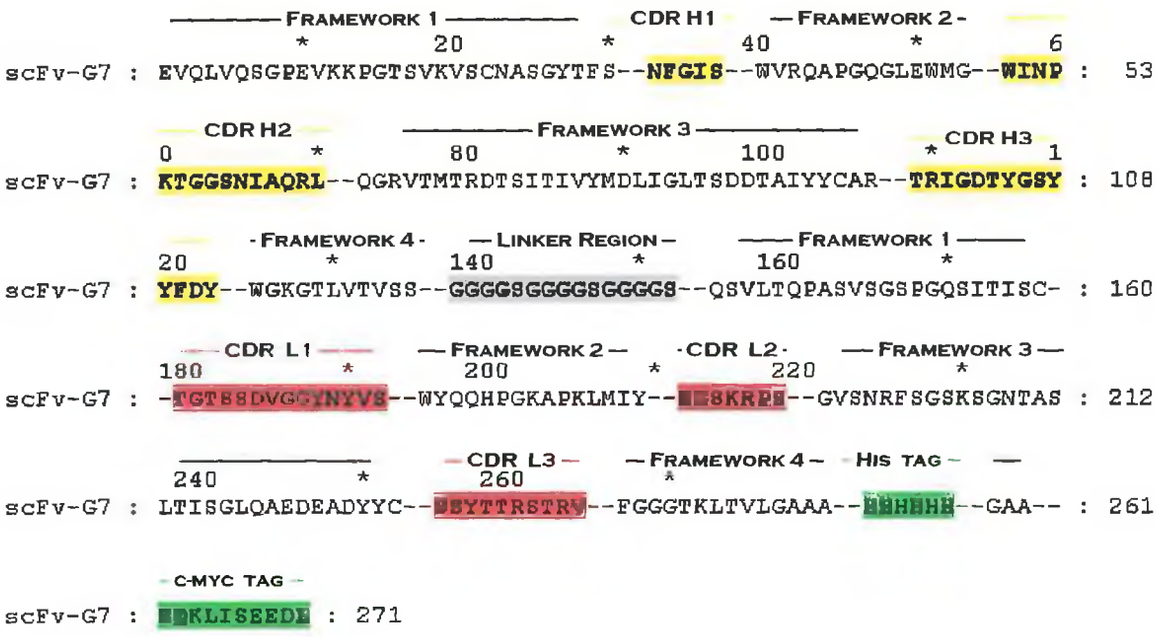


Figure 5.33: *The anti-InlB scFv antibody(clone G7) amino acid sequence. The framework, regions are shown not highlighted while the complementarity determining regions are highlighted in yellow and red representing the heavy and light chain CDR regions, respectively. The fifteen amino acid glycine-serine repeat linker region is shown highlighted in grey. Antibodies expressed in the pCantab6 vector contain a 6 x histidines tag and a c-myc peptide tag for the purification and detection of the scFv antibody. Both affinity tags are shown highlighted in green.*

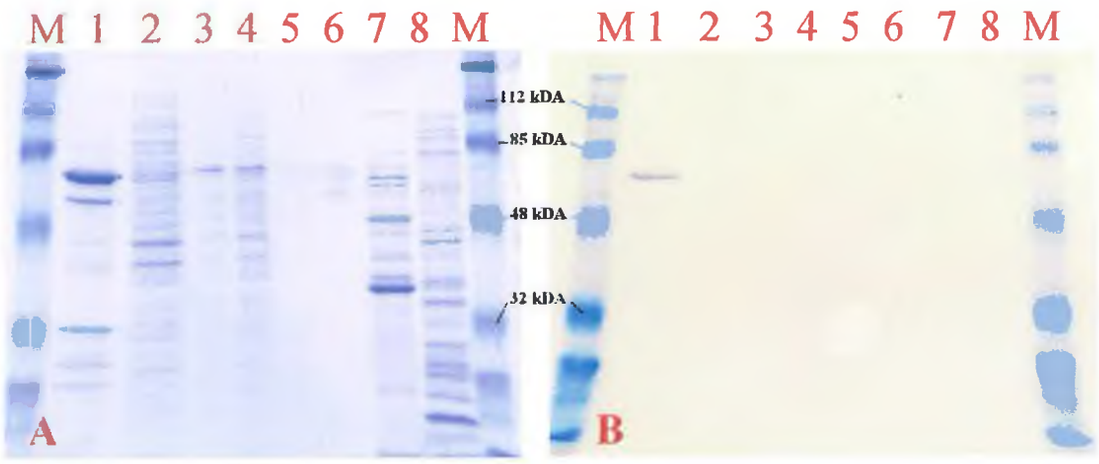


Figure 5.34: *SDS-PAGE and Western blot analysis of Tris-Cl protein extracts from various bacteria. Panel A shows extracts from L. monocytogenes (lane 1), L. innocua 6a (lane 2), L. innocua 6b (lane 3), L. ivanovii (lane 4), L. welshimeri (lane 5), L. seeligeri (lane 6), B. subtilis (lane 7) and E. coli (lane 8). Panel B shows a western blot of each extract shown in panel A probed with the anti-InlB scFv clone G7 and detected with anti-c-myc-HRP monoclonal antibody. A Pierce Blue Ranger pre-stained molecular weight marker (section 2.2.6) was used (lanes M) and the molecular weights indicated.*

Table 5.7: *Percentage cross reactivities of the anti-InlB scFv antibody with various bacteria using the inhibition ELISA format described in section 2.4.4.15.*

Organism	**% Cross reactivity**
L. innocua 6a	Neg.
L. innocua 6b	3.52
L. welshimeri	3.79
L. seeligeri	1.62
L. ivanovii	Neg.
B. subtilis	Neg.
E. coli	Neg.

5.6. *Discussion*

A detailed description of the generation of two antibody libraries developed from the spleens of mice immunised with heat-treated *Listeria monocytogenes* cells and InlB protein extract is presented in this chapter. Phage-scFv antibodies selected from both murine antibody libraries and a naïve human antibody library were characterised by SDS-PAGE, Western blotting and ELISA. Selected scFv antibody genes were sequenced and the complementarity determining regions identified.

The method used in section 5.2 to develop scFv antibody libraries from the spleen cells of mice, was described by Krebber *et al.* (1997a). Splenomic mRNA from immunised mice with an antibody titre of over 1/50,000 for their respective immunogens, was isolated and used as a template for cDNA synthesis. Variable domain genes were amplified from the cDNA template, by primers incorporating all mouse V_H, V_λ and V_κ sequences collected in the Kabat data base (Kabat et al., 1991), combined with some additional extended primer sets (Kettleborough *et al.*, 1993; Ørum *et al.*, 1993 and Zhou *et al.*, 1994). Good amplification of variable light and heavy chain genes (Figure 5.3 and 5.4) was achieved and single chain Fv fragments were assembled in the orientation V_L-$(G_4S)_4$-V_H (where V_H and V_L are the variable heavy and light chains domains, respectively, and $(G_4S)_4$ is the 20 amino acid linker region) by a two fragment Splice by Overlap Extension PCR. A PCR Optimizer™ kit from Invitrogen was used to determine the optimal buffer composition for the SOE PCR. From Figure 5.5 and 5.6, a buffer composition of 7.5 mM $MgCl_2$, pH 9.0 (buffer E) was determined to be optimal for both SOE PCR reactions. Purified pAK100 plasmid and SOE PCR product were restricted with the *SfiI* restriction enzyme allowing directional cloning in to the vector. *SfiI* cuts DNA containing a sequence of GGCCNNNNNGGCC where N can be any nucleotide. Krebber *et al.*(1997a), designed the plasmid to contain two different *SfiI* sites (one site with N nucleotides which hybridise the variable light chain DNA and another site which can hybridise the variable heavy chain DNA) allowing the directional cloning of scFv sequences with a single enzyme. Ligated pAK100-scFv gene sequences were originally transformed into Stratagene supercompetent *E. coli* XL-1 Blue cells but less than ten colonies were observed after transformation. The transformation efficiency of supercompetent cells is severely affected by changes in temperature and the cells could have been damaged during delivery. High efficiency *E. coli* XL-1 Blue cells were therefore produced as described in section 2.4.2.10, and two antibody libraries of approximately 5-6 x 10^3 transformants produced. The size of the generated antibody libraries were very small with a library size of between 10^6 and 10^8 transformants expected. However, due to the pre-immunised nature of the splenomic cells, which contain highly specific mRNA for production of antibodies to

Listeria monocytogenes cells and InlB extract, low levels of efficiency may in fact produce specific scFv antibodies.

Nine phage-scFv clones, six from the InlB extract library and three from the *Listeria monocytogenes* library were selected that reacted with HT- *Listeria monocytogenes* cells in ELISA (sections 5.3.1 and 5.3.2). Alignment of the nine clones revealed that two of the clones (clones D8 and D11) were actually the same clone, while the diversity between each of the remaining clones varied, with a number of clones showing significant similarities. The high degree of similarity between clones selected from two different libraries would indicate that there may have been cross contamination of DNA prior to domain linking by SOE PCR. The Polymerase Chain Reaction is a very sensitive technique and the presence of trace amounts of contaminating DNA can be easily be amplified. A homology model of one of the selected clones (clone G9) was produced and various views of the antigen binding domain presented. The homology model illustrated the importance of the CDR 3 regions of the antibody and showed how not all residues of the CDR regions are involved in antigen binding. Subcloning the selected scFv genes into the pAK400 vector failed to produce soluble scFv antibodies (section 5.3.5). Before pursuing the strategies discussed in section 5.3.5 for the solublisation of scFv antibodies, the tendency of each clone to cross-react with bacterial strains was evaluated in section 5.3.6. Each of the antibodies tested failed to recognise intact *Listeria monocytogenes* cells to the same degree as heat-killed cells. Also, each phage-scFv clone recognised the four non-pathogenic strains of the genus *Listeria* and the Gram positive bacterium *B. subtilis*, showing binding equal to or more than that observed to intact *Listeria monocytogenes* 4b cells.

In pursuit of a species-specific recombinant scFv antibody, a naïve human antibody library, with an antibody diversity of 1.4×10^{10} clones (Vaughan *et al.*, 1996), was bio-panned against *Listeria monocytogenes* cells (section 5.4) and purified recombinant *Listeria monocytogenes* invasion associated proteins, Internalin B (InlB) and p60 (section 5.5). Four rounds of bio-panning were performed with HT-*Listeria monocytogenes* cells and two clones selected that recognised HT-*Listeria monocytogenes* cells in an inhibition ELISA format. Both clones recognised an unknown 38-43 kDa *Listeria monocytogenes* associated protein as observed in Figure 5.22. Sequencing of the scFv genes sequences revealed that both clones were in fact the same clone. The naïve human antibody library used in section 5.4 allows the expression of soluble scFv antibodies without the need for subcloning into an expression vector. However, induction of scFv expression with 1 mM IPTG failed to generate soluble antibody. A Western blot of cytoplasmic extracts from the scFv clone at various times after induction was performed. Single chain Fv fragment

bands were observed at 3 and 4 hours after induction in the denatured cytoplasmic extracts indicating that the majority of the scFv gene product is expressed as insoluble inclusion bodies. The use of phage-scFv antibodies in the development of an inhibition immunoassay for the detection of HT-*Listeria monocytogenes* cells was subsequently evaluated. Phage-scFv antibodies were incubated with cell concentrations from $2x10^{10}$ to $4x10^{4}$ cells/ml as presented in Figure 5.27. The ELISA was repeated using the same phage-scFv antibody supernatant over a four-day period and the sensitivity of the ELISA observed to decrease dramatically with time (from approximately $5x10^{5}$ cells/ml at Day 1 to 5-$6x10^{6}$ cells/ml at Day 4) with coefficients of variance between 12 and 20%. The loss of sensitivity of the inhibition ELISA is thought to be due to the loss of the scFv antibodies from the surface of the phage, which was also observed by Johns, *et al.* (2000). When assayed against intact cells as described in section 2.4.3.11, clone F12 phage-scFv antibodies generated a much decreased signal (over 70% decrease in signal when assayed against intact *Listeria monocytogenes* 4b) and showed no significant increase in reactivity to *Listeria monocytogenes* cells compared to the other bacterial strains tested (Table 5.2).

In order to refine the specificity of scFv antibodies capable of reacting with *Listeria monocytogenes* cells, the naïve human antibody library was bio-panned with purified recombinant InlB (rInlB) and recombinant p60 (rp60). The recombinant proteins were purified from *E. coli* cytoplasmic extracts by immobilised metal affinity chromatography (IMAC) as described in chapter in chapter 4. After 4 rounds of bio-panning with each protein, fifteen clones were selected that reacted with rInlB (Table 5.3) and nine clones selected that reacted with rp60 (Table 5.4). The 24 phage-scFv clones selected were expressed as soluble scFv antibodies as described in section 2.4.4.9, and screened for reactivity against recombinant protein and *Listeria monocytogenes* cells. Only two clones, clones G7 and A1 from round 4 of bio-panning against rInlB, expressed functional scFv antibodies in the supernatant, while the cytoplasmic extracts of five clones (clone D3 form round 4 of bio-panning against rp60, clone H12 from round 3 of bio-panning against rInlB and clones G3, G7 and A1 form round 4 of bio-panning against rInlB) were observed to react with either rp60 or rInlB protein. Only one of the clones, clone D3, seemed to react with immobilised *Listeria monocytogenes* cells, however this was later observed to be non-specific binding of the scFv antibody to the blocking solution. Clone G7 was observed to react against purified rInlB protein in solution using an inhibition ELISA format (Figure 5.29) and showed good expression of scFv antibody in both the supernatant and cytoplasmic extract (Figure 5.28). Therefore, it was selected for the development of an immunoassay to detect InlB protein (and therefore indirectly *Listeria monocytogenes*) as described in section 5.5.2.

An inhibition immunoassay for the detection of InlB protein from *Listeria monocytogenes* cell Tris-Cl extracts was developed as described in section 2.4.4.14. The assay was plotted with a 4 parameter equation using BIAevaluation software and had a linear range of detection of between approximately $3x10^6$ and $1x10^8$ cells/ml. Interday coefficients of variance of between 3.19 and 7.69% were observed which is well within the 20% limit reported by Findlay *et al.* (2000). The percentage accuracies of the curve ranged from 91.35% to 105.08% which indicates that the fitted four parameter equation provides an accurate representation of the sigmoidal relationship between the measured response and the logarithm of concentration observed for the immunoassay. These results show that this anti-InlB antibody generates an accurate, specific and reproducible assay. Demonstration of specificity of an immunoassay for the antigen of interest is critical as most immunoassays are not preceded by extraction of the antigen from the matrix of interest. To assess the binding reactivity of the scFv antibody clone G7 against non-pathogenic strains of bacteria, cross reactivity studies were performed as described in section 2.4.4.15. The scFv clone G7 recognised a single band, representing that of the *Listeria monocytogenes* InlB protein when probed by Western blotting against Tris-Cl extracts from each bacterial strain examined. No significant cross reactivity with the tested Tris-Cl extracts was observed in ELISA indicating the antibody has the potential to specifically detect *Listeria monocytogenes* cells.

To summarise, two antibody libraries were successfully generated from the spleens of mice immunised with *Listeria monocytogenes* cells and InlB protein extract. However, the small size of the libraries can reduce the possibility of selecting high affinity soluble scFv antibodies to their targets. Phage-scFv antibodies were selected from both murine antibody libraries as well as from a naïve human antibody library. Isolation of specific phage clones does not guarantee that you will get soluble scFv expression, as antibody libraries, and in particular immune libraries are riddled with "truncated" clones, some with internal amber stop codons or with frameshifts (personal communication with Prof. Itai Benhar, Tel-Aviv University, Israel). A soluble scFv antibody was selected from the naïve human antibody library that recognised the Internalin B protein. To our knowledge, this antibody is the second recombinant anti-*Listeria monocytogenes* scFv antibody reported in the literature (Benhar *et al.*, 2001 reported using a phage displayed scFv antibody) and first anti-*Listeria* scFv antibody used in soluble form. An inhibition immunoassay was developed with the scFv supernatant that had a limit of detection of approximately 3×10^6 cells/ml. The immunoassay showed excellent precision with CV values less that 8% and showed no significant cross reactivity with the bacterial strains studied.

Chapter 6

Development of a biosensor-based assay for the detection of *Listeria monocytogenes* using surface plasmon resonance

6.1 *Introduction*

Biosensors offer the potential of rapid, accurate and sensitive detection of analytes (e.g. bacteria, proteins and DNA). Surface plasmon resonance biosensors allow the label free detection of biomolecular interactions in "real-time". The pioneers of SPR-based bio sensing were Pharmacia BiosensorAB, now BIAcore AB, who launched the original BIAcore system in 1990 (Liedberg *et al.*, 1995). BIAcore's detection principle relies on surface plasmon resonance (SPR), an electron charge density wave phenomenon that arises at the surface of a metallic film when light is reflected at the film under certain conditions (see section 1.17 for a more detailed description of SPR). Resonance occurs as a result of energy and momentum being transformed from incident photons into surface plasmons, and is sensitive to the refractive index of the medium on the opposite side of the film from the reflected light (Anon., 2001). In a typical BIAcore immunoassay, a ligand, usually an antibody, is chemically attached to a sensor chip surface, consisting of a thin metal film applied to a glass surface. When the sensor is exposed to a sample containing the specific analyte (e.g. Internalin B), the binding of the analyte to the antibody causes a change in the angle of reflected light at the metal surface, which is measured by the BIAcore instrument. The size of the shift is proportional to the quantity of the analyte in the sample.

The work described in this chapter a BIAcore 3000 instrument was used to detect *Listeria monocytogenes* cells in various assay formats. Two polyclonal antibodies, an anti-*Listeria monocytogenes* antibody and an anti-InlB extract antibody (described in chapter 3), were used to directly detect *Listeria monocytogenes* cells while the anti-InlB scFv antibody described in chapter 5, was used to detect *Listeria monocytogenes* InlB extract and, therefore, indirectly to detect *Listeria monocytogenes* cells.

A subtractive inhibition assay was developed to rapidly detect *Listeria monocytogenes* cells in solution using the anti-*Listeria monocytogenes* polyclonal antibody described in chapter 3. Antibody was incubated with various concentrations of *Listeria monocytogenes* cells and free unbound antibody separated from bound antibody by centrifugation. By removing cells and bound antibody from solution with a stepwise centrifugation step (gradually increasing the centrifugation speed in small increments), free unbound antibody could be detected by an immobilised anti-Fab antibody on the sensor chip surface. A decrease in free antibody concentration was observed with increasing *Listeria monocytogenes* cell concentrations. A calibration curve was constructed by plotting the average (n=3) change in response for each *Listeria monocytogenes* cell concentration

standard against the known concentration of *Listeria monocytogenes* cells used in each run.

A polyclonal antibody against InlB extract (also described in chapter 3), was used to develop a direct inhibition assay to accurately detect *Listeria monocytogenes* cells in solution. Protein G-purified antibody was incubated with various concentrations of *Listeria monocytogenes* cells and subsequently injected over a recombinant InlB (described in chapter 4) immobilised CM5 sensor chip surface. A decrease in antibody binding response was observed with increasing *Listeria monocytogenes* cell concentrations and a standard curve generated. Intra- and interday assay variability studies were carried out to evaluate the precision and reproducibility of the assay.

An anti-InlB single chain Fv fragment antibody (clone G7 described in section 5.5.2), was used to develop an inhibition assay to indirectly detect *Listeria monocytogenes.* Antibody incubated with InlB extract from a known concentration of *Listeria monocytogenes* cells, was injected over a recombinant InlB protein immobilised sensor chip surface. The decrease in binding response observed with increasing InlB extract concentrations was recorded and standard curve against equivalent *Listeria monocytogenes* cell numbers (equivalent cell numbers at each InlB extract concentration). However, the choice of sensor chip used for the assay had to be evaluated as the Internalin B protein was observed to non-specifically bind to the dextran matrix due to the strong electrostatic interactions between the negatively charged dextran surface and the cationic protein. It was also observed that the InlB protein also non-specifically bound the dextran free flat carboxymethylated surface of the BIAcore pioneer C1 sensor chip. Nevertheless, the observed non-specific binding to the flat carboxymethylated surface of the C1 sensor chip could be eliminated by over-activation of the sensor chip surface following immobilisation of the recombination InlB with subsequent capping of unreacted groups with 1M ethylene diamine, pH 8.5 (described in section 6.4.1.5). Activation of the carboxymethylated surface under normal conditions (section 2.5.2) usually results in the activation of only 40% of the carboxyl groups on the surface (personal communication with John Butler, BIAcore AB, UK.). Over-activation (activation for double the time) of the sensor chip surface, followed by capping with ethylene diamine (instead of ethanolamine), allows the formation of an amine surface, increasing the overall pH of the surface.

6.2 *Indirect detection of Listeria monocytogenes with an anti-Listeria monocytogenes polyclonal antibody using a subtractive inhibition assay*

The BIAcore biosensor is a commercial biosensor based on the phenomenon of surface plasmon resonance (SPR) (Quinn and O'Kennedy, 1999). The objective of this study was to develop a new "real-time" subtractive inhibition assay to detect low numbers of *Listeria monocytogenes* cells in solution using a BIAcore 3000 biosensor as described in Figure 6.1. By removing cells and bound antibody from solution with a stepwise centrifugation step, free unbound antibody could be detected by an immobilised anti-rabbit Fab antibody on the sensor chip surface. A decrease in free antibody concentration was observed with increasing *Listeria monocytogenes* cell concentrations. Removing the cells and bound antibody by gradually increasing the centrifugation speed was the key to the success of the experiment. Centrifugation at 3000 rpm without the gradual increase in speed (results not shown) seemed to shear the antibodies from the cells, possibly due to the high centrifugal forces, resulting in no inhibition of antibody being observed. Removing cells and antibody by centrifugation as shown in this section, is relatively quick, easy and will allow the detection of cells in more complex and viscous matrices which may cause problems for other reported methods of cell removal such as filtration (Haines and Patel, 1995). In this section a polyclonal rabbit anti-whole *Listeria monocytogenes* cell antibody (described in chapter 3) was mixed with various concentrations of heat-killed *Listeria monocytogenes* cells and free antibody detected using a polyclonal goat anti-rabbit Fab antibody immobilised on the sensor chip surface.

6.2.1 *Preconcentration of the goat anti-rabbit Fab antibody on to the CM5 sensor chip surface*

Low ionic strength buffers, such as 10 mM sodium acetate, allow the electrostatic adsorption of positively charged proteins (proteins in low ionic buffer at a pH below the proteins pI) to the negatively charged dextran surface, maximising "preconcentration" of protein to the sensor surface for subsequent immobilisation and thus, increasing the potential yield of immobilised ligand. To maximise the preconcentration of the goat anti-rabbit Fab antibody to the CM dextran surface, 50 µg/ml of antibody, diluted in 10 mM sodium acetate buffer at various pH increments (Figure 6.2) were injected over a blank CM dextran surface and the degree of preconcentration recorded. From Figure 6.2, optimal antibody preconcentration was observed in sodium acetate buffer, pH 4.3, which was used for all subsequent immobilisations of the goat anti-rabbit Fab antibody.

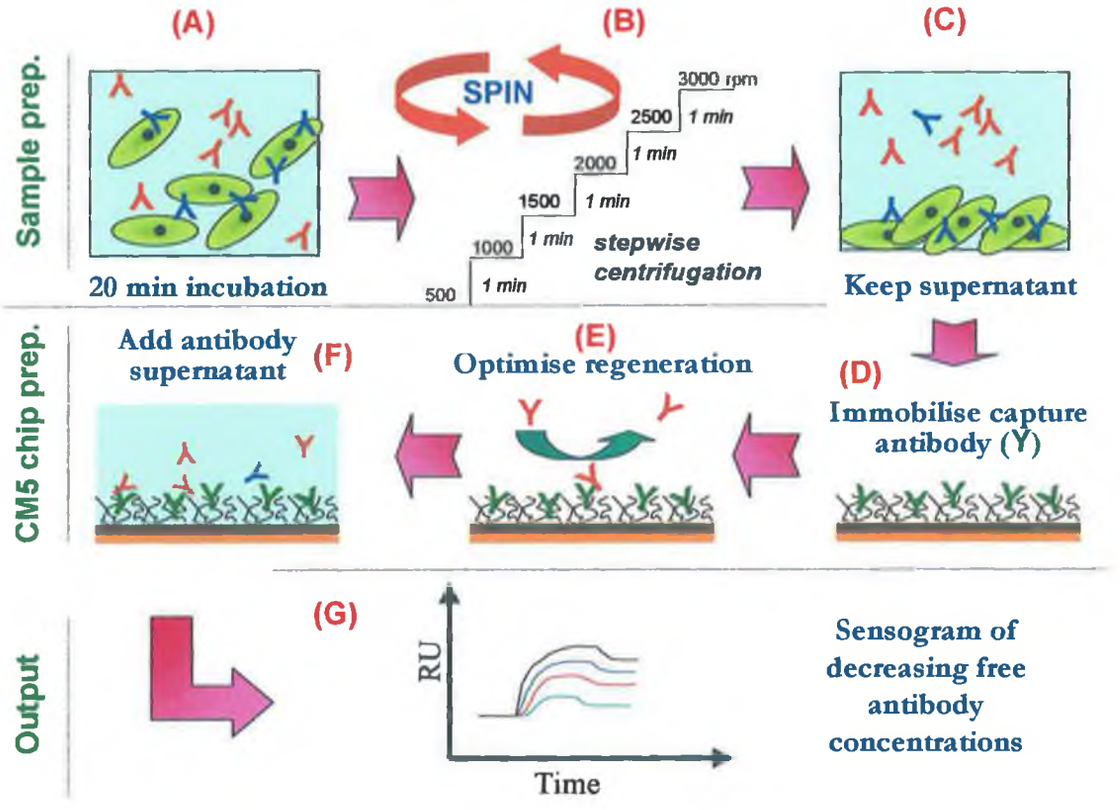


Figure 6.1: *Illustration of the subtractive inhibition assay used to indirectly detect Listeria monocytogenes cells using a BIAcore 3000 instrument.*

(A) *Various concentrations of Listeria monocytogenes cells are incubated with an anti-Listeria monocytogenes antibody for twenty minutes as described in section 2.5.4.*

(B) *Cells and cell bound antibodies are separated from unbound antibody by centrifugation in a stepwise manner, gradually increasing the centrifugation speed at one minute intervals.*

(C) *Unbound antibody (supernatant) is carefully removed and later detected using a goat anti-rabbit Fab antibody immobilised on a BIAcore CM5 sensor surface (panel F).*

(D) *A goat anti-rabbit Fab antibody is immobilised on the surface of a CM5 chip as described in section 2.5.2.*

(E) *The surface regeneration conditions are optimised and the surface binding capacity determined for a number of binding-regeneration cycles.*

(F) *Antibody supernatants from (C) above are passed over the anti-Fab immobilised surface and the response observed.*

(G) *A sensorgram of increasing free antibody concentrations with decreasing Listeria monocytogenes cell numbers is observed and a standard curve plotted.*

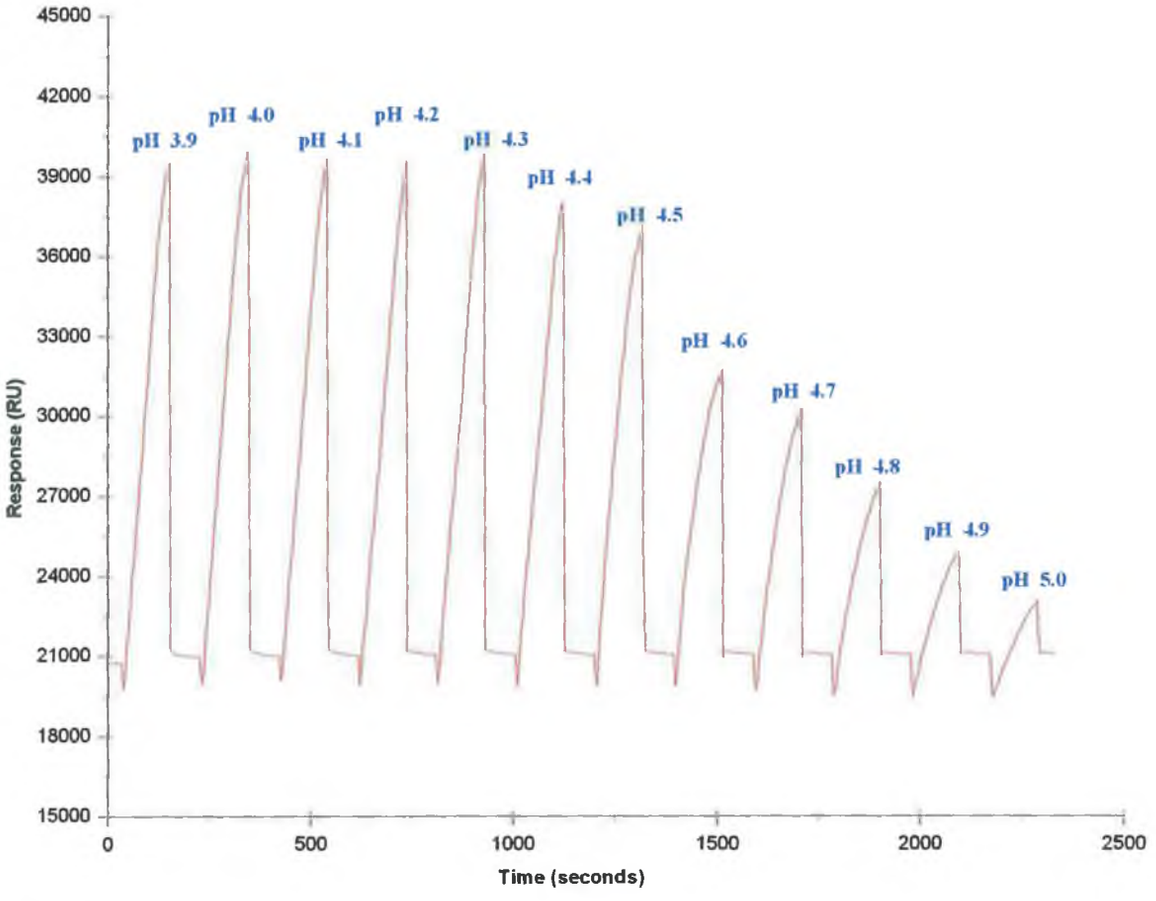


Figure 6.2: *Typical preconcentration of the goat anti-rabbit Fab antibody to the CM-dextran sensor chip surface. Solutions of 50 μg/ml of antibody in 10 mM sodium acetate buffer at various pH increments (indicated on the sensorgram shown here) were passed over an inactivated CM-dextran surface at 10 μl/minute for a period of 2 minutes. The low ionic strength of the acetate buffer favours the electrostatic attraction between the negatively charged dextran layer and the positively charged protein (i.e. below its isoelectric point). The degree of preconcentration was measured from the response prior to the end of each sample injection with the ionic strength of the HBS running buffer (150 mM) sufficient for the removal of electrostatically attracted antibody from the surface. The optimal pH for the immobilisation of the anti-rabbit Fab antibody onto the CM-dextran chip surface was observed to be pH 4.3.*

6.2.2 *Immobilisation of antibody on to the CM dextran sensor chip surface*

Immobilisation of ligand to a CM5 sensor chip surface involves activation of the surface, followed by ligand preconcentration/immobilisation and finally, surface deactivation. Even though there are a wide variety of immobilisation chemistries available for the coupling of ligands to the CM dextran surface, depending on the residues available for coupling, the most commonly employed strategy is the use of EDC (N-ethyl-N'-(dimethylaminopropyl)carbodiimide) and NHS (N-hydroxysuccinimide) chemistry. Using the carbodiimide EDC, the CM dextran carboxyl groups can be transformed into active ester functional groups in the presence of NHS. The surface NHS esters can then react with suitable amino groups in the protein and the surface deactivated or "capped" by the injection of 1M ethanolamine hydrochloride (pH 8.5), which also serves to remove non-covalently bound protein by reducing the electrostatic attraction between the protein and the CM-dextran.

Immobilisation of the goat anti-rabbit Fab antibody to the dextran surface was performed as described in section 2.5.2. 50 μg/ml of goat anti-rabbit Fab antibody, diluted in sodium acetate buffer, pH 4.3, was injected over the EDC/NHS activated sensor chip surface for 45 minutes at 5 μl/minute. Unreacted sites were blocked with a 7 minute injection of 1M ethanolamine hydrochloride (pH 8.5) at 5 μl/minute. Approximately 23,000 RU of antibody was immobilised on the surface (Figure 6.3).

To assess the non-specific binding of the anti-*Listeria monocytogenes* polyclonal antibody to the CM dextran surface, antibody was sequentially injected over the immobilised anti-rabbit Fab antibody surface and a blank CM-dextran surface (Figure 6.4) and the change in response following injection recorded. Injection of anti-*Listeria monocytogenes* polyclonal antibody over both surfaces resulted in approximately 900 response units of binding to immobilised anti-Fab surface while negligible binding was observed to the blank surface.

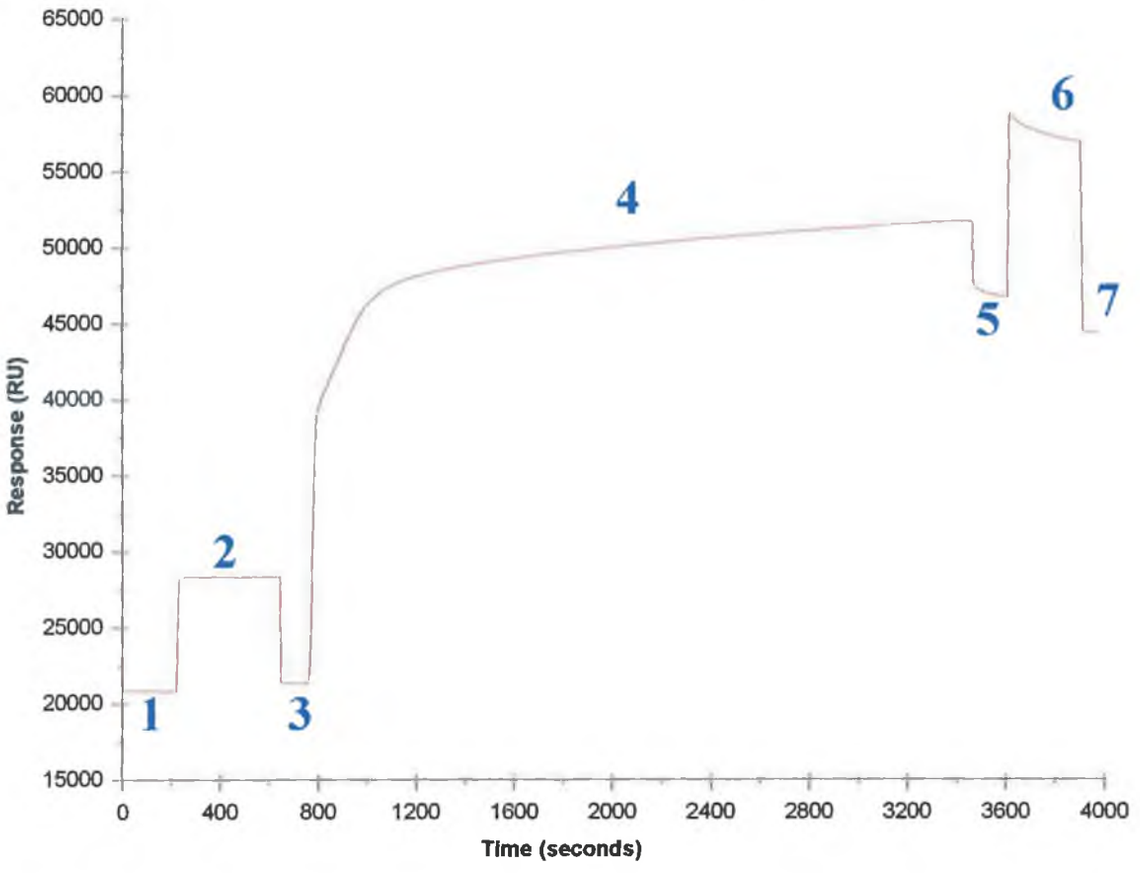


Figure 6.3: *Typical immobilisation of the goat anti-rabbit Fab antibody onto a CM dextran chip surface.*

(1) *HBS buffer is initially passed over the sensor chip surface and the baseline response recorded.*

(2) *The CM dextran surface is then activated with 0.05 M NHS/0.2 M EDC mixture resulting in a large increase in SPR signal due primarily to a bulk refractive index change.*

(3) *HBS buffer is again passed over the now activated surface and a small increase in the baseline is observed.*

(4) *A 50 μg/ml solution of antibody diluted in 10 mM sodium acetate buffer, pH 4.3, is then passed over the activated surface for a period of 45 minutes.*

(5) *HBS buffer is again passed over the surface and the amount of bound ligand recorded.*

(6) *Deactivation of the surface NHS-esters is performed by injecting 1M ethanolamine, pH 8.5 over the surface for 7 minutes, which also serves to elute non-covalently bound antibody.*

(7) *Approximately 23,000 RU of goat anti-rabbit Fab antibody remain bound to the surface after the immobilisation procedure.*

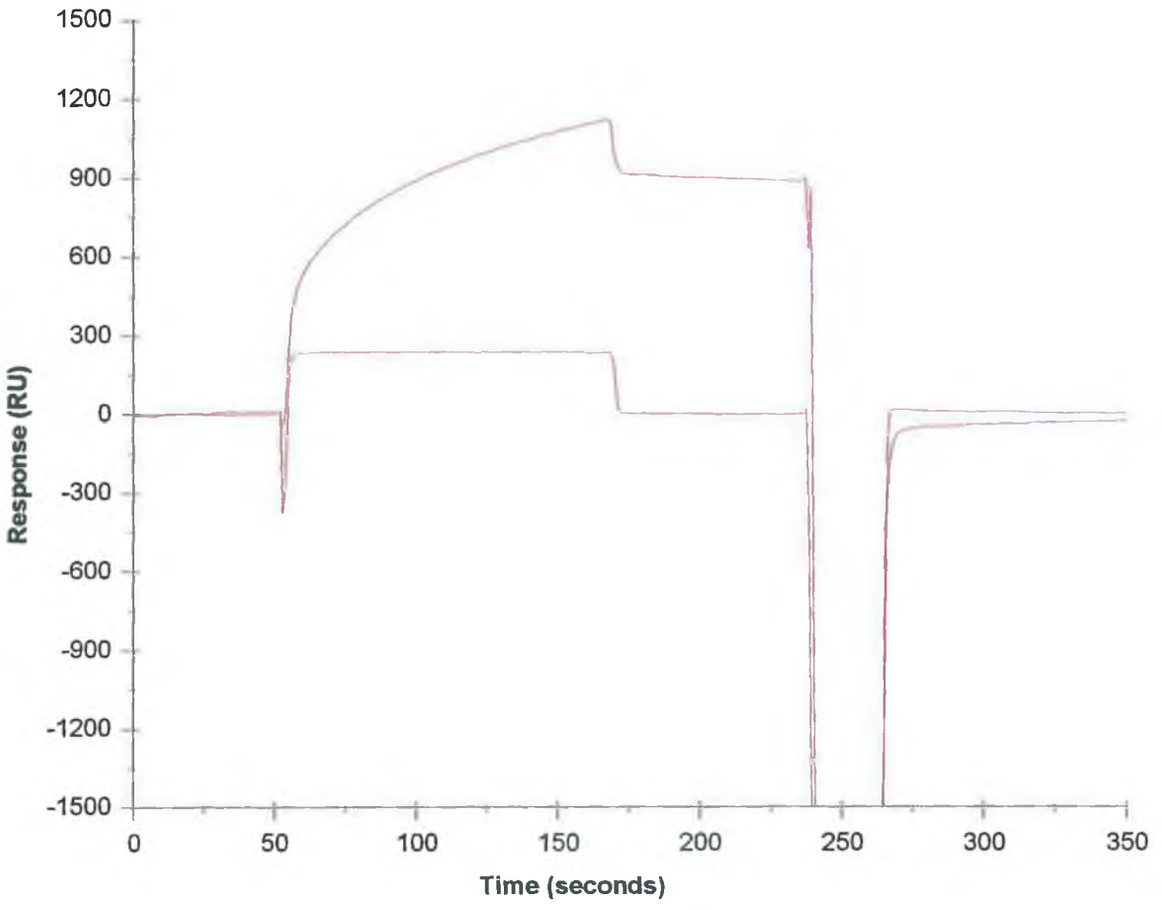


Figure 6.4: *Overlay plot showing the simultaneous injection of the anti-Listeria monocytogenes polyclonal antibody over a goat anti-rabbit Fab antibody immobilised surface (—) and a blank CM dextran surface (—). Protein G affinity- purified anti-Listeria monocytogenes antibody (1/300 dilution) was simultaneously injected over an anti-rabbit Fab antibody surface and a blank CM dextran surface for 2 minutes at 10 µl/minute. No significant binding to the blank surface was observed while approximately 900 RU of anti-Listeria monocytogenes antibody was captured on the immobilised anti-rabbit Fab surface. Bound antibody could be dissociated with a 30 second pulse of 20 mM NaOH and the baseline restored with the injection of HBS running buffer over the surface.*

6.2.3 *Regeneration studies of the immobilised anti-Fab antibody surface*

Regeneration conditions affect the performance and life-time of the sensor chip surface and therefore optimum regeneration is essential for the analysis of large numbers of samples, to evaluate the reproducibility of each measurement and to reduce the cost of the assay. The binding capacity of the anti-rabbit Fab antibody immobilised surface was determined by a series of binding and surface regeneration sequences to assess at which point the binding capacity deviated outside the desired performance range of <20% suggested by Wong *et al.* (1997). Protein G affinity-purified anti-*Listeria monocytogenes* antibody was injected over the immobilised antibody surface for 90 seconds at 10 µl/minute and the surface regenerated with 30 seconds pulses of 20 mM HCl and 20 mM NaOH. The binding-regeneration cycles were repeated for 60 cycles resulting in less than 13% decrease in binding capacity from the first to the last cycle studied (Figure 6.5). The immobilised anti-Fab surface studied in this section went through over 100 binding cycles over a two week period before a significant (<20% original binding capacity) decrease in binding capacity was observed.

6.2.4 *Development of a subtractive inhibition assay for the detection of Listeria monocytogenes cells*

To determine the working range of the inhibition assay, serial dilutions of *Listeria monocytogenes* cells in PBS were incubated with the anti-*Listeria monocytogenes* polyclonal antibody as described in section 2.5.4. The samples containing unbound antibody (stepwise centrifugation supernatant) were then passed over the surface of the sensor chip immobilised with the goat anti-rabbit Fab antibody in random order, followed by regeneration of the surface using a 30 second pulses of 20 mM HCl and 20 mM NaOH. A typical overlay plot demonstrating the decrease in binding of the anti-*Listeria monocytogenes* polyclonal antibody to the immobilised anti-Fab antibody surface with increasing *Listeria monocytogenes* cell concentrations is presented in Figure 6.6. A calibration curve was constructed by plotting the average (n=3) change in response for each *Listeria monocytogenes* cell standard against the known concentration of *Listeria monocytogenes* cells used in each run. The range of detection for the anti-*Listeria monocytogenes* antibody was found to be between 1×10^8 and 1×10^6 cells/ml (Figure 6.7).

In order to determine the accuracy and the intermediate precision of the subtractive BIAcore Inhibition immunoassay, intra- and interday assay variability studies were

performed. Three sets of standards were prepared and incubated with antibody before being assayed. The results obtained from intra- and interday assay studies are shown in Table 6.1. The coefficients of variation (CV's) were determined to assess the precision of the analytical method, expressing standard deviation as a percent function of the mean. These values ranged from 0.59 to 4.80% and 0.14 to 4.00% as observed for the intra- and interday studies, respectively, indicating that the assay had a very good degree of precision. The percentage accuracies values obtained in the linear part of the interday assay (Table 6.1) indicate the fitted four parameter curve provides an accurate representation of the measured response in the linear range of the assay (percentage accuracy values from 92 to 108%).

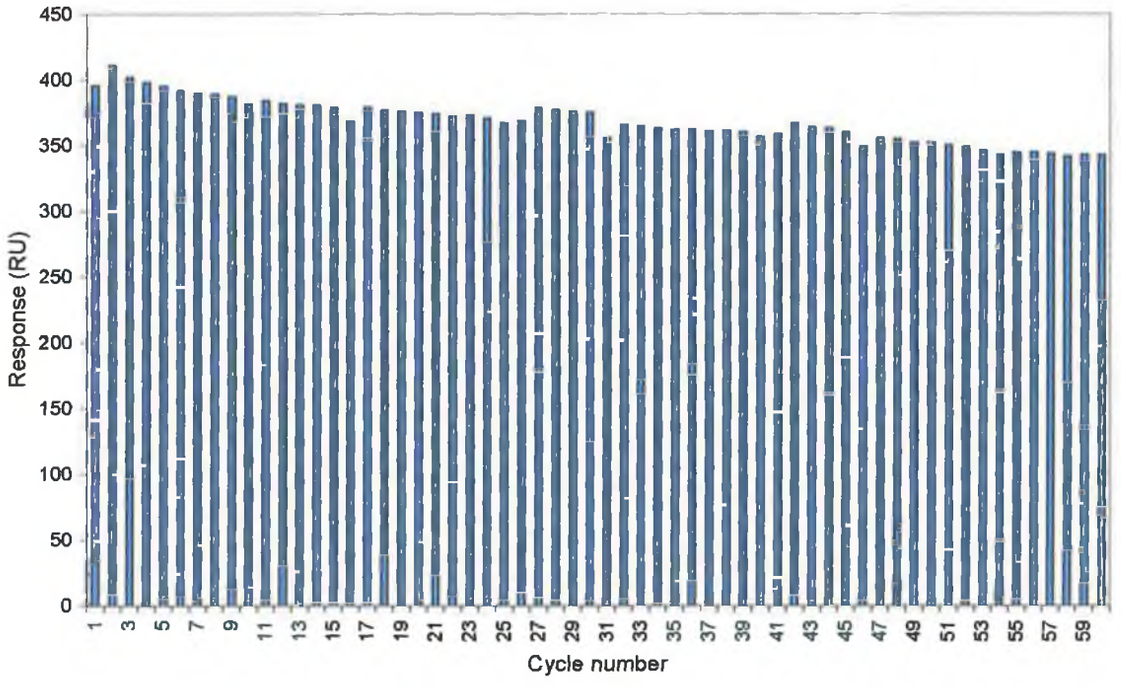


Figure 6.5: *Regeneration study of the binding capacity of the immobilised anti-Fab antibody surface. A 90 second injection of 10 μg/ml Protein G affinity-purified anti-Listeria monocytogenes polyclonal antibody was passed over the immobilised anti-rabbit Fab antibody sensor surface and bound antibody regenerated with a 30 second pulse of 20 mM HCl followed by a 30 second pulse of 20 mM NaOH. The binding-regeneration cycle was repeated for 60 cycles and the degree of binding plotted as shown. The binding capacity decreased by 13% from the first binding-regeneration cycle to the last cycle studies (cycle 60) which is within the limit of <20% suggested by Wong et al. (1997).*

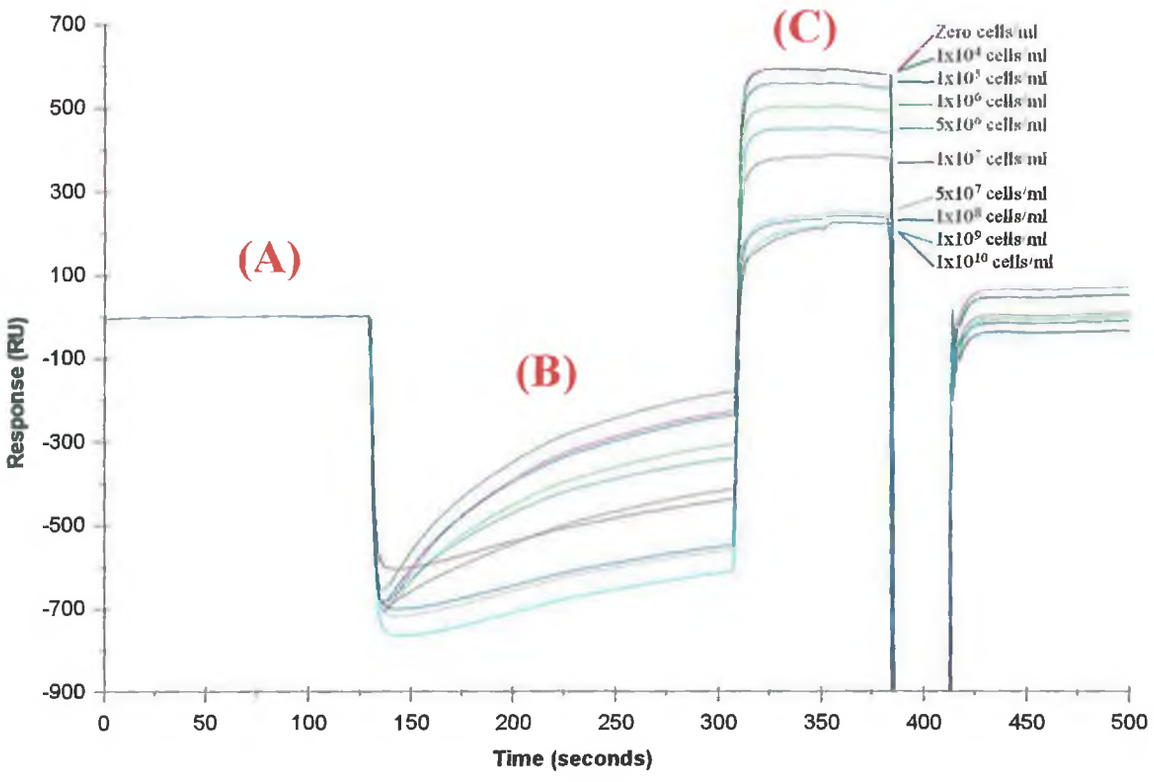


Figure 6.6: *Overlay plot demonstrating the decrease in binding of affinity purified anti-Listeria monocytogenes polyclonal antibody to the immobilised anti-Fab antibody surface with increasing Listeria monocytogenes cell concentrations. (A) shows the normalised baselines of each sensorgram when HBS is injected over the sensor chip surface, (B) shows the binding of increasing free antibody with decreasing Listeria monocytogenes cell concentrations, while (C) shows the base line shift when buffer is passed over the surface after each sample injection. The concentration of Listeria monocytogenes cells used in each binding cycle is indicated beside each sensorgram.*

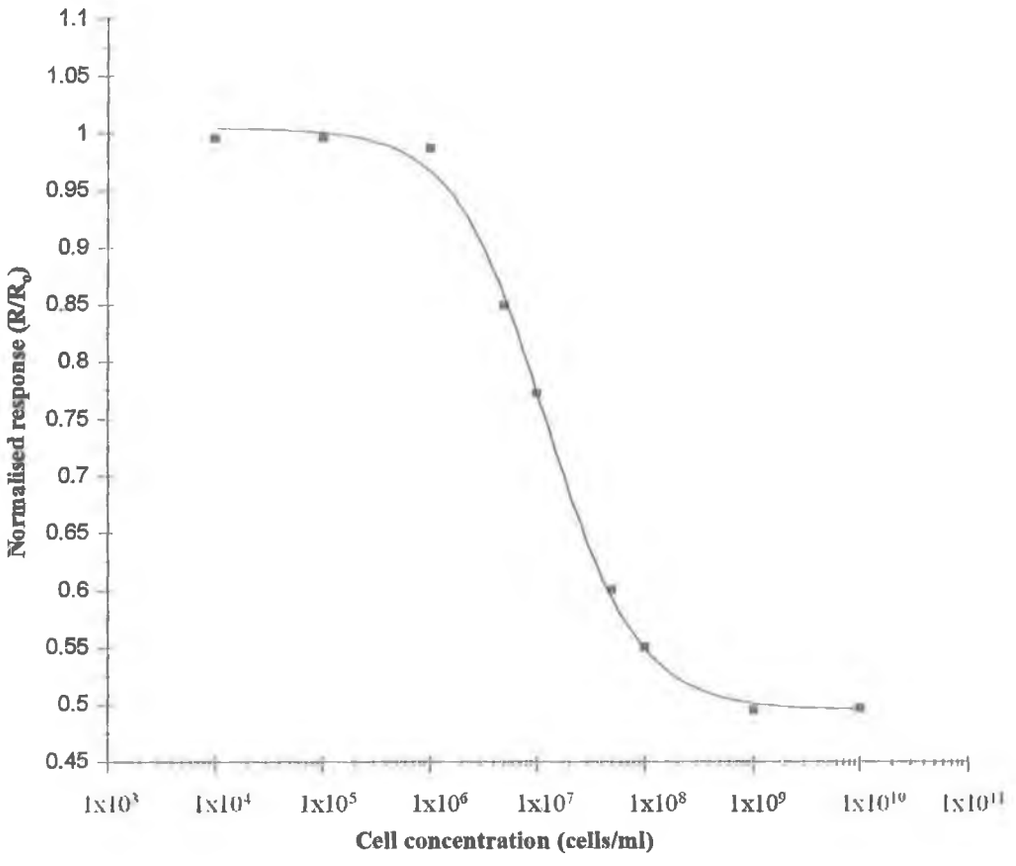


Figure 6.7: *Interday assay calibration curve for the anti-Listeria monocytogenes antibody. A 1/250 dilution of the anti-Listeria monocytogenes antibody was incubated with various Listeria monocytogenes cell concentrations and unbound antibody injected over an immobilised anti-rabbit Fab antibody surface as described in section 2.5.4 . A 4-parameter equation was fitted to the data set using BIAevaluation 3.1 software. The inter-assay means and coefficients of variation are tabulated in Table 6.1. Each point on the curve is the mean of three replicate measurements analysed over three days.*

Table 6.1: *Intra- and interday assay coefficients of variation (CV's) and percentage accuracies for the anti-Listeria monocytogenes polyclonal antibody used in the subtractive inhibition assay described in section 2.5.4. Coefficients of variance (a quantitative measure of precision) was calculated using the equation % CV= (S.D./Mean) x 100 where for intraday studies, the standard deviation (S.D.) is computed from replicate (3 replicates) analyses within a single validation run and for intermediate precision (interday), the S.D. is computed from replicate (3 replicates) analyses over 3 validation runs on 3 separate days. The rows with a "-" represent that no value could be obtained as the data point did not fit the calibration curve.*

	Intraday			***Interday***		
Concentration (cells/ml)	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**
1×10^{10}	1.29×10^{9}	1.60	187	4.23×10^{9}	4.00	158
1×10^{9}	-	0.59	-	1.12×10^{10}	0.14	-
1×10^{8}	9.36×10^{7}	1.18	106	9.73×10^{7}	2.49	103
5×10^{7}	4.85×10^{7}	0.89	103	4.61×10^{7}	2.79	108
1×10^{7}	9.17×10^{6}	3.53	108	1.02×10^{7}	0.18	98
5×10^{6}	6.44×10^{6}	1.01	71	5.41×10^{6}	2.63	92
1×10^{6}	2.57×10^{5}	4.80	174	4.52×10^{5}	1.50	155
1×10^{5}	3.30×10^{4}	3.52	167	2.10×10^{5}	2.74	-
1×10^{4}	4.06×10^{5}	1.42	-	2.19×10^{5}	3.26	-

6.3 *Direct detection of Listeria monocytogenes with an anti-InlB extract polyclonal antibody*

The polyclonal anti-InlB extract antibody presented in chapter 3 was also used in the development of BIAcore inhibition assay (Figure 6.8) to detect *Listeria monocytogenes* cells as described in section 2.5.5. Immobilised metal affinity chromatography (IMAC)-purified recombinant Internalin B (InlB) protein (described in chapter 4) was immobilised on the a CM5 sensor chip surface (section 6.3.2) and protein G-purified anti-InlB extract antibody, incubated with decreasing concentrations of HT-*Listeria monocytogenes* cells, injected over the surface. The shift in binding response after each injection was recorded and the results presented in section 6.3.4.

6.3.1 *Preconcentration of the purified recombinant InlB protein on to the CM dextran sensor chip surface*

As described in section 6.2.1, low ionic strength buffers, such as 10 mM sodium acetate, allow the electrostatic adsorption of positively charged proteins to the negatively charged CM dextran CM5 sensor chip surface. To determine the degree of preconcentration of purified recombinant InlB protein to the sensor chip surface prior to immobilisation, 50 μg/ml of recombinant InlB protein diluted in sodium acetate buffer (pH 3.9 to 5.0) was injected over a blank CM5 sensor chip surface and the degree of binding recorded. However, due to the high isoelectric point of the InlB protein (pI ~10.2) the protein has an overall positive charge in the BIAcore running buffer (HBS buffer, pH 7.4, containing 10 mM HEPES, 150 mM NaCl, 3.4 M EDTA, and 0.05% (v/v) Tween 20) and does not dissociate from the sensor chip surface. Figure 6.9 shows a sensorgram of the preconcentration of the recombinant InlB protein diluted in 10 mM sodium acetate buffer, pH 4.6, to a blank CM5 sensor chip surface. From the sensorgram it can be seen that after a 30 second injection of recombinant InlB protein in sodium acetate buffer, pH 4.6, approximately 16,000 RU of protein is electrostatically bound to the sensor chip surface (labelled B on the sensorgram). After an eight minute injection of HBS buffer over the surface (labelled C on the sensorgram) approximately 12,000 RU of protein still remains electrostatically bound to the CM dextran surface which is only removed by the injection of 20 mM NaOH (labelled D on the sensorgram). Due to the very high electrostatic binding observed in Figure 6.9, subsequent immobilisations were performed in sodium acetate buffer pH 4.6 without further evaluation of preconcentration at different pH increments.

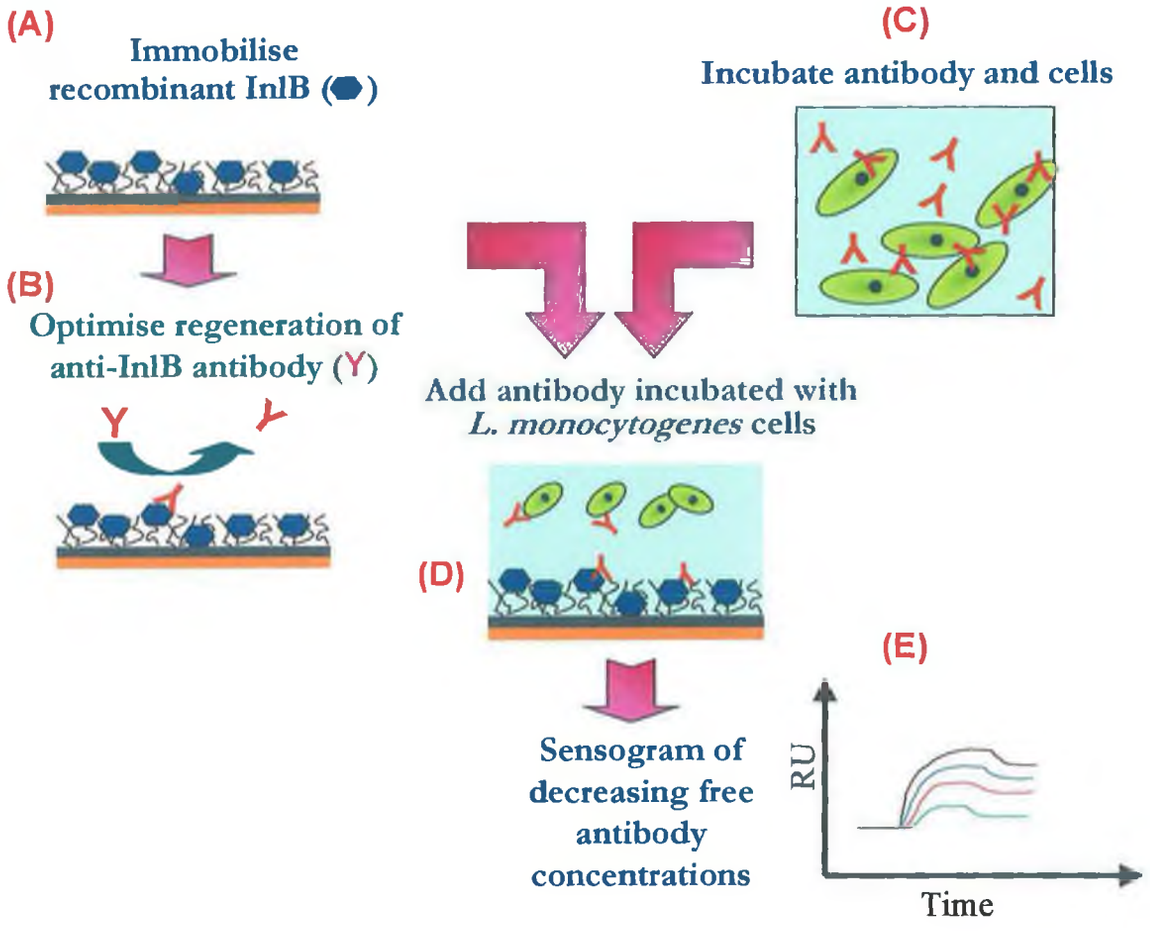


Figure 6.8: *Illustration of the BIAcore inhibition assay used to detect Listeria monocytogenes cells with the polyclonal anti-InlB extract antibody.*

(A) *Purified recombinant InlB (rInlB) protein is immobilised on the surface of a CM5 chip as described in section 2.5.2.*

(B) *The surface regeneration conditions are optimised and the surface binding capacity determined for a number of binding-regeneration cycles.*

(C) *Various concentrations of Listeria monocytogenes cells are incubated with an anti-InlB extract antibody for twenty minutes as described in section 2.5.5.*

(D) *Antibody and cells from (C) above are passed over the rInlB immobilised surface and the response observed.*

(E) *A sensorgram of increasing free antibody concentrations with decreasing Listeria monocytogenes cell numbers is observed and a standard curve plotted.*

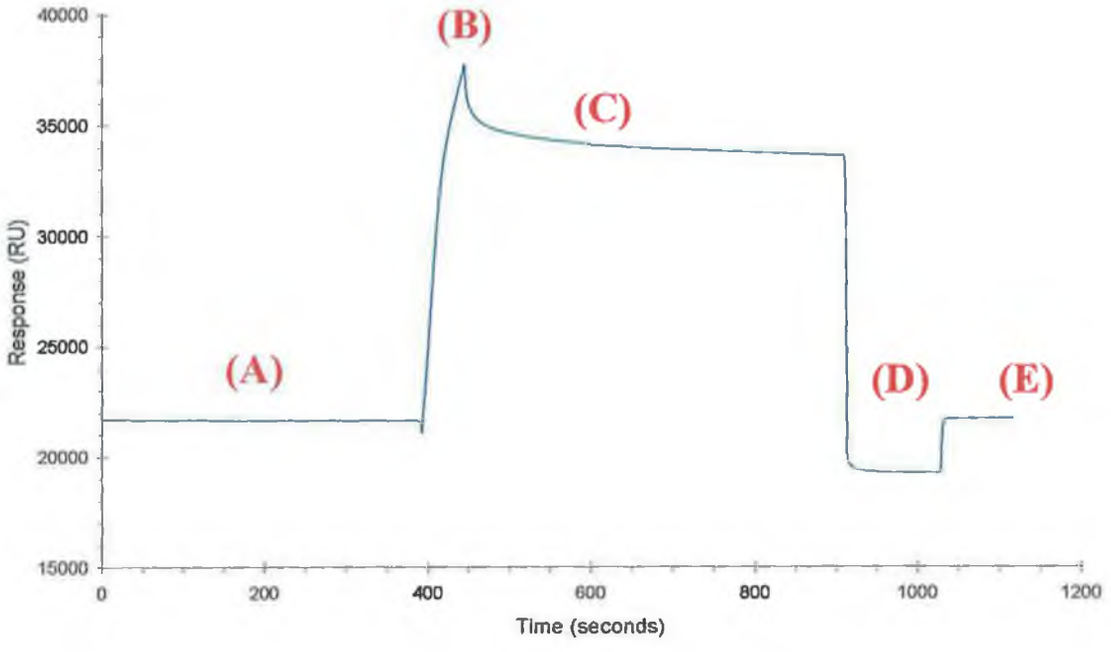


Figure 6.9: *Sensorgram representing the preconcentration of purified recombinant InlB protein on to a blank CM dextran sensor surface.*

(A) *Sensorgram baseline with running buffer (HBS buffer) injected over a blank surface.*

(B) *30 second injection of 50 µg/ml recombinant InlB protein in sodium acetate buffer, pH 4.6.*

(C) *8 minute injection of HBS running buffer over the blank surface.*

(D) *Dissociation of electrostatically bound recombinant InlB protein by the injection with 20 mM NaOH.*

(E) *Injection of HBS running buffer over the surface returns the baseline to its original value.*

6.3.2 *Immobilisation of the recombinant InlB protein on to the CM dextran sensor chip surface*

Purified recombinant InlB protein was immobilised onto the CM dextran surface of a CM5 sensor chip as described in section 2.5.2. 50 μg/ml of affinity-purified recombinant InlB protein, diluted in sodium acetate buffer, pH 4.6, was injected over the EDC/NHS activated sensor chip surface for 45 minutes at 5 μl/minute. Unreacted sites were blocked with a 7 minute injection of 1M ethanolamine hydrochloride (pH 8.5) at a rate of 5 μl/minute. Approximately 10,000 RU of recombinant InlB was successfully immobilised on the surface as shown in Figure 6.10.

6.3.3 *Regeneration studies of a rInlB immobilised surface with a polyclonal anti-InlB antibody*

The binding capacity of the recombinant InlB immobilised surface was determined by a series of binding and surface regeneration sequences to assess at which point the binding capacity deviated outside the desired performance range of <20% suggested by Wong *et al.* (1997). Protein G affinity-purified anti-InlB extract antibody was injected over the immobilised recombinant InlB surface for 2 minutes at 10 μl/minute and the surface regenerated with a 30 second pulse of 20 mM NaOH. The binding-regeneration cycles were repeated for 45 cycles resulting in less than 2% decrease in binding capacity from the first to the last cycle studied (Figure 6.11). The immobilised recombinant InlB surface studied in this section went through over 200 binding cycles over a one month period before a significant (<20% original binding capacity) decrease in binding capacity was observed.

6.3.4 *Development of a BIAcore inhibition assay for the detection of Listeria monocytogenes*

As described in Figure 6.11, the first injection of antibody in each cycle resulted in a decrease in observed binding response and therefore an inhibition assay was developed by discarding the first value in each cycle and taking an average of the remaining three replicates. To determine the working range of the inhibition assay, serial dilutions of *Listeria monocytogenes* cells in PBS were incubated with the anti-InlB extract polyclonal antibody as described in section 2.5.5. The samples containing antibody and *Listeria monocytogenes* cells were passed over the surface of the sensor chip immobilised with purified recombinant InlB protein in random order and the binding response recorded.

Surface bound antibody was regenerated from the surface using a 30 second pulse of 20 mM NaOH. A typical overlay plot demonstrating the decrease in binding of the anti-InlB polyclonal antibody to the immobilised recombinant InlB surface with increasing *Listeria monocytogenes* cell concentrations is presented in Figure 6.12. A calibration curve was constructed by plotting the average (n=3) change in response for each *Listeria monocytogenes* cell standard against the known concentration of *Listeria monocytogenes* cells used in each run. The range of detection for the anti-InlB extract antibody was found to be between approximately 1×10^8 and 2×10^5 cells/ml (Figure 6.13).

In order to determine the accuracy and the intermediate precision of the presented BIAcore inhibition immunoassay, intra- and interday assay variability studies were performed. Three sets of standards were prepared and incubated with antibody before being assayed. The results obtained from intra- and interday assay studies are shown in Table 6.2. The coefficients of variation (CV's) were determined to assess the precision of the analytical method, expressing standard deviation as a percent function of the mean. These values ranged from 0.53 to 4.55% and 2.98 to 7.70% as observed for the intra- and interday studies, respectively, indicating that the assay had a very good degree of precision. The percentage accuracies values obtained in the linear part of the interday assay (Table 6.2) indicate the fitted four parameter curve provides an accurate representation of the measured response in the linear range of the assay (percentage accuracy values from 93 to 108%).

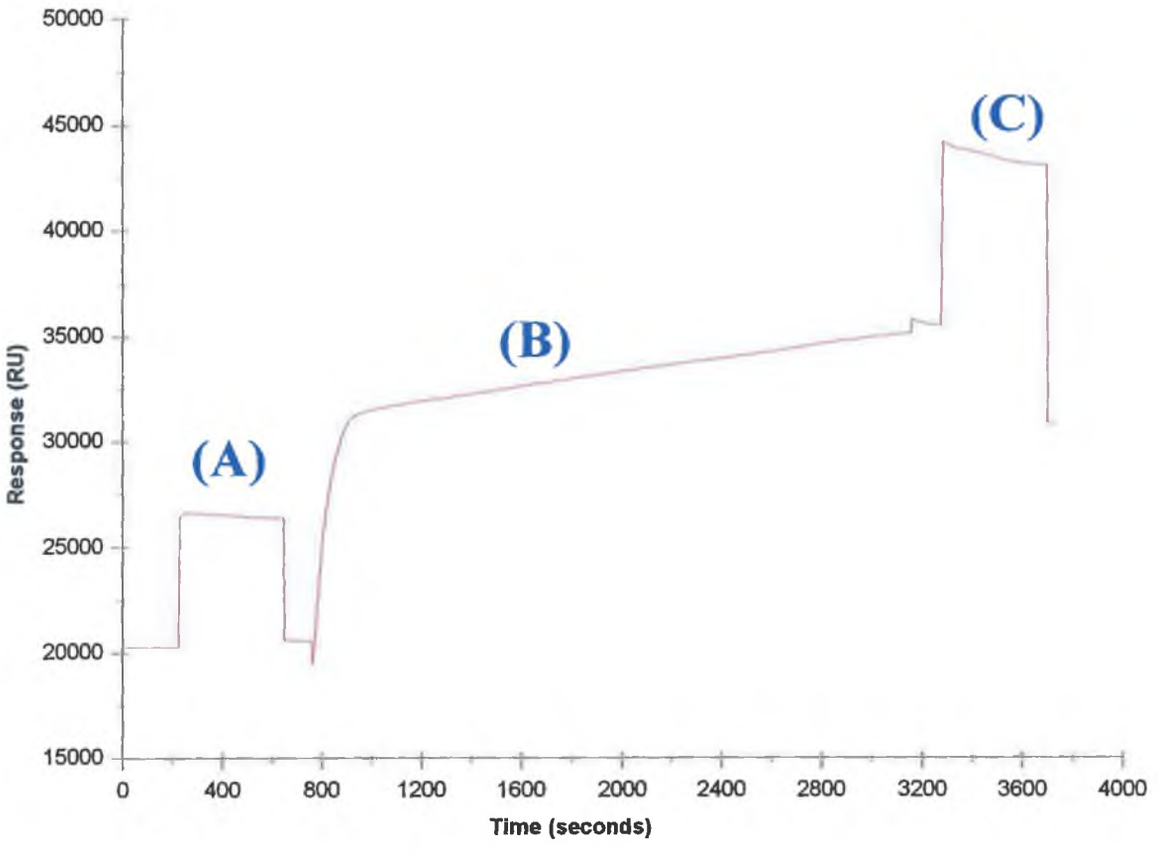


Figure 6.10: *Sensorgram showing the immobilisation of recombinant InlB protein to a CM5 sensor chip surface.*

(A) *HBS buffer is initially passed over the sensor chip surface and the baseline response recorded. The CM dextran surface is then activated with 0.05 M NHS/0.2 M EDC mixture resulting in a large increase in SPR signal due primarily to a bulk refractive index change. HBS buffer is again passed over the now activated surface and a small increase in the baseline is observed.*

(B) *A 50 μg/ml solution of purified recombinant InlB protein diluted in 10 mM sodium acetate buffer, pH 4.6, is then passed over the activated surface for a period of 45 minutes.*

(C) *Deactivation of the surface NHS-esters is performed by injecting 1M ethanolamine, pH 8.5, over the surface for 7 minutes, which also serves to elute non-covalently bound antibody. Approximately 10,000 RU of recombinant InlB protein remained bound to the surface after the described immobilisation procedure.*

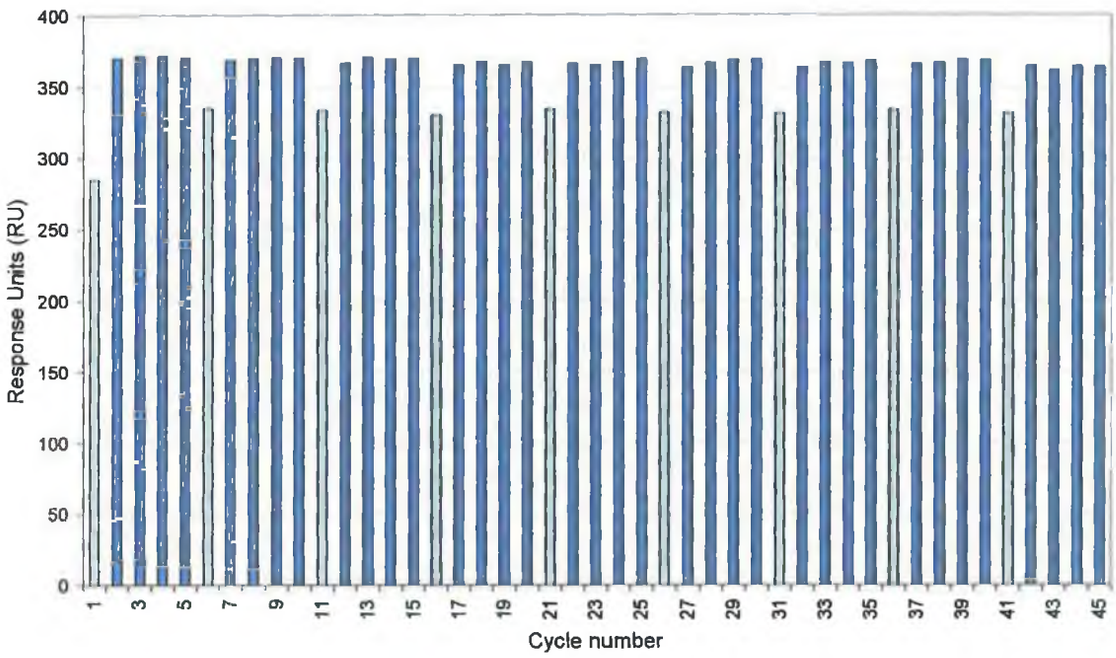


Figure 6.11: *Bar chart showing 45 binding-regeneration cycles of the polyclonal anti-InlB extract antibody to the recombinant InlB immobilised surface. Nine cycles of five 2 minute injections of a 1/30 dilution of purified anti-InlB extract antibody were injected over the recombinant InlB immobilised sensor chip surface and regenerated with a 30 second pulse of 20 mM NaOH. A large decrease in binding of antibody to the immobilised recombinant InlB surface was observed for the first injection of each of the nine binding cycles (shown in grey on the presented bar chart). This seemed to be a phenomenon of the BIAcore instrument when the program changes cycles, as it was also observed in Figures 6.5 (4 cycles of 15 injections) and 6.20 (4 cycles of 10 injections) which used different ligands and number of binding cycles. According to BIAcore, the pattern observed could be due to the fact that there is a time error in sthe switch valve indicator and effectively what is seen in the first injection is the loss of a small fraction of injected sample. (personal communication with Ron Wolbert, Application Specialist, BIAcore International SA). Excluding the first injection in each cycle, the binding capacity decreased by less than 2% over the 45 antibody injections, indicating that the surface can be reproducibly regenerated and is suitable for the development of an inhibition assay using the described antibody. The sensor surface went through greater than 200 regenerations before a significant (>20%) decrease in binding capacity was observed.*

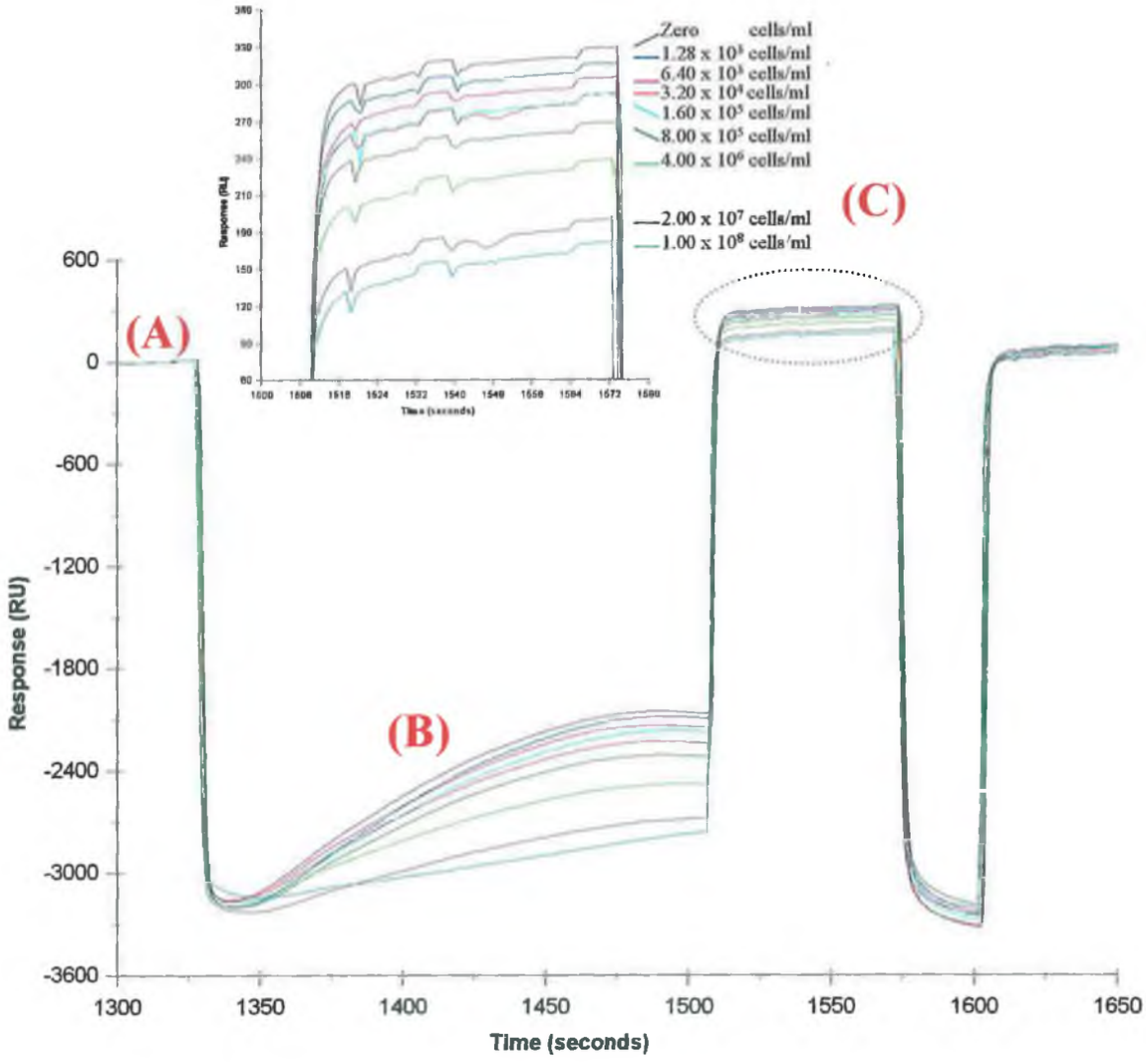


Figure 6.12: *Overlay plot demonstrating the decrease in binding of affinity purified anti-InlB extract polyclonal antibody to the immobilised recombinant InlB surface with increasing Listeria monocytogenes cell concentrations.*

(A) *Shows the normalised baselines of each sensorgram when HBS is injected over the sensor chip surface.*

(B) *Shows the binding of increasing free antibody with decreasing Listeria monocytogenes cell concentrations.*

(C) *Shows the base line shift when buffer is passed over the surface after each sample injection. The concentration of Listeria monocytogenes cells used in each binding cycle is indicated beside each sensorgram.*

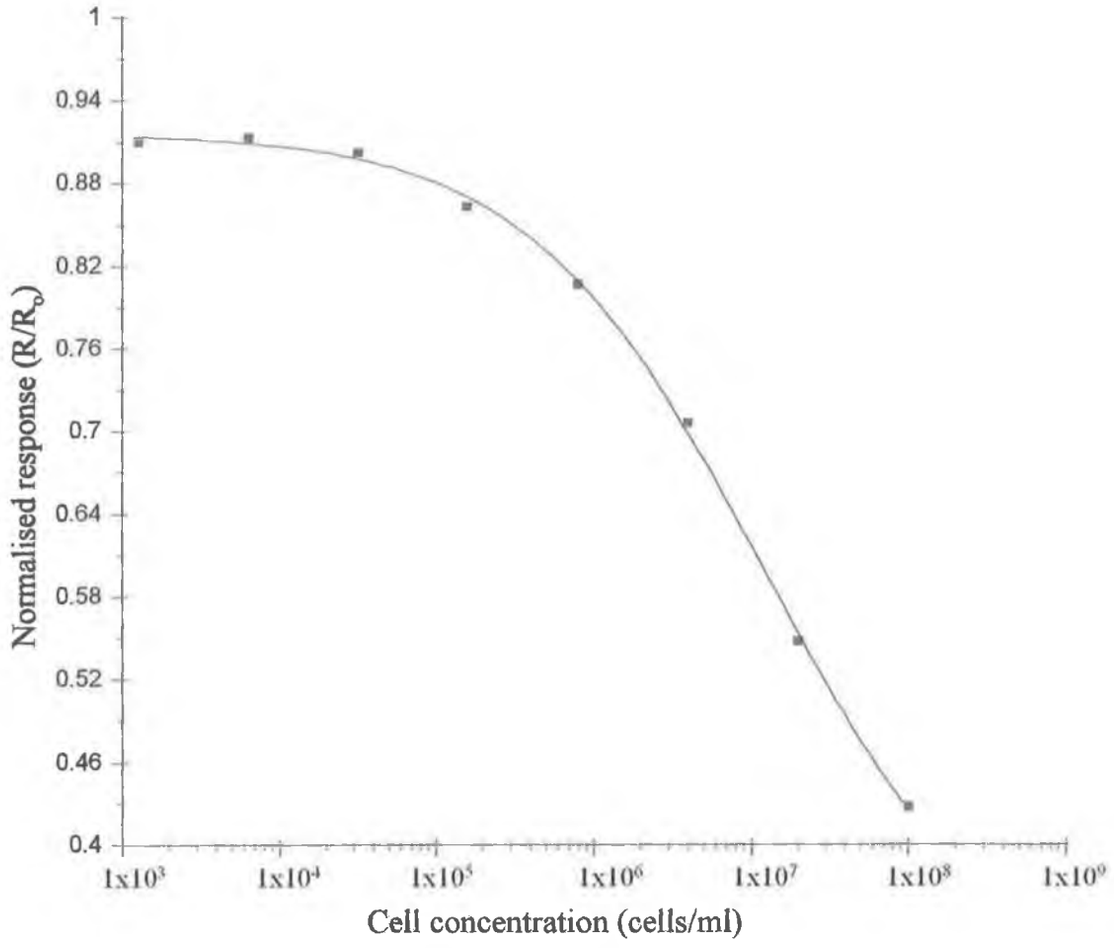


Figure 6.13: *Interday assay calibration curve for the anti-InlB extract antibody. A 1/30 dilution of the anti-InlB extract antibody was incubated with various Listeria monocytogenes cell concentrations injected over an immobilised recombinant InlB surface as described in section 2.5.5. A 4-parameter equation was fitted to the data set using BIAevaluation 3.1 software. The inter-assay means and coefficients of variance are tabulated in Table 6.2. Each point on the curve is the mean of three replicate measurements analysed over three days.*

Table 6.2: *Intra- and interday assay coefficients of variation (CV's) and percentage accuracies for the anti-InlB extract polyclonal antibody used the BIAcore inhibition assay described in section 2.5.5. Coefficients of variance (a quantitative measure of precision) was calculated using the equation % CV= (S.D./Mean) x 100 where for intraday studies, the standard deviation (S.D.) is computed from replicate (3 replicates) analyses within a single validation run and for intermediate precision (interday), the S.D. is computed from replicate (3 replicates) analyses over 3 validation runs on 3 separate days. The rows with a "-" represent that no value could be obtained as the data point did not fit the calibration curve.*

	Intraday			***Interday***		
Concentration (cells/ml)	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**
1.00×10^8	1.04×10^8	0.53	95.85	9.73×10^7	5.53	102.69
2.00×10^7	1.96×10^7	1.35	101.88	2.13×10^7	7.70	93.59
4.00×10^6	4.00×10^6	2.88	100.06	3.66×10^6	6.23	108.61
8.00×10^5	8.28×10^5	4.55	96.50	8.34×10^5	2.45	95.79
1.60×10^5	1.40×10^5	3.12	112.67	2.08×10^5	4.91	70.30
3.20×10^4	8.69×10^3	4.30	172.85	1.99×10^4	3.23	137.94
6.40×10^3	-	3.29	-	1.86×10^3	2.98	170.86
1.28×10^3	4.15×10^4	2.35	-	4.04×10^3	3.60	-

6.4 *Indirect detection of Listeria monocytogenes with an anti-InlB recombinant single chain Fv fragment antibody*

As described in section 5.5.2, a human single chain Fv (scFv) fragment antibody capable of binding the *Listeria monocytogenes* invasion-associated protein, Internalin B (InlB), was selected from a naïve human antibody library. However, the antibody did not recognise *Listeria monocytogenes* cells indicating that it may bind the protein in a region that is associated with the *Listeria monocytogenes* cell membrane (e.g. in the glycine-tryptophan repeat region). To overcome this problem, InlB protein was extracted from the cell membrane as described in section 2.2.1.2, and used in an inhibition ELISA (Figure 6.14) to indirectly detect *Listeria monocytogenes* cells (sections 2.4.4.14 and 5.5.2). In this section, a BIAcore inhibition assay was developed using the responses obtained from the injection of the anti-InlB scFv antibody incubated with decreasing *Listeria monocytogenes* InlB enriched extract, over an immobilised recombinant InlB sensor chip surface.

6.4.1 *Evaluation of various sensor chip surface chemistries*

BIAcore CM5, C1, NTA and HPA sensor chips were evaluated for their possible use in the development of an inhibition assay to detect *Listeria monocytogenes* InlB extracts in solution.

6.4.1.1 *Evaluation of a CM5 sensor chip*

A BIAcore sensor chip surface consists of a glass support on which a gold film has been deposited. For most applications the gold layer is covered with a non-crosslinked carboxylated dextran hydrogel coupled through an alkyl thiol linker layer. The BIAcore CM5 sensor chip is a general purpose chip, with an extremely stable carboxymethylated dextran surface allowing high binding capacities and coupling via a wide range of chemistries.

To evaluate the use of the CM5 sensor chip for the development of an assay to detect *Listeria monocytogenes* InlB extract, approximately 10,000 response units of recombinant InlB protein were immobilised on the CM dextran surface as described in section 2.5.2. Dilutions of InlB extract and anti-InlB scFv antibody were then passed over the surface and the change in response recorded. Figure 6.15 shows the response change observed from a 1 minute injection of a 1/5 dilution of InlB extract in PBS over a recombinant InlB immobilised CM5 sensor chip surface. 2875 RU of InlB extract remained non-specifically

bound to the sensor chip surface upon re-injection of HBS running buffer over the surface. The magnitude of non-specific binding far exceeded that of the specific binding of the anti-InlB scFv antibody (253 RU) to the recombinant InlB surface indicating that the CM5 sensor chip surface is not suitable for the development of the described assay. As described in section 6.3.1, Internalin B has a very high pI and is positively charged in acidic or neutral buffers, resulting in electrostatic interactions between the protein and the negatively charged carboxymethylated dextran matrix. Figure 6.16 shows the injection of InlB extract over a recombinant InlB CM5 sensor surface in PBS containing different salt concentrations. From the sensorgram it can be seen that buffers with a NaCl concentration of 0.5 M eliminate the electrostatic interactions between the sensor surface and the InlB protein. However, at these NaCl concentrations, binding of the anti-InlB scFv antibody to InlB is also hindered (results not shown) and therefore a more suitable sensor chip system is required.

6.4.1.2 *Evaluation of a NTA sensor chip*

The BIAcore NTA chip contains pre-immobilised nitrilotriacetic acid (NTA) on the dextran layer. The NTA chip chelates metal ions such as Ni^{2+}, leaving coordination sites free for binding histidines residues in recombinant proteins tagged with polyhistidine. The NTA chip controls the orientation of the ligand component for optimal site exposure and allows the single regeneration of the sensor surface with EDTA.

To investigate the possible use of the BIAcore NTA chip for use in the described assay, the NTA surface was activated with $NiCl_2$ and the anti-InlB scFv antibody immobilised (1139.6 RU) to the surface via its polyhistidine tag as shown in Figure 6.17. *Listeria monocytogenes* InlB extract was then passed over the anti-InlB antibody immobilised surface resulting in 304 RU of extract binding to the immobilised antibody surface. The surface was regenerated with 100 mM EDTA and the same concentration of InlB extract passed over a now blank NTA surface. 303.8 RU of InlB extract (Figure 6.17) remained bound to the blank NTA-dextran sensor chip surface upon addition of HBS which was equivalent to that observed over the anti-InlB scFv antibody surface. The observed results indicate that BIAcore sensor chips containing a dextran layer should not be used when trying to detect positively charged proteins, such as InlB.

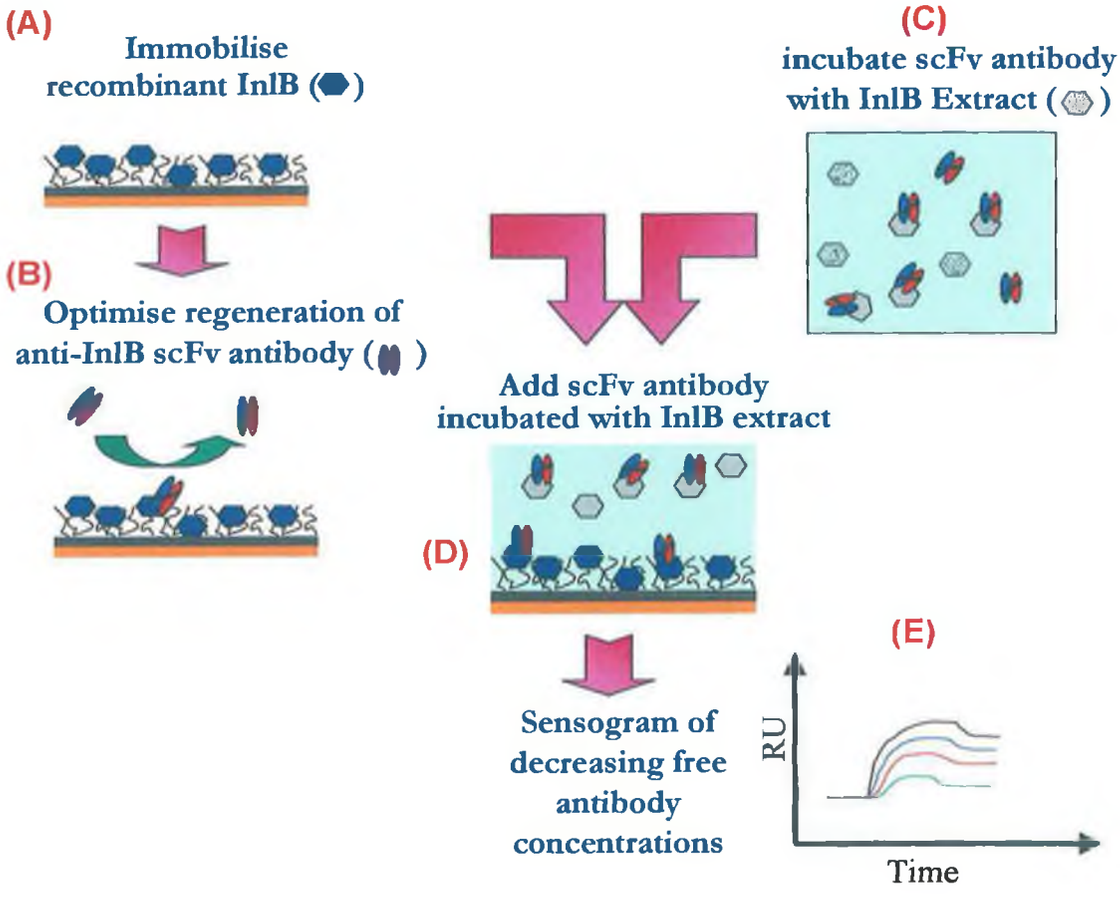


Figure 6.14: *Illustration of the BIAcore inhibition assay used to indirectly detect Listeria monocytogenes cells with the anti-InlB scFv antibody.*

(A) *Purified recombinant InlB (rInlB) protein is immobilised on the surface of a CM5 chip as described in section 2.5.2.*

(B) *The surface regeneration conditions are optimised and the surface binding capacity determined for a number of binding-regeneration cycles.*

(C) *Various concentrations of Listeria monocytogenes InlB extracts are incubated with an anti-InlB scFv antibody as described in section 2.5.6.*

(D) *Antibody and extract from (C) above are passed over the rInlB immobilised surface and the response observed.*

(E) *A sensorgram of increasing free antibody concentrations with decreasing Listeria monocytogenes cell numbers is observed and a standard curve plotted.*

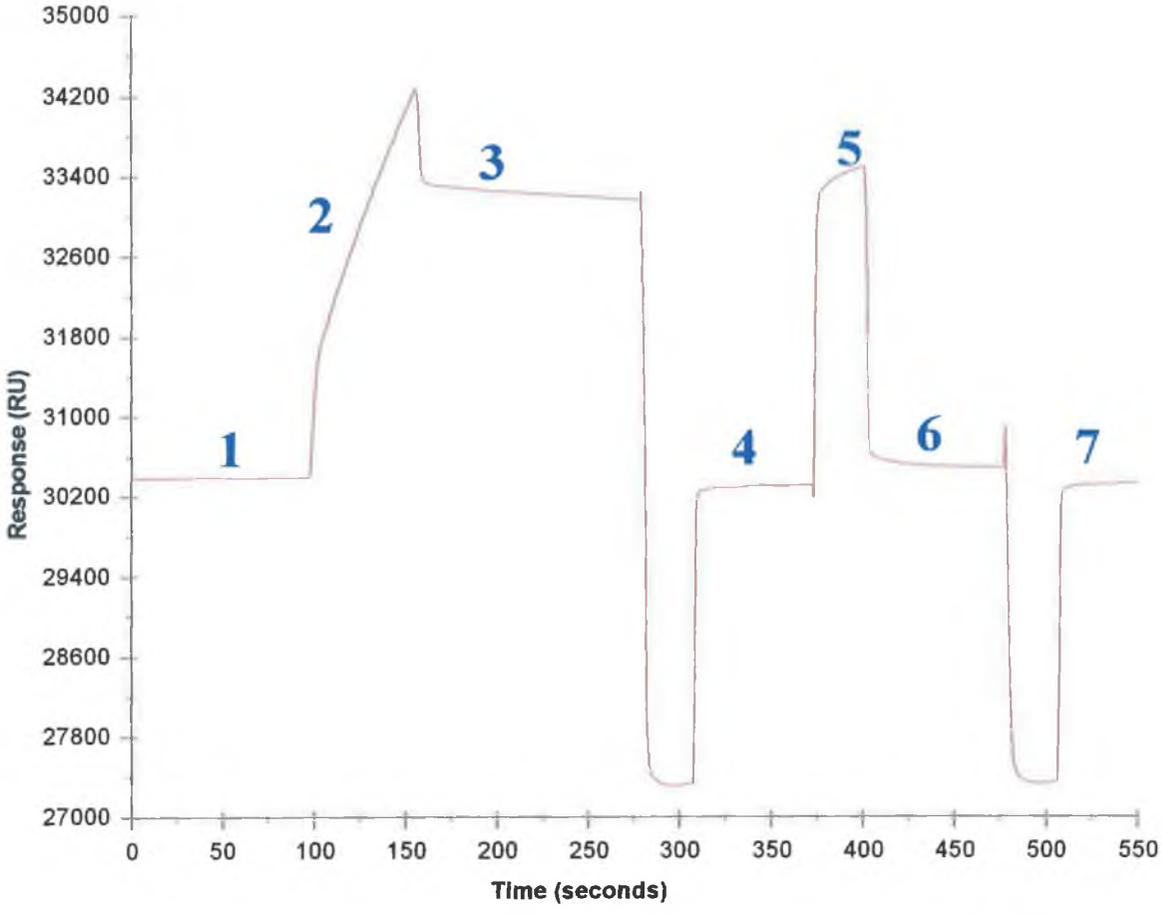


Figure 6.15: *Sensorgram showing the injection of Listeria monocytogenes-enriched InlB extract and anti-InlB scFv antibody over a recombinant InlB immobilised CM5 sensor chip surface. The numbers indicated on the sensorgram represent:*

(1) *HBS buffer injected over the recombinant InlB surface.*

(2) *1 minute injection of a 1/5 dilution of the InlB extract in PBS.*

(3) *HBS buffer injected over the recombinant InlB surface. The baseline difference between (1) and (3) represents the amount of InlB extract (2875.1 RU) that is electrostatically bound to the sensor chip surface.*

(4) *30 second injection of 20 mM NaOH followed by HBS injected over the surface.*

(5) *30 second injection of a 1/4 dilution of anti-InlB scFv antibody supernatant in PBS.*

(6) *HBS buffer injected over the recombinant InlB surface. The baseline difference between (4) and (6) represents the amount of antibody (253.3 RU) that is bound to the recombinant InlB-immobilised sensor chip surface.*

(7) *30 second injection of 20 mM NaOH followed by HBS injected over the surface.*

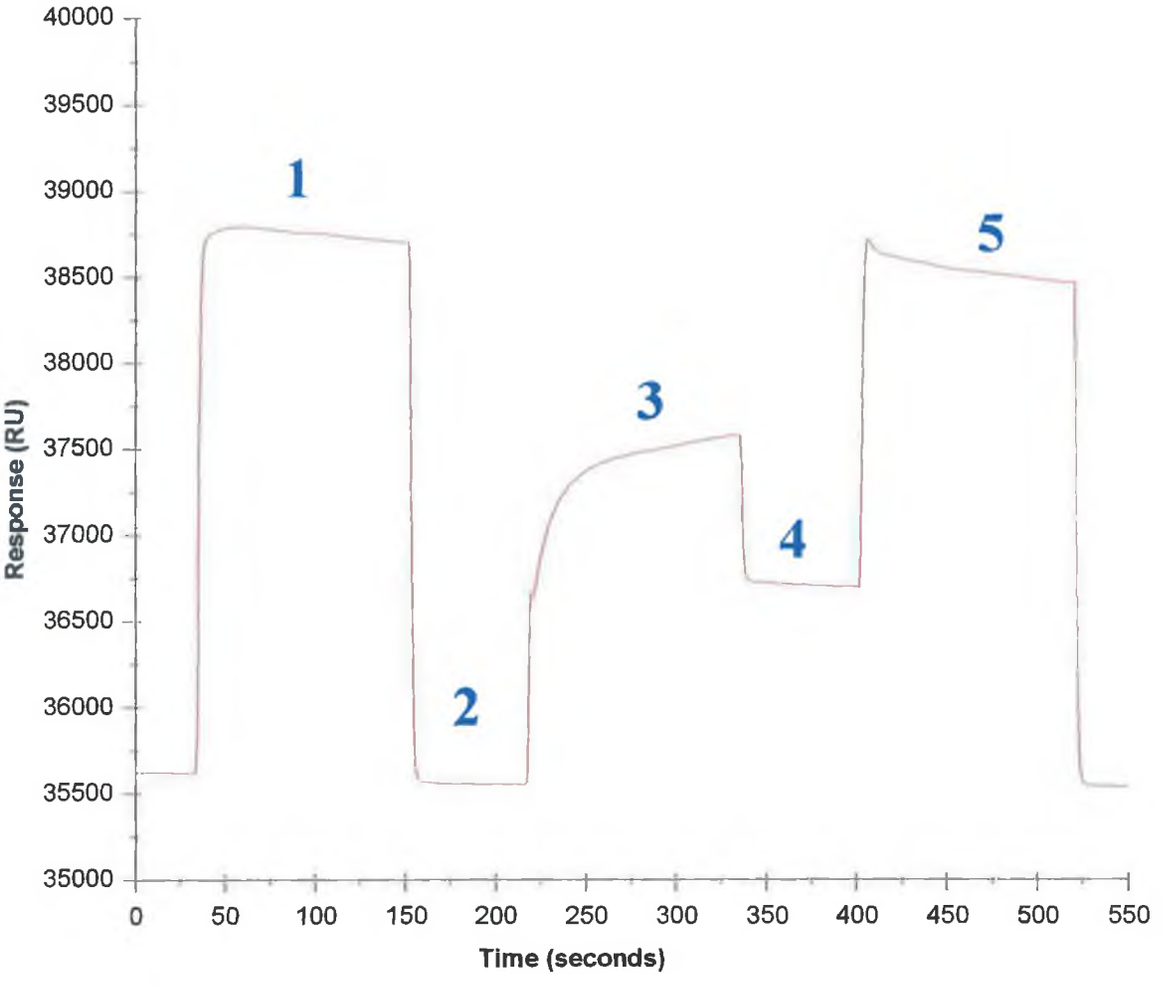


Figure 6.16: *Sensorgram showing the effect of NaCl concentration of the binding of Listeria monocytogenes InlB extract to a recombinant InlB immobilised CM5 sensor chip surface. The numbers indicated on the sensorgram represent the following:*

(1) *A 2 minute injection of a 1/20 dilution of InlB extract in PBS containing 0.5 M NaCl (-73.8 RU change in baseline).*

(2) *HBS buffer injected over the recombinant InlB surface.*

(3) *A 2 minute injection of a 1/20 dilution of InlB extract in normal PBS (0.15 M NaCl; 1154.1 RU of extract non-specifically bound to the recombinant InlB surface).*

(4) *HBS buffer injected over the recombinant InlB surface.*

(5) *Surface regenerated with a 2 minute injection of PBS containing 0.5 M NaCl.*

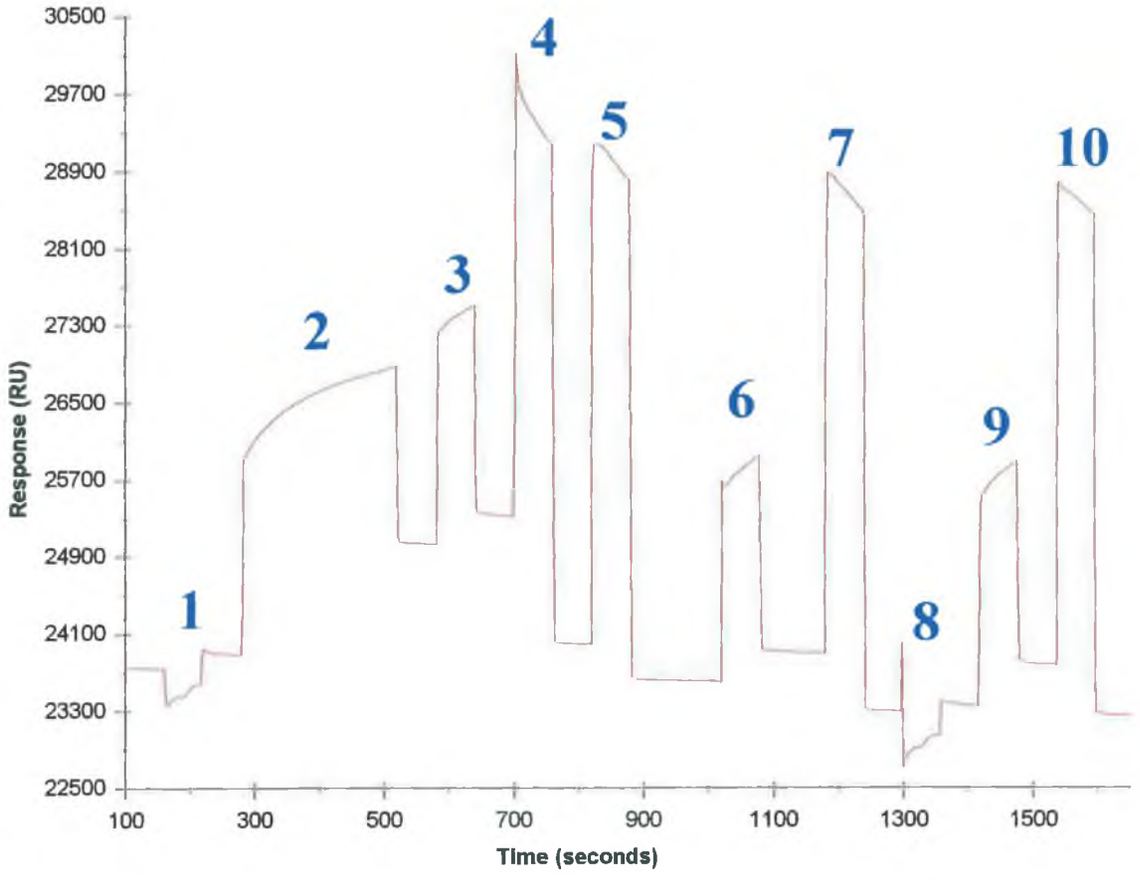


Figure 6.17: *Sensorgram showing the injection of Listeria monocytogenes-enriched InlB extract and anti-InlB scFv antibody over a recombinant InlB-immobilised NTA sensor chip surface. The numbers indicated on the sensorgram represent:*

(1) *The NTA sensor chip, on which nitrilotriacetic acid is covalently linked to a carboxy-methylated dextran matrix, was activated with a pulse of $NiCl_2$, forming a chelating complex with NTA which binds polyhistidine peptides.*

(2) *5 minute injection of an anti-InlB scFv antibody (1139.6 RU of antibody immobilised).*

(3) *HBS buffer injected over the scFv immobilised surface followed by an injection of a 1/50 dilution of InlB extract leaving 304.0 RU of extract bound to the immobilised scFv antibody surface.*

(4) *+* ***(5)*** *Regeneration of the immobilised scFv antibody surface with 100 mM EDTA solution resulting in the chelating of the Ni^{2+} and subsequently releasing histidines bound peptides from the NTA surface.*

(6) *HBS buffer injected over the regeneration NTA surface followed by an injection of a 1/50 dilution of InlB extract leaving 303.8 RU of extract bound to the blank surface.*

(7) *1 minute injection of 100 mM EDTA removes non-specifically bound material.*

(8) *The NTA surface was again activated with a pulse of $NiCl_2$, forming a chelating complex with NTA.*

(9) *A 1 minute injection of a 1/50 dilution of InlB extract leaving 387.4 RU of extract bound to the activated NTA surface.*

(10) *Regeneration of the activated NTA surface with a 100 mM EDTA solution.*

6.4.1.3 *Evaluation of a C1 sensor chip*

The BIAcore C1 sensor chip has a carboxymethylated surface that supports the same immobilisation chemistry as CM dextran surfaces but lacks the dextran matrix. The surface lacks the hydrophilic character and has a much lower immobilisation capacity than the CM dextran chips. However, the chip is useful in cases where the dextran matrix interferes with the interaction being studied.

A sensorgram showing the injection of *Listeria monocytogenes* enriched InlB extract and anti-InlB scFv antibody over a recombinant InlB immobilised C1 sensor chip surface is shown in Figure 6.18. Recombinant InlB was immobilised on the sensor chip surface as described in section 2.5.2 and anti-InlB scFv antibody subsequently injected over the sensor chip surface. A 6 minute injection of a 1/6 dilution of anti-InlB scFv antibody in PBS resulted in a baseline shift of 642.3 RU. However, injection of a 1/3 dilution of InlB extract incubated with an equal volume anti-InlB antibody resulted in a much higher baseline shift of 1749.9 RU. Also a 6 minute injection of a 1/6 dilution of InlB extract in PBS resulted in a baseline shift of 1806 RU indicating that the InlB extract non-specifically binds to the sensor chip surface. This result was not expected as the chip contains no dextran layer. However, the surface is still carboxymethylated, even though it has a slightly more hydrophobic nature (lacks the hydrophilic character of the dextran layer) which could possibly result in the non-specific binding of the positively charged membrane associated InlB protein. Under these conditions, the BIAcore C1 sensor chip does not provide a feasible surface for the development of an inhibition assay to detect InlB extract.

6.4.1.4 *Evaluation of a HPA sensor chip*

The BIAcore HPA chip surface is composed of long-chain alkanethiol molecules that form a flat, quasi-crystalline hydrophobic surface for working with membrane-associated ligands. The hydrophobic surface is inherently "sticky" and will adsorb a large variety of molecules and proteins and therefore can be used to mimic ELISA conditions, such as those used in section 5.5.2. Figure 6.19 shows the injection of *Listeria monocytogenes* enriched InlB extract and anti-InlB scFv antibody over a recombinant InlB-immobilised HPA sensor chip surface. Recombinant InlB protein was hydrophobically absorbed to the sensor chip surface by injecting 50 µg/ml of recombinant InlB protein over a clean HPA chip surface for 100 minutes at 5 µl/minute and blocked with 50 µg/ml of ovalbumin for 20 minutes at 5 µl/minute. As presented in Figure 6.19, injection of InlB extract incubated

with anti-InlB scFv antibody (71.3 RU) resulted in a decrease in observed response compared to that observed from same concentration of antibody diluted in PBS (149 RU). The inhibition observed indicates that hydrophobically absorbing the recombinant InlB protein to the sensor surface followed by blocking (mimicking ELISA) could potentially be used for the generation of an inhibition assay with both the anti-InlB scFv antibody and InlB extract. However, when the injection of both samples (antibody-extract and antibody in PBS) was repeated over the surface (Figure 6.19, labelled 3-6) the binding capacity of the surface decreased drastically limiting the feasibility of the use of the HPA chip surface for used in a development of an accurate and cost effective immunoassay.

6.4.1.5 *Reduction of the overall negative charge of a C1 sensor chip surface by over-activation of the surface carboxyl groups and capping with ethylene diamine*

As concluded in sections 6.4.1.1 and 6.4.1.2, BIAcore sensor chips containing a carboxymethylated dextran surface are not suitable for use with highly positively charged proteins such as InlB due to the high electrostatic interactions observed. However, the BIAcore C1 chip (section 6.4.1.3) containing no dextran layer also displayed electrostatic interactions between the InlB protein and the carboxymethylated flat surface resulting in high undesirable non-specific binding. In order to attempt to decrease the overall negative charge on the sensor chip surface, the surface was over-activated with a double injection of EDC/NHS (according to BIAcore, standard activation procedures only activate approximately 40% of the carboxyl groups on the surface) and the surface capped with 1 M ethylene diamine, pH 8.5, after immobilisation of the recombinant InlB protein. Figure 6.20 shows a sensorgram of the injection of the anti-InlB scFv antibody and InlB extract over an recombinant InlB immobilised C1 sensor chip surface that has been over-activated with two 7 minute injections of EDC/NHS and capped with 1 M ethylene diamine, pH 8.5. A 1 minute injection of anti-InlB antibody over the recombinant InlB immobilised surface resulted in a baseline shift of 796.8 RU units compared to 131.9 RU observed from a 1/5 dilution of InlB extract incubated with the same concentration of antibody over the same surface (Figure 6.20). This result indicates the potential of the described recombinant InlB surface for use in the development of an assay to detect InlB extract using the anti-InlB scFv antibody.

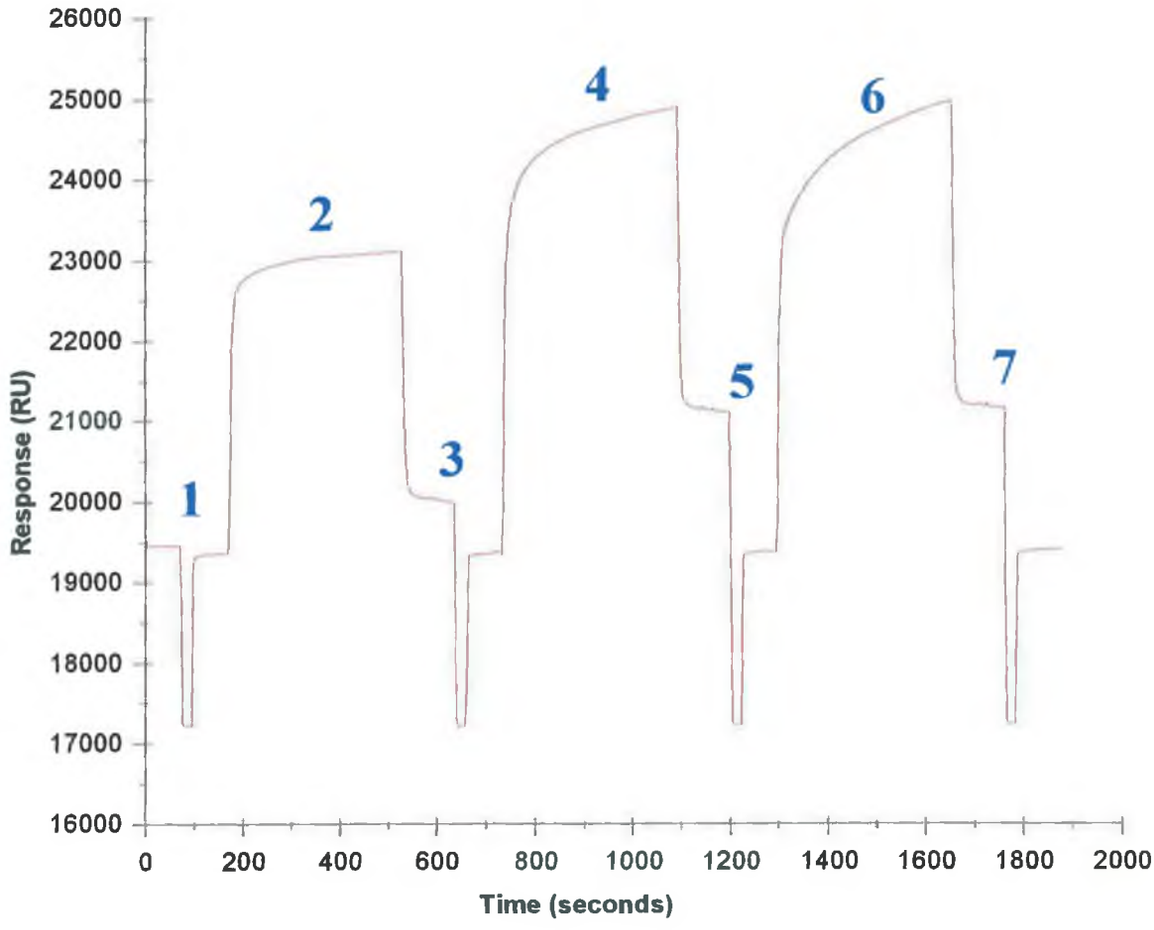


Figure 6.18: : *Sensorgram showing the injection of Listeria monocytogenes-enriched InlB extract and anti-InlB scFv antibody over a recombinant InlB immobilised C1 sensor chip surface. The numbers indicated on the sensorgram represent:*

(1) *HBS buffer injected over the recombinant InlB surface followed by a short injection of 20 mM NaOH.*

(2) *6 minute injection of 1/6 dilution of the anti-InlB scFv antibody supernatant over an immobilised recombinant InlB surface (642.3 RU of antibody bound).*

(3) *HBS buffer injected over the recombinant InlB surface followed by a short injection of 20 mM NaOH.*

(4) *6 minute injection of 1/3 dilution of the anti-InlB scFv antibody supernatant incubated with a 1/3 dilution of InlB extract over an immobilised recombinant InlB surface (1749.9 RU of antibody/InlB extract bound).*

(5) *HBS buffer injected over the recombinant InlB surface followed by a short injection of 20 mM NaOH.*

(6) *6 minute injection of a 1/6 dilution of InlB extract over an immobilised recombinant InlB surface (1806.0 RU of InlB extract bound).*

(7) *HBS buffer injected over the recombinant InlB surface followed by a short injection of 20 mM NaOH.*

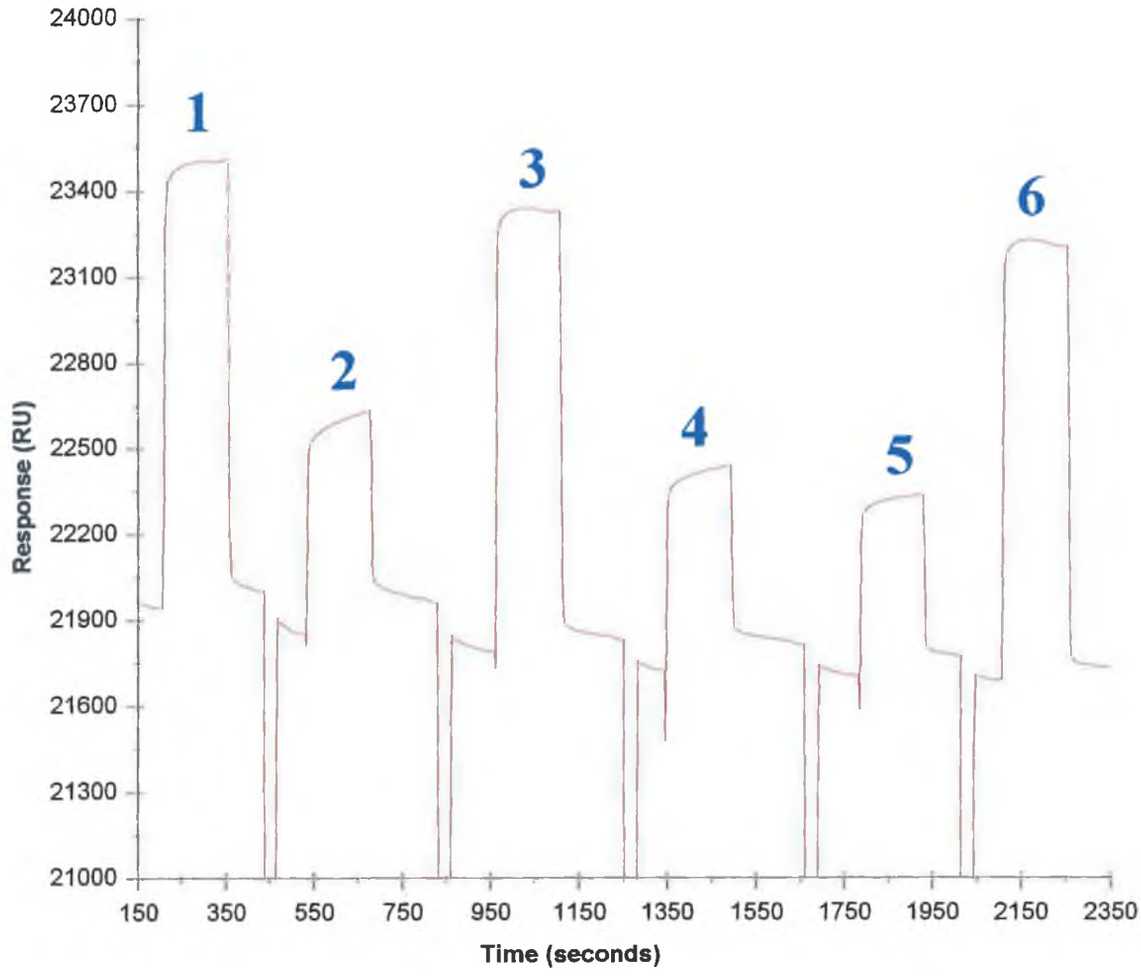


Figure 6.19: : *Sensorgram showing the injection of Listeria monocytogenes-enriched InlB extract and anti-InlB scFv antibody over a recombinant InlB-immobilised HPA sensor chip surface. The numbers indicated on the sensorgram represent:*

(1) *2.5 minute injection of a 1/3 dilution of scFv antibody incubated with a 1/5 dilution of InlB extract resulting in a baseline shift of 71.3 response units. The surface is regenerated with a 30 second pulse of 20 mM NaOH.*

(2) *2.5 minute injection of a 1/3 dilution of scFv antibody incubated with an equal volume of PBS resulting in a baseline shift of 149.0 response units. The surface is regenerated with a 30 second pulse of 20 mM NaOH.*

(3) *Injection of (1) repeated resulting in a baseline shift of 48.1 response units.*

(4) *Injection of (2) repeated resulting in a baseline shift of 115.5 response units.*

(5) *Injection of (2) and (4) repeated resulting in a baseline shift of 81.8 response units.*

(6) *Injection of (1) and (3) repeated resulting in a baseline shift of 50.9 response units.*

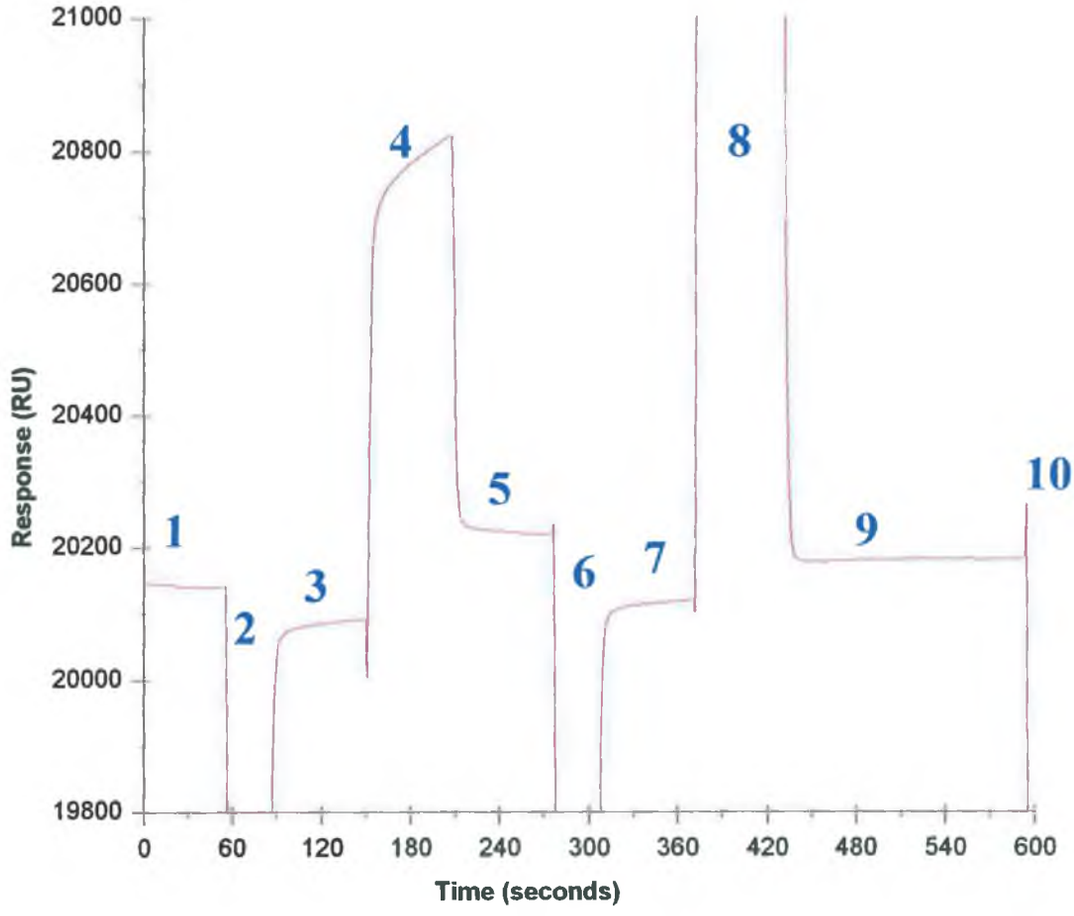


Figure 6.20: *Sensorgram showing the injection of the anti-InlB scFv antibody and InlB extract over an recombinant InlB immobilised C1 sensor chip surface that has been over-activated with two 7 minute injections of EDC/NHS and capped with ethylene diamine instead of ethanolamine. The numbers indicated on the sensorgram represent:*

(1) *HBS buffer injected over the recombinant InlB surface.*

(2) *30 second injection of 20 mM NaOH.*

(3) *HBS buffer injected over the recombinant InlB surface.*

(4) *1 minute injection of a 1/2 dilution of the anti-InlB scFv culture supernatant in PBS.*

(5) *HBS buffer injected over the recombinant InlB surface. The difference baseline between (3) and (5) indicates the amount of antibody bound to the immobilised InlB surface (796.8 RU).*

(6) *30 second injection of 20 mM NaOH.*

(7) *HBS buffer injected over the recombinant InlB surface.*

(8) *Injection of the scFv antibody incubated with a 1/5 dilution of InlB extract diluted in PBS injected over the recombinant InlB immobilised surface. The difference in baseline between (7) and (9) indicates the amount of antibody bound to the recombinant InlB immobilised surface (131.9 RU).*

(9) *HBS buffer injected over the recombinant InlB surface.*

(10) *30 second injection of 20 mM NaOH.*

6.4.2 *Immobilisation of recombinant InlB protein on to a BIAcore C1 sensor chip surface*

Purified recombinant InlB protein was immobilised onto the carboxymethylated surface of a C1 sensor chip as briefly described in section 6.4.1.5. 50 µg/ml of affinity-purified recombinant InlB protein, diluted in sodium acetate buffer, pH 4.6, was injected over the EDC/NHS over-activated sensor chip surface for 45 minutes at 5 µl/minute. Unreacted sites were blocked with a 7 minute injection of 1M ethylene diamine (pH 8.5) at 5 µl/minute. Approximately 1,100 RU of recombinant InlB was successfully immobilised on the surface as shown in Figure 6.21.

6.4.3 *Regeneration studies of the anti-InlB scFv antibody*

The binding capacity of the recombinant InlB immobilised surface was determined by a series of binding and surface regeneration sequences to assess at which point the binding capacity deviated outside the desired performance range of <20% suggested by Wong *et al.* (1997). Anti-InlB scFv antibody was injected over the immobilised antibody surface for 2 minutes at 10 µl/minute and the surface regenerated with a 30 second pulse of 20 mM NaOH. The binding-regeneration cycles were repeated for 40 cycles resulting in a decrease of approximately 20% in binding capacity from the first to the last cycle studied (Figure 6.22). This result indicates that the surface can be used for up to 40 binding-regeneration cycles before the decrease binding capacity exceeds the desired performance range suggested by Wong *et al.* (1997).

6.4.4 *Development of a BIAcore inhibition assay for the indirect detection of Listeria monocytogenes using an anti-InlB scFv antibody*

To determine the working range of the inhibition assay, serial dilutions of *Listeria monocytogenes* InlB extracts in PBS were incubated with the anti-InlB scFv antibody as described in section 2.5.6. The samples containing antibody and *Listeria monocytogenes* InlB extract were passed over the surface of the sensor chip immobilised with purified recombinant InlB protein in random order and the binding response recorded. Surface bound antibody was removed from the surface using a 30 second pulse of 20 mM NaOH. A typical overlay plot demonstrating the decrease in binding of the anti-InlB scFv antibody to the immobilised recombinant InlB surface with increasing *Listeria monocytogenes* InlB extract concentrations is presented in Figure 6.23. A calibration curve was constructed by plotting the average (n=3) change in response for each *Listeria*

monocytogenes InlB extract standard against the known equivalent concentration of *Listeria monocytogenes* cells used in each run. The range of detection for the anti-*Listeria monocytogenes* antibody was found to be between approximately 6.6 x 10^8 and 2 x 10^7 cells/ml (Figure 6.24). The limit of detection (approximately 2 x 10^7 cells/ml) observed in Figure 6.24 is approximately 10 fold higher than that observed in ELISA (2 x 10^6 cells/ml) with the same antibody (section 5.5.3, Figure 5.32) which was not expected.

In order to determine the accuracy and the intermediate precision of the presented BIAcore Inhibition immunoassay, intra- and interday assay variability studies were performed. Three sets of standards were prepared and incubated with antibody before being assayed. The results obtained from intra- and interday assay studies are shown in Table 6.3. The coefficients of variation (CV's) were determined to assess the precision of the analytical method, expressing standard deviation as a percent function of the mean. These values ranged from 0.46 to 5.62% and 1.41 to 9.58% as observed for the intra- and interday studies, respectively, indicating that the assay had a very good degree of precision. The percentage accuracies values obtained in the linear part of the interday assay (Table 6.3) indicate the fitted four parameter curve provides an accurate representation of the measured response in the linear range of the assay (percentage accuracy values from 94.46 to 106.84%).

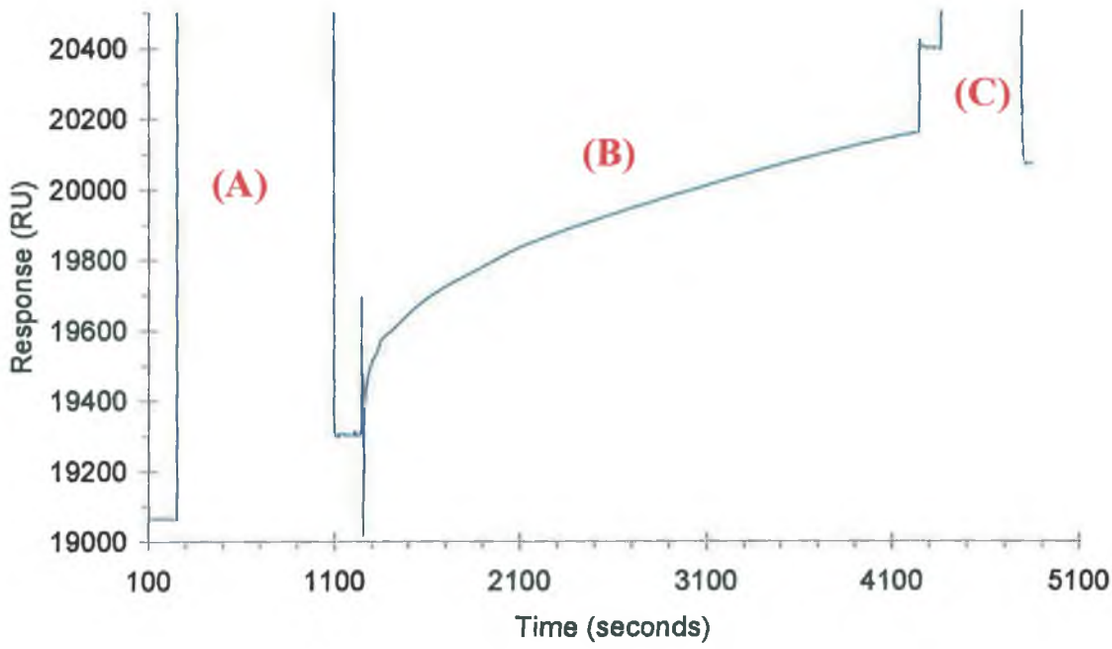


Figure 6.21: *Sensorgram showing the immobilisation of recombinant InlB protein to a C1 sensor chip surface by over-activation of the carboxymethylated surface followed by capping of the unreacted groups with 1M ethylene diamine, pH 8.5 instead of 1M ethanolamine at the same pH.*

(A) *HBS buffer is initially passed over the sensor chip surface and the baseline response recorded. The carboxymethylated surface is then activated with 0.05 M NHS/0.2 M EDC mixture resulting in a large increase in SPR signal due primarily to a bulk refractive index change. HBS buffer is again passed over the now activated surface and a small increase in the baseline is observed.*

(B) *Purified recombinant InlB protein (50 μg/ml) diluted in 10 mM sodium acetate buffer, pH 4.6, is then passed over the activated surface for a period of 45 minutes.*

(C) *Deactivation of the surface NHS-esters is performed by injecting 1M ethylene diamine, pH 8.5, over the surface for 7 minutes, which also serves to elute non-covalently bound antibody. Approximately 1100 RU of recombinant InlB protein remained bound to the surface after the described immobilisation procedure.*

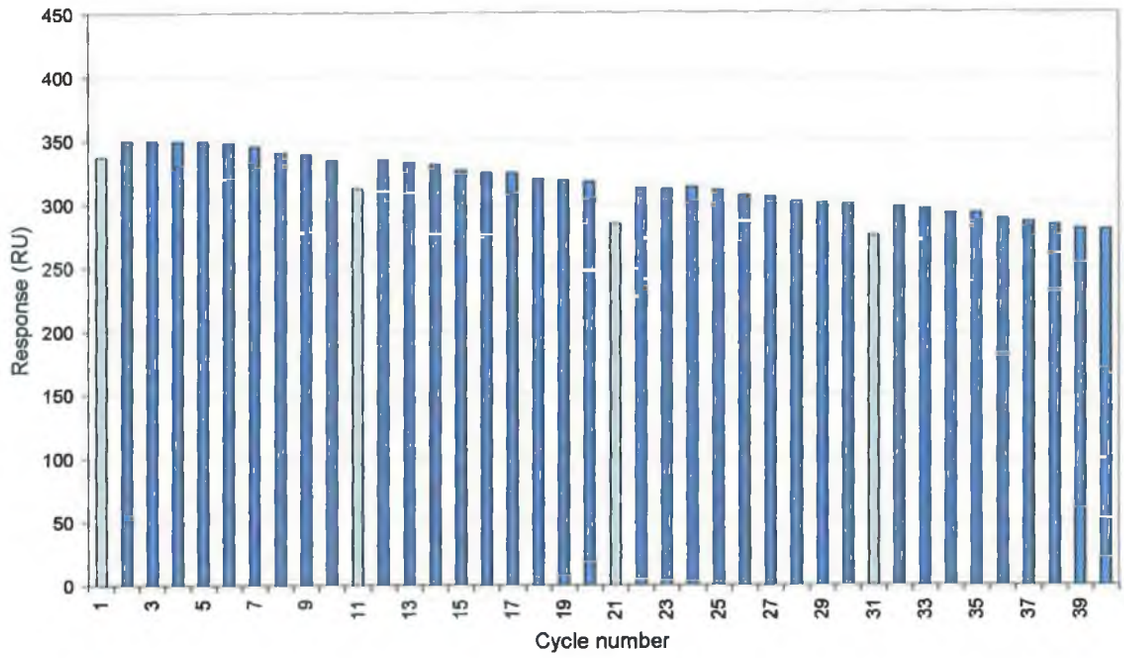


Figure 6.22: *Bar chart showing 40 binding-regeneration cycles of the anti-InlB scFv antibody to the recombinant InlB immobilised C1 sensor chip surface. Four cycles of ten 2 minute injections of a 1/10 dilution of anti-InlB scFv antibody supernatant were injected over the recombinant InlB immobilised sensor chip surface and regenerated with a 30 second pulse of 20 mM NaOH. A large decrease in binding of antibody to the immobilised recombinant InlB surface was observed for the first injection of each of the four binding cycles (shown in grey on the presented bar chart). As previously discussed, this pattern could be due to the fact that the matrix swells when in contact with different pHs, and effectively what is seen in the first injection is a conditioning of the matrix. Excluding the first injection in each cycle, the binding capacity decreased by approximately 20% over the 40 antibody injections which is just within the limits described by Wong et. al. (1997).*

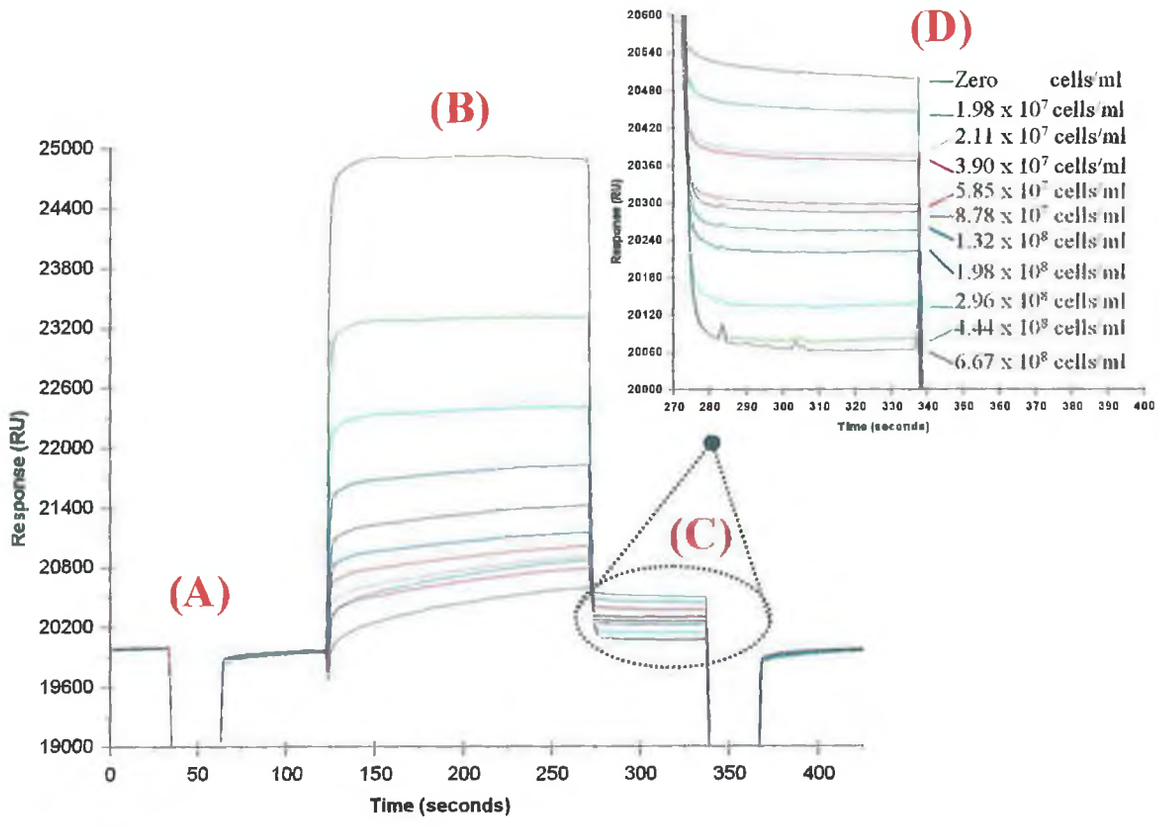


Figure 6.23: *Overlay plot demonstrating the decrease in binding of anti-InlB scFv antibody to the immobilised recombinant InlB surface with increasing Listeria monocytogenes InlB extract concentrations.*

(A) *Shows the normalised baselines of each sensorgram when HBS is injected over the sensor chip surface after a brief injection of 20 mM NaOH.*

(B) *Shows the binding of increasing free antibody with decreasing Listeria monocytogenes InlB extract concentrations.*

(C) *Shows the base line shift when buffer is passed over the surface after each sample injection.*

(D) *Shows an enlarged view of (C) with the equivalent concentration of Listeria monocytogenes cells used in each binding cycle indicated beside each sensorgram.*

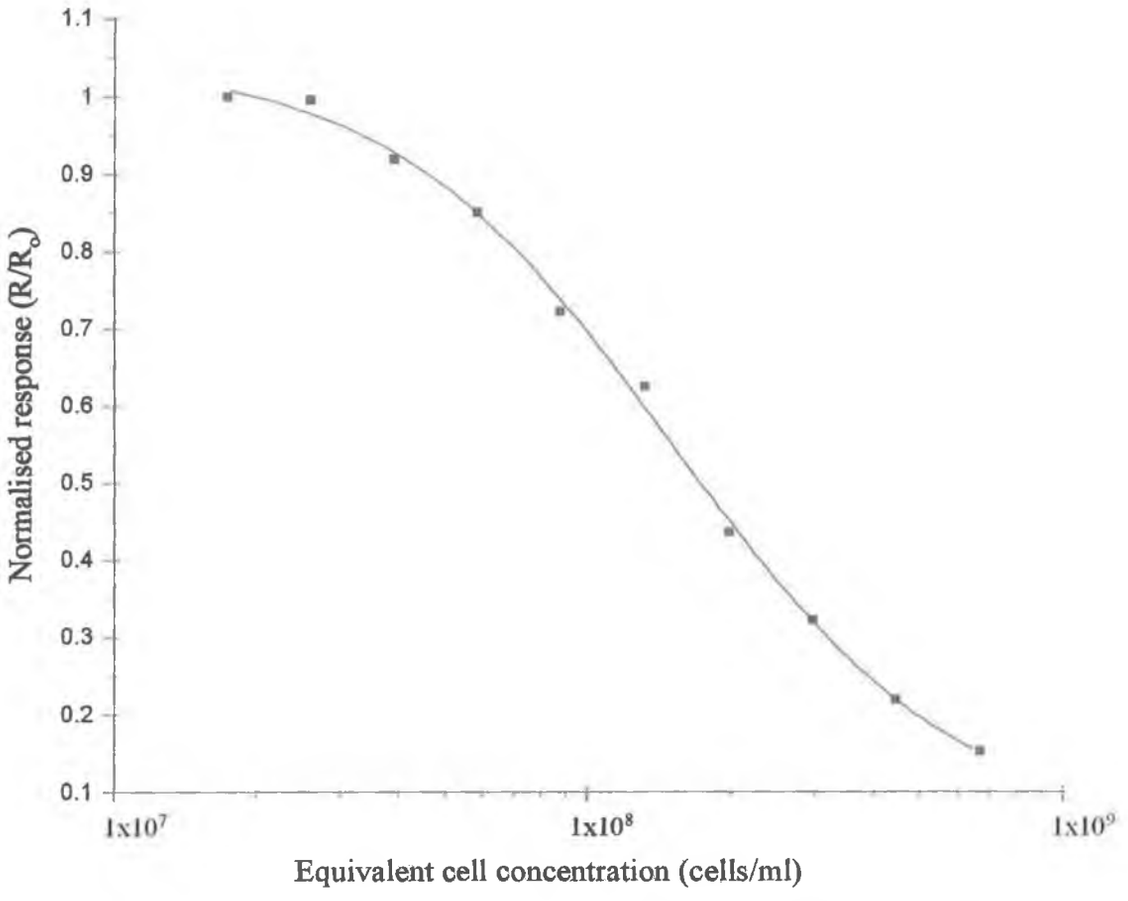


Figure 6.24: *Interday assay calibration curve for the anti-InlB scFv antibody based SPR assay. A 1/5 dilution of the anti-InlB extract antibody was incubated with various Listeria monocytogenes cell concentrations injected over an immobilised recombinant InlB surface as described in section 2.5.6. A 4-parameter equation was fitted to the data set using BIAevaluation 3.1 software. The inter-assay means and coefficients of variance are tabulated in Table 6.3. Each point on the curve is the mean of three replicate measurements analysed over three days.*

Table 6.3: *Intra- and interday assay coefficients of variation (CV's) and percentage accuracies for the anti-InlB scFv antibody used the BIAcore inhibition assay described in section 2.5.6. Coefficients of variance (a quantitative measure of precision) was calculated using the equation % CV= (S.D./Mean) x 100. For intraday studies, the standard deviation (S.D.) is computed from replicate (3 replicates) analyses within a single validation run, while for intermediate precision (interday), the S.D. is computed from replicate (3 replicates) analyses over 3 validation runs on 3 separate days.*

	Intraday			***Interday***		
Concentration (cells/ml)	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**	**Back-calculated concentration (cells/ml)**	**CV's %**	**% Accuracies**
6.67×10^8	6.36×10^8	1.17	104.65	6.59×10^8	9.06	101.22
4.44×10^8	4.58×10^8	0.59	96.93	4.47×10^8	1.41	99.43
2.96×10^8	2.88×10^8	1.65	102.61	2.94×10^8	9.13	100.62
1.98×10^8	2.19×10^8	0.72	89.45	2.06×10^8	9.25	95.74
1.32×10^8	1.19×10^8	1.15	109.74	1.23×10^8	9.58	106.94
8.78×10^7	8.72×10^7	0.46	100.69	9.23×10^7	7.86	94.92
5.85×10^7	5.79×10^7	5.62	101.02	5.82×10^7	2.44	100.54
3.90×10^7	4.61×10^7	1.57	81.91	4.12×10^7	6.64	94.46
2.60×10^7	2.96×10^7	1.41	86.04	2.11×10^7	2.31	118.86
1.74×10^7	7.69×10^6	5.25	155.79	1.98×10^7	4.22	86.10

6.5 *Discussion*

This chapter describes the development of three BIAcore assays to detect *Listeria monocytogenes* using both polyclonal and recombinant single chain Fv fragment antibodies. Each of the three assays described, was developed using a BIAcore 3000 instrument and intra- and interday studies carried out to determine the precision and reproducibility of each assay. A 4-parameter equation was fitted to each standard curve using BIAevaluation software and the percentage accuracies calculated.

The polyclonal anti-*Listeria monocytogenes* antibody described in chapter 3 was used to develop a subtractive inhibition assay for the detection of *Listeria monocytogenes* cells (section 6.2). As described in section 2.5.4, by removing cells and bound antibody from solution with a stepwise centrifugation step, free unbound antibody could be detected by an immobilised anti-rabbit Fab antibody on the sensor chip surface. To maximise the preconcentration of the anti-rabbit Fab antibody to the CM5 sensor chip surface, 50 μg/ml of antibody, diluted in 10 mM sodium acetate buffer at various pH increments (Figure 6.2) were injected over a blank CM dextran surface and the degree of preconcentration recorded. The optimal antibody concentration was observed in sodium acetate pH 4.3 buffer, which was used for all subsequent immobilisations of the anti-rabbit Fab antibody. Approximately 23,000 response units (RU) of anti-rabbit Fab antibody were immobilised (Figure 6.3) on the surface of a CM5 sensor chip surface using EDC and NHS chemistry as described in section 2.5.2. Regeneration studies were carried out on the immobilised anti-rabbit Fab antibody surface to evaluate the performance and lifetime of the sensor chip surface. Binding regeneration cycles were repeated for 60 cycles (Figure 6.5) resulting in less than 13% decrease in binding capacity from the first to the last cycle studied which is well within the limits suggested by Wong *et al.* (1997). An important part of any immunoassay is the cost of the assay. A stable sensor chip surface not only provides the bases for the production of an accurate immunoassay but also can reduce the overall cost of the assay by allowing multiple samples to run over the same assay surface. The immobilised anti-Fab surface studied in section 6.2.3, went through over 100 binding cycles over a two week period before a significant (<20% original binding capacity) decrease in binding capacity was observed.

To determine the working range of the assay, samples containing unbound antibody (stepwise centrifugation supernatant) were injected over the anti-rabbit Fab antibody immobilised surface in random order (Figure 6.6) and a calibration curve constructed. The range of detection of the anti-*Listeria monocytogenes* antibody was found to be between 1

x 10^8 and 1 x 10^6 cells/ml (Figure 6.7) which is equivalent to that observed in ELISA for the same antibody (Figure 3.9). Intra- and interday assay variability studies were performed to determine the accuracy and the intermediate precision of the subtractive inhibition assay. The percentage coefficients of variation (CV's) values ranged from 0.59 to 4.80% and 0.14 to 4.00% as observed for the intra- and interday studies, respectively, indicating that the assay had a very good degree of precision. These coefficients of variation values were significantly lower than those observed in ELISA (Table 3.2) due to the automated nature of the BIAcore biosensor system. The percentage accuracies obtained in the linear part of the interday assay (Table 6.1) indicate the fitted 4-parameter curve provides an accurate representation of the measured response in the linear range of the assay.

Section 6.3 describes the development of an inhibition assay for the direct detection of *Listeria monocytogenes* using the anti-InlB extract polyclonal antibody described in chapter 3. Protein G-purified anti-InlB extract antibody, incubated with decreasing concentrations of *Listeria monocytogenes* cells, was injected over an IMAC purified recombinant Internalin B protein (described in chapter 4) immobilised CM5 sensor chip surface. Prior to immobilisation, the degree of preconcentration of the recombinant InlB protein to the sensor chip surface was determined. As described in section 6.3.1 the recombinant InlB protein electrostatically bound the sensor chip surface and could only be removed with an injection of 20 mM NaOH. Due to the high electrostatic interactions observed, subsequent immobilisations of the recombinant InlB protein were performed in sodium acetate buffer, pH 4.6. Approximately 10,000 RU of recombinant InlB was successfully immobilised on the surface of a CM5 sensor chip as shown in Figure 6.9. Regeneration studies, involving 45 binding-regeneration cycles were performed and a decrease of less than 2% in binding capacity from the first to the last cycle observed. This result indicates that the surface is very stable and should provide an accurate platform for the development of an assay to detect *Listeria monocytogenes* cells. However, it was also observed that the first injection of each assay cycle was significantly lower than subsequent injections containing the same sample (Figure 6.11). According to BIAcore this pattern could be due to the fact that there is a time error in the instruments switch valve indicator and was is observed in actually a reduction in injected sample volume. Therefore, an inhibition assay was developed by discarding the first value in each cycle and taking an average of the remaining three replicates. The range of detection for the anti-InlB extract antibody was found to be between approximately 1 x 10^8 and 1-2 x 10^5 cells/ml as shown in Figure 6.13. The observed limit of detection was approximately 10 fold lower than that observed in ELISA with the same antibody (Figure 3.7) highlighting

the sensitivity of the BIAcore 3000 automated biosensor. The intra- and interday assay coefficients of variation values were determined to assess the precision of the analytical method. These values ranged from 0.53 and 4.55% and 2.98 and 7.70% as observed for the intra- and interday studies respectively. The percentage accuracies of the assay indicate that the fitted 4-parameter equation provides an accurate representation of the measured response in the linear range of the assay. Overall the assay showed good sensitivity and the sensor chip surface was very stable over the number of cycles studied.

A BIAcore inhibition assay was also developed to indirectly detect *Listeria monocytogenes* using the anti-InlB scFv antibody described in section 5.5.2. However, the antibody did not recognise *Listeria monocytogenes* cells indicating that it may be specific for a region of the protein which is involved in the association of the protein to the *Listeria monocytogenes* cell membrane. Therefore, InlB protein was extracted from the *Listeria monocytogenes* cell surface as described in section 2.2.1.2, and used to develop a BIAcore inhibition assay using the responses obtained from the injection of the anti-InlB scFv antibody incubated with decreasing concentrations of *Listeria monocytogenes* InlB extract over an immobilised recombinant InlB sensor chip surface. Due to the cationic nature of the InlB protein, various BIAcore sensor chips were evaluated for use in the described assay. It was observed that the InlB extract seemed to non-specifically bind to the dextran surface of the CM5 and NTA sensor chips (sections 6.4.1.1 and 6.4.1.2) limiting their use for further development. It was observed that high salt concentrations (Figure 6.15) could eliminate the electrostatic interactions of the cationic InlB protein to the anionic surfaces but such high salt concentrations also seriously hindered the binding of the recombinant scFv antibody to the sensor surface. In order to mimic ELISA conditions, recombinant InlB protein was adsorbed onto the surface of a HPA chip as described in section 6.2.1.4. As presented in Figure 6.19, an injection of InlB extract incubated with anti-InlB scFv antibody (71.3 RU) resulted in a decrease in observed response compared to that observed from same concentration of antibody diluted in PBS (149 RU). However, when the injection of both samples (antibody-extract and antibody in PBS) was repeated over the surface (Figure 6.19, labelled 3-6) the binding capacity of the surface decreased drastically limiting the feasibility of the use of the HPA chip surface for used in a development of an accurate and cost effective immunoassay.

Using the standard EDC/NHS immobilisation procedure (section 5.5.2), the *Listeria monocytogenes* InlB extract was also observed to non-specifically bind to the flat carboxymethylated surface of a C1 sensor chip (section 6.4.1.3). In order to decrease the overall negative charge on the sensor surface, the surface was over-activated with a double

injection of EDC/NHS and recombinant InlB immobilised followed by capping with 1M ethylene diamine, pH 8.5. A 1 minute injection of anti-InlB scFv antibody over the recombinant InlB immobilised surface resulted in a baseline shift of 796.8 RU units compared to 131.9 RU observed from a 1/5 dilution of InlB extract incubated with the same concentration of antibody indicating the potential of the described method in the development of an assay to detect InlB extract using the anti-InlB scFv antibody.

The binding capacity of the recombinant InlB immobilised surface was determined by a series of binding and surface regeneration sequences to assess at which point the binding capacity deviated outside the desired performance range of <20% suggested by Wong *et al.* (1997). 40 binding-regeneration cycles were performed resulting in a decrease of approximately 20% in binding capacity from the first to the last cycle studied (Figure 6.22). This finding indicates that the surface can be used for only 40 binding-regeneration cycles before the decrease binding capacity exceeds the desired performance range. Therefore, an assay developed using this method will not be cost effective (only 160 samples per chip can be performed) and will lack the accuracy observed from assays with more stable surfaces such as in section 6.3. However, as a number of sensor chips had already been evaluated and because the antibody had shown good specificity towards *Listeria monocytogenes* cells (section 5.5.5), it was decided that the development of an assay using the described surface could still be a valuable tool for the species-specific detection of *Listeria monocytogenes* even if not cost effective. A calibration curve was constructed by plotting the average (n=3) change in response for each *Listeria monocytogenes* InlB extract standard against the known equivalent concentration of *Listeria monocytogenes* cells used in each run. The range of detection for the anti-*Listeria monocytogenes* antibody was found to be between approximately 6.6×10^8 and 2×10^7 cells/ml (Figure 6.24). The limit of detection (approximately 2×10^7 cells/ml) observed in Figure 6.24 is approximately 10 fold higher than that observed in ELISA (2×10^6 cells/ml) with the same antibody (section 5.5.3, Figure 5.32) which was not expected. The coefficients of variation (CV's) were determined to assess the precision of the analytical method, expressing standard deviation as a percent function of the mean. These values ranged from 0.46 to 5.62% and 1.41 to 9.58% as observed for the intra- and interday studies, respectively, indicating that the assay had a good degree of precision. Even with the automated nature of the BIAcore instrument, the observed coefficient of variation values were still slightly higher than those obtained in ELISA for the same antibody (section 5.5.3), which is probably due to the unstable nature of the sensor chip surface. The percentage accuracies values obtained in the linear part of the interday assay (Table 6.3) indicate the fitted four parameter curve provides an accurate representation of the

measured response in the linear range of the assay (percentage accuracy values from 94.46 to 106.84%).

To summarise, three BIAcore inhibition assays were developed with two polyclonal antibodies described in chapter 3 and with an scFv antibody described in chapter 5. The three assays described use novel formats for the SPR-based detection of *Listeria monocytogenes* and may have wider applications for the study of other bacteria and large molecules. In terms of sensitivity and reproducibility the inhibition assay developed with the anti-InlB antibody proved to be the most sensitive assay (Limit of detection of approximately 2 x 10^5 cells/ml) and could be regenerated over 200 times before significant decrease in binding capacity was observed. This assay proved more sensitive than the other SPR-based detection formats for microorganisms discussed in section 1.18 and was further characterised in terms of reproducibility and accuracy (Intra- and interday assay variability studies performed). In general, the stability of the sensor surface depends on the immobilised ligand with non-protein molecules such as carbohydrate or haptens like progesterone (3600 regeneration cycles, Gillis et al. 2003) being most stable. According to BIAcore (personnel communication with John Butler, BIAcore application specialist) protein immobilised sensor surface tend to stable for between 100 and 200 regeneration cycles. The described anti-InlB polyclonal inhibition assay was stable for over 200 regeneration cycles which compares very well with other protein immobilised surfaces such as that described by Rasooly, 2001 (70 regenerations). However, as described in section 3.2.7, both the anti-InlB extract and anti-*Listeria monocytogenes* polyclonal antibodies are not species specific and cross react with a number of the other members of the genus *Listeria* (Table 3.5). On the other hand, the BIAcore assay developed with the anti-InlB scFv antibody, was not as sensitive nor as cost effective as the anti-InlB extract polyclonal antibody based assay but it was shown to specifically bind *Listeria monocytogenes* InlB extract (section 5.5.5). Therefore, depending on the desired assay requirements (e.g. species or genus-specific detection or low cost assay) the BIAcore assays described in this chapter can be reliably and accurately used to monitor the interaction of anti-*Listeria monocytogenes* related antibodies and cells in "real-time".

Chapter 7

Overall Conclusions

7.1 *Overall conclusions*

The aim of this thesis was to develop an immunoassay for the rapid and specific detection of *Listeria monocytogenes* cells. The strategy used involved the production and characterisation of polyclonal and recombinant single chain Fv antibody fragments to *Listeria monocytogenes* cells and invasion-associated proteins. The *Listeria monocytogenes* invasion-associated proteins, Internalin B (InlB) and p60, were cloned into *E. coli* and expressed in the cytoplasm of the cells. IMAC purified InlB and p60 proteins were used in the selection of recombinant scFv antibodies from a naïve human antibody library. Antibodies, were characterised by ELISA, SDS-PAGE and Western blotting and used in the development of a biosensor-based immunoassay for the detection of *Listeria monocytogenes*.

Chapter 3 describes the production and characterisation of two polyclonal antibodies generated against HT-*Listeria monocytogenes* cells and InlB protein extract. Antibodies were crudely purified by saturated ammonium sulphate precipitation and further purified by protein G affinity chromatography. Purified IgG was used for the development of an inhibition ELISA to detect *Listeria monocytogenes* cells. Inhibition ELISAs were developed with both antibodies in PBS and chocolate milk. As discussed in chapter 3, chocolate milk was reported to have been the source of a *Listeria monocytogenes* food poisoning outbreak in Illinois in 1994. Intra- and interday studies were performed to evaluate the precision and reproducibility of each assay developed. Assays developed with both antibodies proved to be accurate and reproducible with intra- and interday coefficients of variation of typically less than 10-12%. Cross reactivity studies were performed with both antibodies against a number of bacterial strains. Two methods for evaluating the percentage cross reactivity of each antibody were evaluated in section 3.2.7. The observed percentage cross reactivity of each antibody was calculated by using a ratio of concentrations (Figure 3.15) and a ratio of observed inhibition (Figure 3.16) yielding large differences in calculated percentage cross reactivity values. Both antibodies discussed were observed to cross react with the other *Listeria* strains to different degrees indicating that neither antibody can be used for the specific detection of *Listeria monocytogenes* cells from a heterogeneous population of bacterial cells. However, it was discussed that by varying the assay format, and by utilising either of the antibodies as a capture antibody in conjunction with a detection antibody with a more refined specificity (monoclonal antibody), both antibodies have great potential to be valuable reagents for the development of an immunoassay for the reliable detection of *Listeria monocytogenes* cells from a mixed population. Both antibodies were successfully further utilised for the

identification of *Listeria monocytogenes* recombinant invasion-associated proteins (chapter 4) and for the rapid genus-specific detection of *Listeria* cells using surface plasmon resonance (SPR) described in chapter 6.

The availability of large quantities of *Listeria monocytogenes*-specific antigen is a prerequisite for the generation of species specific anti-*Listeria monocytogenes* antibodies. Chapter 4 describes the expression and purification of the *Listeria monocytogenes* invasion-associated proteins, Internalin B (InlB) and p60, in *E. coli* XL10 Gold cells. Genomic DNA was extracted from *Listeria monocytogenes* cells and the InlB and p60 gene sequences amplified by PCR using specific primer sequences. Amplified gene products were cloned into a TA cloning vector (Invitrogen) and subsequently sub-cloned into the pQE-60 expression vector (QIAgen). InlB and p60 gene products were expressed in *E. coli* XL10 Gold cells and purified by immobilised metal affinity chromatography (IMAC). Purified protein was characterised by SDS-PAGE and the immunoreactivity of each protein with anti-*Listeria monocytogenes* antibodies determined. As discussed in section 4.3, both the recombinant p60 (rp60) and recombinant InlB (rInlB) proteins could be specifically detected with monoclonal antibodies raised against the native form of the protein and against the fused 6xHis tag, indicating that both proteins can be used for the generation of *Listeria monocytogenes*-specific antibodies. This result also indicates that the 6xHis tag is not hidden in the protein structure and no proteolytic activity has occurred at the C-terminus of the protein. The detection of both recombinant proteins by ELISA (Figure 4.15 and 4.16) shows the immunoreactivity of the recombinant proteins in native conditions and indicates that both proteins can be used in the development of a specific immunoassay or for the generation of specific antibodies by immunisation or by bio-panning of a combinatorial antibody library.

The selection of single chain Fv (scFv) fragment antibodies from a naïve human antibody library and from two murine antibody libraries generated from the spleens of mice immunised with *Listeria monocytogenes* cells and InlB protein extract is described in chapter 5. Two antibody libraries were produced using the system described by Krebber *et al.* (1997a) and nine phage-scFv antibodies selected against *Listeria monocytogenes* cells. All nine scFv genes were sequenced, aligned and examined for cross reactivity with a number of bacterial strains. All nine phage-scFv antibodies showed tendencies to cross react with the other bacterial strains tested and therefore were not further characterised.

A naïve human antibody library, with an antibody diversity of 1.4×10^{10} clones (Vaughan *et al.*, 1996), was bio-panned against *Listeria monocytogenes* cells (section 5.4) and

purified recombinant *Listeria monocytogenes* invasion associated proteins, Internalin B (InlB) and p60 (section 5.5). Two clones were selected when bio-panned with *Listeria monocytogenes* that recognised HT-*Listeria monocytogenes* cells in an inhibition ELISA format. Sequencing of the scFv genes sequences revealed that both clones were in fact the same clone. When assayed against intact cells as described in section 2.4.3.11, the phage-scFv antibody generated a much decreased signal (over 70% decrease in signal when assayed against intact *Listeria monocytogenes* 4b) and showed no significant increase in reactivity to *Listeria monocytogenes* cells compared to the other bacterial strains tested.

In order to refine the specificity of scFv antibodies capable of reacting with *Listeria monocytogenes* cells, the naïve human antibody library was also bio-panned with purified recombinant InlB (rInlB) and recombinant p60 (rp60). Twenty four phage-scFv clones were selected and expressed as soluble scFv antibodies and screened for reactivity against recombinant protein and *Listeria monocytogenes* cells. Only two clones expressed functional scFv antibodies in the supernatant, while the cytoplasmic extracts of five clones were observed to react with either rp60 or rInlB protein. One scFv antibody (clone G7) was observed to react against purified rInlB protein in solution using an inhibition ELISA format and showed good expression of scFv antibody in both the supernatant and cytoplasmic extract. Therefore, it was selected for the development of an immunoassay to detect InlB protein (and therefore indirectly *Listeria monocytogenes*) as described in section 5.5.2. The generated inhibition assay showed good intra- and interday coefficients of variation values, and the scFv antibody did not cross react with the bacterial strains examined.

Chapter 6 describes the generation of three biosensor-based immunoassays using the two polyclonal antibodies and the anti-InlB scFv antibody described in chapters 3 and 5, respectively. The polyclonal anti-*Listeria monocytogenes* antibody was used to develop a subtractive inhibition BIAcore assay to detected *Listeria* cells. An anti-rabbit Fab antibody was immobilised on the surface of a CM5 sensor chip and used to detect free rabbit polyclonal anti-*Listeria monocytogenes* antibodies. Decreasing concentrations of *Listeria monocytogenes* cells were incubated with anti-*Listeria monocytogenes* antibody, and unbound antibody removed by centrifugation. Gradually increasing the centrifugation speed in a stepwise manner allowed the separation of cells with bound antibody from free unbound antibody. Antibody supernatant was passed over the immobilised anti-rabbit Fab surface and decrease in free antibody observed with increasing cell concentrations. The sensor surface proved to be stable for over 100 binding cycles, with the developed assay

proving accurate and reproducible with intra- and interday coefficient of variation values less than 5% obtained.

An inhibition assay for the direct detection of *Listeria monocytogenes* was also developed using the anti-InlB extract polyclonal antibody described in chapter 3. Protein G-purified anti-InlB extract antibody was incubated with decreasing concentrations of *Listeria monocytogenes* cells for 1 hour at 37°C as described in section 2.5.5. Antibody and cells were then injected over a CM5 sensor chip surface that had been previously immobilised with IMAC-purified recombinant Internalin B protein (described in chapter 4). Regeneration studies, involving 45 binding-regeneration cycles were performed and a decrease of less than 2% in binding capacity from the first to the last cycle observed. This result indicated that the surface was very stable and should provide an accurate platform for the development of an assay to detect *Listeria monocytogenes* cells. Percentage coefficient of variation values of less than 8% were observed for the intra- and interday assay variability studies. Overall the assay showed good sensitivity (better than other reported SPR-based assays for microorganisms described in section 1.18) and the sensor chip surface was very stable (200 regeneration cycles compared to 70 cycles reported by Rasooly, 2001) over the number of cycles studied.

A BIAcore inhibition assay was also developed to indirectly detect *Listeria monocytogenes* using the anti-InlB scFv antibody described in section 5.5.2. InlB protein was extracted from the cell membrane of *Listeria monocytogenes* cells and used to develop a BIAcore inhibition assay. An anti-InlB scFv antibody incubated with decreasing concentrations of *Listeria monocytogenes* InlB extract was injected over an immobilised recombinant InlB sensor chip surface and the change in observed response recorded. Various sensor chips and immobilisation procedures were evaluated as the InlB extract was observed to non-specifically bind to the negatively charged dextran surface. Over activation of the surface of a C1 sensor chip, containing no dextran layer, and capping with ethylene diamine was deduced to be the best approach for the preparation of an immunoassay to detect the cationic InlB extract. The assay surface was not as stable as the previously described assays as the surface could be used for only 40 binding-regeneration cycles before the decrease binding capacity exceeded the desired performance range of 20%. The coefficients of variation (CV's) were determined to assess the precision of the analytical method, with intra- and interday assays values of less than 10% obtained, indicating that the assay had a good degree of precision.

Overall, the work described in this thesis involved the generation of polyclonal and recombinant scFv antibodies to *Listeria monocytogenes* cells and invasion-associated protein. Antibodies were purified and characterised by ELISA, SDS-PAGE and Western blotting. The *Listeria monocytogenes* invasion associated proteins, InlB and p60, were cloned and expressed in *E. coli* providing an available of pure antigen for the selection of protein specific antibodies and for use in immunoassay development. Three BIAcore inhibition assays were developed with two polyclonal antibodies described in chapter 3 and with an scFv antibody described in chapter 5.

An evaluation of the literature for detection of *Listeria monocytogenes* reveals three main detection platforms which can differ in sensitivity, robustness and confirmation time. Cultural methods such as described by Scotter et al. (2001a,b), have proven to be the "gold standard" for the detection of *Listeria monocytogenes*. However, cultural methods can be too time consuming, taking up to a week in some cases to yield confirmation of a result. Nucleic acid-based and immunological-based detection systems have become more widely used in recent years (see Table 1.3 for list of recent nucleic acid-based assays for the detection of *Listeria monocytogenes* and Tables 1.4 and 1.6 for a list of antibody-based detection platforms for *Listeria monocytogenes*). Nucleic acid-based detection systems have been reported to be extremely sensitive with detection limits of less than 10 cfu/ml units reported by a number of research groups (Kaclikova et al., 2003; Hein et al., 2001 and O'Connor et al., 2000). When compared with immunological detection systems such as those presented in Table 1.6 or discussed in section 1.18, nucleic acid-based assays have proven more sensitive. However, a direct comparison of sensor-based and nucleic-based detection systems is not so clear cut. Nucleic acid-based detection can be sensitive to a number of environmental contaminants present in food therefore pre-enrichment of sample is required before sample analysis. The reported limits of detection listed in Table 1.6 for recent optical and electrochemical sensor-based detection systems for *Listeria monocytogenes* are falsely high compared to reported nucleic acid-based systems (Table 1.3) as no pre-enrichment is reported. Pre-enrichment of large food samples (e.g. current regulations for the need to detect 1 cell in 25g of food) is required to isolate the desired target and realistically is a prerequisite for both nucleic-acid and immunological-based detection of microorganisms. In conjunction with sensitivity, important assay criteria for the detection of a target analyte are speed, cost and complexity. Detection of pathogens in "real-time" after sample enrichment can drastically shorten assay time, a requirement for the routine quality control of semi-perishable foods. With the exception of real-time PCR, nucleic-acid-based and most immunological detection methods (with the exception of dipstick type assays) still require several hours to yield results after enrichment. Real-time

biosensor-based systems are generally more robust and less complex than nucleic acid-based systems and can be used for multiple sample analysis (over 200 cycles observed with the anti-InlB polyclonal antibody) allowing the "on-site" detection of pathogens with "unskilled" personnel at low cost. Therefore, the choice of a suitable detection method for *Listeria monocytogenes* will depend on factors such as the sensitivity need (PCR tend to be slightly more sensitive than biosensor-based systems and will therefore require shorter enrichment times), complexity (immunoassay-based systems tend to be more user-friendly than nucleic acid-based systems and therefore can be performed with "unskilled" personnel), enrichment steps (both nucleic acid-based and immunological-based assays require enrichment to accurately detect low numbers of pathogens in large food samples), robustness (nucleic acid-based systems are sensitive to environmental contaminants are only suitable for laboratory-based detection while handheld antibody-sensor systems or dipstick type assays can used for field testing) and cost (will depend on the specific format used). Therefore, depending on the desired assay requirements (e.g. species or genus-specific detection) the BIAcore assays described in this thesis can be reliably and accurately used to monitor the interaction of anti-*Listeria monocytogenes* related antibodies and cells in "real-time".

The work presented in this thesis could be further developed by transferring the described BIAcore assays to a miniature and portable SPR sensor platform, such as the Spreeta SPR biosensor, for use in environment testing. Ligand fishing using magnetic particles coated with anti-*Listeria* antibodies (such as the anti-whole *Listeria monocytogenes* cell polyclonal antibody) could be used to capture and concentrate low numbers of *Listeria monocytogenes* cells from large sample volumes. Using antibody engineering techniques such as those described in section 1.15, the described anti-InlB scFv antibody could be affinity maturated to increase its sensitivity and stability, enhancing its capabilities for the indirect detection of *Listeria monocytogenes* cells.

Chapter 8

References

Adams, G.P. and Schier, R. (1999). Generating improved single-chain Fv molecules for tumour targeting. *J. Immunol. Methods*, **231**:249-260.

Akerstrom, B. and Bjork, L. (1986). A physiochemical study of protein G, a molecule with unique immunoglobulin G-binding properties. *J. Biol. Chem.,* **261**:10240-10247.

Akerstrom, B. Brodin, T., Reis, T. and Bjorck, L. (1985). Protein G: a powerful tool for binding and detection of monoclonal polyclonal antibodies. *J. Immunol.,* **135**:2589-2592.

Alfthan, K. (1998). Surface plasmon resonance biosensors as a tool in antibody engineering. *Biosens. Bioelectron.,* **13**:653-663.

Almeida, P.F. and Almeida, R.C.C. (2000). A PCR protocol using *inl* gene as a target for specific detection of *Listeria monocytogenes. Food Control,* **11**:97-101.

Amstutz, P., Forrer, P., Zahnd, C. and Plückthun, A. (2001). *In vitro* display technologies: novel developments and applications. *Curr. Opin. Biotechnol.,* **12**:400-405.

Anon., (2001). Surface plasmon resonance. *BIAcore Technology Note 1*, 15th September, p1-4.

Anon. (1997). Food poisoning, Listeriosis, and Febrile Gastroenteritis. *Nutr. Rev.,* **55**:57-60.

Artault, S., Blind, J.L., Delaval, J., Dureuil, Y., Gaillard, N. (2001) Detecting *Listeria monocytogenes* in food. *Int. Food Hygiene,* **12**:23.

Azzazy, H.M.E. and Highsmith, W.E. Jr. (2002). Phage display technology: clinical applications and recent innovations. *Clin. Biochem.*, **35**:425-445.

Bhagwat, A.A. (2003). Simultaneous detection of *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* strains by real-time PCR. *Int. J. Food Microbiol.,* 84:217-224.

Barbas, C. F. III, Burton, D.R., Scott, J.K. and Silverman, G.J (2001). In *Phage Display: A laboratory manual*. Cold Spring Harbor Laboratory Press, New York, USA.

Beckerle, M.C. (1998). Spatial control of actin filament assembly: Lessons from Listeria. *Cell,* **95**:741-748.

Benhar, I., Eshkenazi, I., Neufeld, T., Opatowsky, J., Shaky, S. and Rishpon, J. (2001). Recombinant single chain antibodies in bioelectrochemical sensors. *Talanta,* **55**:899-907.

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

Bernardini, M.L, Mounier, J., d'Hauteville, H., Coquis-Rondon, M., Sansonetti, PJ. (1989). Identification of icsA, a plasmid locus of *Shidella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. *Proc. Natl. Acad. Sci. (USA),* **86**:3867-71.

Bhunia, A.K. (1997). Antibodies to *Listeria monocytogenes*. *Crit. Rev. Microbiol.,* **23**:77-107.

Bierne, H. and Cossart, P. (2002). InlB, a surface protein of *Listeria monocytogenes* that behaves as an invasin and a growth factor. *J. Cell Sci.,* **115**:3357-3367.

Bierne, H., Gouin, E., Roux, P., Caroni, P., Yin, H.L. and Cossart, P. (2001). A role for cofilin and LIM kinase in *Listeria*-induced phagocytosis. *J. Cell Biol.,* **155**:101-112.

Bille, J. (1990). Epidemiology of human listeriosis in Europe with special reference to the Swiss outbreak, p71-74, in *Foodborne Listeriosis* eds. Miller, A.J., Smith, J.L. and Somkuli, G.A. Elsevier, Amsterdam, Netherlands.

Bjorck, L. and Kronvall, G. (1984). Purification and some properties of Streptococcal protein G, a novel IgG-binding reagent. *J. Immunol.,* **133**:969-974.

Bokken, G.C.A.M., Corbee, R.J., van Knapen, F. and Bergwerff, A.A. (2003). Immunochemical detection of *Salmonella* group B, D and E using an optical surface plasmon resonance biosensor. *FEMS Microbiol. Lett.,* **222**:75-82.

Borucki, M.K., Krug, M.J., Muraoka, W.T. and Call, D.R. (2003). Discrimination among *Listeria monocytogenes* isolates using a mixed genome DNA microarray. *Vet. Microbiol.*, **92**:351-362.

Branden, C. and Tooze, J (1991). Recognition of foreign molecules by the immune system, p179-199, in *Introduction to Protein Structure*. Garland Publishing Inc., New York, USA.

Braun, L., Dramsi, S., Dehoux, P., Bierne, H., Lindahl, G., Cossart, P. (1997). InlB: an invasion protein of *Listeria monocytogenes* with a novel type of surface association. *Mol. Microbiol.*, **25**:285-294.

Braun, L., Ghebrehiwet, B. and Cossart, P. (2000). gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of *Listeria monocytogenes. EMBO J.*, **19**:1458-1466.

Brennan, J., Dillon, P. and O'Kennedy, R. (2003). Production, purification and characterisation of genetically derived scFv and bifunctional antibody fragments capable of detecting illicit drug residues. *J. Chromatogr. B*, **786**:327-342.

Brigham-Burke, M., Edwards, J.R. and O'Shannesy, D.J. (1992). Detection of receptor-ligand interactions using surface plasmon resonance: model studies employing the HIV-1 gp120/CD4 interaction. *Anal. Biochem.*, **205**:125-131.

Bsat, N, Weidmenn, M, Czajka, J., Barany, F., Piani, M., Batt, C.A. (1994). Food safety applications of nucleic acid based assays. *Food Technol.*, **48**:142-145.

Bubert, A., Schubert, P., Kohler, S., Frank, R. and Goebel, W. (1994). Synthetic peptides derived from the *Listeria monocytogenes* p60 protein as antigens for the generation of polyclonal antibodies specific for secreted cell-free *L. monocytogenes* p60 proteins. *Appl. Environ. Microbiol.*, **60**:3120-3127.

Burmester, J. and Plückthun, A. (2001). Construction of scFv Fragments from Hybridoma or Spleen Cells by PCR Assembly, p19-40, in *Antibody Engineering*, Kontermann, R. and Dübel, S. (eds.), Springer Lab Manual, Springer-Verlag, Berlin/Heidelberg, Germany.

Cabanes, D., Dehoux, P., Dussurget, O., Frangeul, L. and Cossart, P. (2002). Surface proteins and the pathogenic potential of *Listeria monocytogenes. Trends Microbiol.,* **10**:238-245.

Cahill, D., Roben, P., Quinlan, N. and O'Kennedy, R. (1995). Production of antibodies, p2057-2066, in *Encyclopedia of Analytical Science,* Townsend, A. (ed.), Academic Press , New York, USA.

Call, D.R., Borucki, M.K. and Loge, F.J. (2003). Detection of bacterial pathogens in environmental samples using DNA microarrays. *J. Microbiol. Methods,* **53**:235-243.

Caponi, L. and Migliorini, P. (1999), Immunoblotting, p32-46, in *Antibody usage in the lab*. Springer lab manual, Springer, Berlin, Germany.

Carlier, M.F., Laurent, V., Santolini, J., Melki, R., Didry, D.,Hong, Y., Xia, G-X., Chua, N-H., Pontaloni, D. (1997). actin depolymerising factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *J. Cell Biol.,* **136**:1307-1323.

Carlier, M-F. (1998). Control of actin dynamics. *Curr. Opin. Cell Biol.,* **10**:45-51.

Casalvilla, R., Duenas, M., Ayala, M., Cruz, S., Cruz, L., Buurman, W. A. and Gavilondo, J.V. (1999). A bacterial single-chain Fv antibody fragment that inhibits binding of its parental anti-E-Selectin monoclonal antibody to activated human endothelial cells. *J. Biotechnol.*, **72**:1-12.

Chaplin, M.F. and Bucke, C. (1990), in *Enzyme Technology,* 1st Ed., Cambridge University Press, UK.

Choi, W. S. and Hong, C-H. (2003). Rapid enumeration of *Listeria monocytogenes* in milk using competitive PCR. *Int. J. Food Microbiol.*, 84:79-85.

Cooper, J.A. and Schafer, D.A. (2000). Control of actin assembly and disassemble at filament ends. *Curr. Opin. Cell Biol.,* **12**:97-103.

Cossart, P. (2000). Actin-based motility of pathogens: the Arp2/3 complex is a central player. *Cell. Microbiol.,* **2**:195-205.

Cossart, P. and Bierne, H. (2001a). The use of host cell machinery in the pathogenesis of *Listeria moncotyogenes. Curr. Opin. Immunol.,* **13**:96-103.

Cossart, P. (2001b). Met, the HGF-SF receptor: another receptor for *Listeria monocytogenes. Trends Microbiol.,* **9**:105-107.

Cossart, P. (2002). Molecular and cellular basis of the infection by *Listeria monocytogenes*: an overview. *Int. J. Med. Microbiol.,* **291**:401-409.

Cossart, P., Pizarro-Cerda, J. and Lecuit, M. (2003). Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions. *Trends Cell. Biol.,* **13**:23-31.

Dale, J. W. (1998), in *Molecular Genetics of Bacteria,* 3rd Ed., John Wiley and Sons. West Sussex, UK.

Dalton, C.B., Austin, C.C, Sobel, J., Hayes, P.S., Bibb, W.F., Graves, L.M., Swaminathan, B., Proctor, M., Griffin, P.M. (1997). An outbreak of Gastroenteritis and fever due to *Listeria monocytogenes* in milk. *New Engl. J. Med.*, **336**:100-105.

Daly, S.J., Keating, G.J., Dillon, P.P., Manning, B.M. and O'Kennedy, R. (2000). Development of surface plasmon resonance immunoassay for aflatoxin B1. *J. Agric. Food Chem.,* **48**:5097-5104.

Daly, S.J.; Dillon, P.P.; Brennan, J.; Dunne, L.; Fitzpatrick, J.; O'Kennedy, R. (2001). Production and analytical applications of scFv antibody fragments. *Anal. Lett.*, **34**:1799-1827.

Davis, N.G., Boeke, J.D., and Model, P. (1985). Fine structure of membrane anchor domain. *J. Molec. Biol.,* **181**:111-121.

De Boer, E. and Beumer, R.R. (1999). Methodology for detection and typing of foodborne microorganisms. *Int. J. Food Microbiol.,* **50**:119-130.

Delves, P.J. (1997). General Introduction, in *Antibody production- essential techniques,* p1-12, Rickwood, D. (Ed.), John Wiley and Sons, West Sussex, UK.

Deneer, H.G., and Boychuk, I., (1992). Species Specific Detection of *Listeria monocytogenes* by DNA Amplication. *Appl. Enviro. Micro.*, **57**:606-609.

De Valk, H., Vaillant, V., Jacquet, C., Rocourt, J., Le Querrec, F., Stainer, F., Quelquejeu, N., Pierre, O., Pierre, V., Descenclos, J.-C. and Goulet, V. (2001). Two consecutive nationwide outbreaks of listeriosis in France, October 1999-February 2000. *Am. J. Epidemiol.*, **154**:944-950.

Dillon, P.P., Daly, S.J., Manning, B.M. and O'Kennedy, R. (2003). Immunoassay for the determination of morphine-3-glucuronide using a surface plasmon resonance-based biosensor. *Biosens. Bioelectron.*, **18**:217-227.

Doganay, M. (2003). Listeriosis: clinical presentation. *FEMS Immunol. Medical Microbiol.*, **1485**;1-3.

Dold, F.G., Sanger, J.M., Sanger, J.W. (1994). Intact alpha actinin molecules are needed for both the assembly of actin into tails and the locomotion of *Listeria monocytogenes* inside infected cells. *Cell Motil. Cytoskel.*, **28**:97-107.

Domann, E., Wehland, J., Rohde, M., Pistor, S., hartl, M., Goebel, W., Leimeister-Wachter, M., Wuenschner, M., and Chakraborty, T. (1992). A novel bacterial gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to proline-rich region of vinculin. *EMBO J.*, **11**:1981-1990.

Donnelly, C.W. (2001). *Listeria monocytogenes:* A continuing challenge. *Nutrition Rev.*, **59**:183-194.

Dower, W.J. and Mattheakis, L.C. (2002). *In vitro* selection as a powerful tool for the applied evolution of proteins and peptides. *Curr. Opin. Chem. Biol.*, **6**:390-398.

Doyle, M.P., and Schoeni, J.L. (1987). Comparison of procedures for isolating *Listeria monocytogenes* in soft, surface ripened cheese. *J. Food Prot.* **50**:4-6.

Dramsi, S., Biswas I, Maguin, E., Braun, L., Mastroeni, P., Cossart, P. (1995). Entry of *Listeria monocytogenes* into hepatocytes requires expression of InlB, a surface protein of the internalin multigene family. *Mol. Microbiol.*, **16**:251-61.

Dramsi, S., Dehoux, P., Lebrun, M., Goossens, P., Cossart, P. (1997). Identification of four new members of the internalin multigene family in *Listeria monocytogenes* strain EGD. *Infect. Immunity,* **65**:1615-25.

Dübel, S. and Kontermann, R. (2001). Recombinant antibodies, p3-18, in *Antibody Engineering*, Dübel, S. and Kontermann, R. (Eds.), Springer lab manuals, Germany.

Dunbar, S.A., Vander-Zee, C.A., Oliver, K.G., Karem, K.L. and Jacobson, J.W. (2003). Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP™ system. *J. Immunol. Methods,* **53**:245-252.

Findlay, J. W . A., Smith, W. C., Lee, J. W., Nordblom, G. D., Das, I., DeSilva, D. S., Khan, M. N. and Bowsher, R. R. (2000). Validation of immunoassays for bioanalysis: a pharmaceutical industry perspective. *Pharmaceutical Biomed. Anal.*, **21**:1249-1273.

Finlay, B.B. and Cossart, P. (1996). Exploitation of mammalian host cell functions by bacterial pathogens. *Science,* **276**:718-725.

Fischetti, V.A., Pancholi, V., and Schneewind, O. (1990). Conservation of hexa peptide sequence in the anchor region of surface proteins from Gram positive cocci. *Mol. Microbiol.,* **4**:1603-1605.

Fitzpatrick, J., Fanning, L., Hearty, S., Leonard, P., Manning, B.M., Quinn, J.G. and O'Kennedy R. (2000). Applications and recent developments in the use of antibodies for analysis. *Anal. Lett.,* **33**:263-2609.

Fitzpatrick, J., Manning, B.M. and O'Kennedy, R. (2003). Development of ELISA and sensor-based assays for the detection of ethynyl estradiol in bile. *Food Agric. Immunol.,* **15**:55-64.

Fleming, D.W., Cochi, S.L., MacDonald, K.L., Brondum, J., Hayes, P. S., Plikaytis, B. D., Holmes, M.B., Audurier, A., Broome, C.V., Reingold, A. L. (1985). Pasteurised milk as a vehicle of infection in an outbreak of Listeriosis. *New Engl. J. Med.,* **312**;7:404-407.

Fratamico, P.M., Strobaugh, T.P., Medina, M.B. and Gehring, A.G. (1998). Detection of *Escherichia coli* O157:H7 using a surface plasmon resonance biosensor. *Biotechnol. Tech.,* **12**:571-576.

Gaillard, J-L., Berche, P., frehel, C., Gouin, E., Cossart, P. (1991). Entry of *Listeria monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of the surface antigens from Gram-positive cocci. *Cell,* **65**:1127-41.

Geginat, G., Nichterlein, T., Kretschmar, M., Schenk, S., Hof, H., Lalic-Multhaler, M., Goebel, W. and Bubert, A. (1999). Enhancement of the *Listeria monocytogenes* p60-specific CD4 and CD8 T-cell memory by non-pathogenic *Listeria innocua. J. Immunol.,* **162**:4781-4789.

Gillis, E.H., Gosling, J.P., Sreenan, J.M. and Kane, M. (2002). Development and validation of a biosensor-based immunoassay for progesterone in bovine milk. *J. Immunol. Methods,* **267**:131-138.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couve, E., de Daruvar, A., Dehoux, P., Domann, E., Dominguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Entian, K.D., Fsihi, H., Portillo, F.G., Garrido, P., Gautier, L., Goebel, W., Gomez-Lopez, N., Hain, T., Hauf, J., Jackson, D., Jones, L.M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueno, E., Maitournam, A., Vicente, J.M., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Perez-Diaz, J.C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vazquez-Boland, J.A., Voss, H., Wehland, J., Cossart, P. (2001). Comparative genomics of Listeria species. *Science,* **294**:849-852.

Glockshuber, R., Malia, M., Pfitzinger, I. and Plückthun, A. (1990). A comparison of strategies to stabilise immunoglobulin Fv-fragments. *Biochemistry,* **29**:1362-1367.

Goepel, W. (1991). Chemical sensing, molecular electronics and nanotechnology: interface technologies down to the molecular scale. *Sensor. Actuat. B,* **4**:7-21

Goepel, W. and Heiduschka, P. (1995). Interface analysis in biosensor design. *Biosens. Bioelectron.,* **10:**853-883.

Goldberg, M.B. (2001). Actin-based motility of intracellular microbial pathogens. *Microbiol. Mol. Biol. Rev.,* **65**:595-626.

Goldfine, H. and Wadsworth, S.J., (2002). Macrophage intercellular signalling induced by *Listeria monocytogenes. Microb. Infect.*, **4**:1335-1343.

Goldman, E.R., Pazirandeh, M.P., Mauro, J.M., King, K.D., Frey, J.C. and Anderson, G.P. (2000). Peptide-displayed peptides as biosensor reagents. *J. Mol. Recognition*, **13**:382-87.

Goldschmidt-Clermont, P.J., Furman, M.I., Wachsstock, D., Safer, D., Nachmias, V.T., Pollard, J.M (1992). The control of actin nucleotide exchange by thymosin beta-4 and prfilin: a potential regulatory mechanism for actin polymerisation in cells. *Mol. Biol. Cell.*, **3**:1015-1024.

Golsteyn Thomas, E.J., King, R.K., Burchak, J. and Gannon, V.P.J. (1991). Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef using the polymerase chain reaction (PCR). *Appl. Enviro. Micro.*, **57**:2576-2580.

Goodridge, L. and Griffiths, M. (2002). Reporter bacteriophage assays as a means to detect foodborne pathogenic bacteria. *Food Res. Int.*, **35**:863-870.

Haines, J. and Patel, P.D. (1995). Detection of food borne pathogens using BIA. *Biacore AB Biotechnology note*, p. 31.

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

Hannig, G. and Makrides, S.C. (1998). Strategies for optimising heterologous protein expression in *Escherichia coli. Trends Biotechnol.*, **16**:54-60.

Harvey, J., and Gilmour, A. (1992). Occurrence of *Listeria* in raw milk in Ireland. *J. Appl. Bacteriol.*, **72**:119-125.

Hayhurst, A. and Harris, W. J. (1999). *Escherichia coli* skp chaperone co-expression improves solubility and phage display of single-chain antibody fragments. *Protein Expression and Purification*, **15**:336-343.

Hayhurst, A., Happe, S., Mabry, R., Zwphyr, K., Iverson, B. L. and Georgiou, G. (2003). Isolation and expression of recombinant antibody fragments to the biological warfare pathogen *Brucella melitensis*. *J. Immunol. Methods*, **276**:185-196.

Hein, I., Klein, D., Lehner, A., Bubert, A., Brandl, E. and Wagner, M. (2001). Detection and quantification of the *iap* gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. *Res. Microbiol.*, **152**:37-46.

Heinzen, R.A., Hayes, S.F., Peacock, M.G., Hackstadt., T. (1993). Directional Actin Polymerisation associated with spotted fever group Rickettsia infection of Vero cells. *Infect. Immunity*, **61**:1926-1935.

Hermiston, M.L. and Gordon, J.I. (1995). *In vivo* analysis of cadherin function in mouse small intestinal enterocytes: essential role in adhesion, maintenance of differentiation and regulation of programmed cell death. *J. Cell. Biol.*, **129**:489-506.

Hess, J., Gentshev, I., Szalah, G., Ladel, C., Bubert, A., Goebel, W. and Kaufmann, S. (1995). *Listeria monocytogenes* p60 supports host cell invasion by and *in vivo* survival of attenuated *Salmonella typhimurium*. *Infect. Immunity*, **63**:2047-2053.

Hof, H. (2003). History and epidemiology of listeriosis. *FEMS Immunol. Medical Microbiol.*, **1489**:1-4.

Holliger, P. and Winter, G. (1993a). Engineering bispecific antibodies. *Curr. Opin. Biotechnol.*, **4**:446-449.

Holliger, P., Prospero, T. and Winter, G. (1993b). "Diabodies": small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. (U.S.A.)*, **90**:6444-6448.

Hollingsworth, P. (1999). A proactive approach to safety issues. *Food Technol.*, **53**:26.

Homola, J., Yee, S.S. and Gauglite, G. (1999). Surface plasmon resonance sensors: review. *Sensor. Actuat. B*, **54**:3-15.

Hoogenboom, H.R.; de Bruïne, A.P.; Hufton, S.E.; Hoet, R.N.; Arends, J-W.; Roovers, R.C. (1998). Antibody phage display technology and its applications. *Immunotechnology*, **4**:1-20.

Hsih, H-Y. and Tsen, H-Y. (2001). Combination of Immunomagnetic separation and polymerase chain reaction for the simultaneous Detection of *Listeria monocytogenes* and salmonella spp. in food samples. *J. Food Prot.,* 64(11):1744-1750.

Hudson, L. and Hay, F.C. (1989). In *Practical Immunology*. 3rd ed., Blackwell Scientific Publications, UK.

Hudson, J.A., Lake, R.J., Savill, M.G., Scholes, P. and McCormick, R.E. (2001). Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic separation followed by polymerase chain reaction. *J. Applied Microbiol.,* 90:614-621.

Hudson, P.J. (1998). Recombinant antibody fragments. *Curr. Opin. Biotechnol.,* **9**:395-402.

Huston, J.S., Leuinson, D., Mudgett, H.M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. and Oppermann, H. (1988). Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli. Proc. Natl. Acad. Sci. (U.S.A.),* **85**:5879-5883.

Ingianni, A., Floris, M., Palomba, P., Madeddu, M.A., Quartuccio, M. and Pompei, R. (2001). Rapid detection of *Listeria monocytogenes* in foods, by a combination of PCR and DNA probe. *Mol. Cellular Probes,* **15**:275-280.

Inoue, H., Nojima, H. and Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, **96**:23-28.

Invitski, D., Abdel-Hamid, I., Atanasov, P. and Wilkins, E. (1999). Biosensors for the detection of pathogenic bacteria. *Biosens. Bioelectron.,* **14**:599-624.

Iqbal, S.S., Mayo, M.W., Bruno, J.G., Bronk, B.V., Batt, C.A., Chambers, P. (2000) A review of molecular recognition technologies for detection of biological threat agents. *Biosens. Bioelectron.,* **15**:549-578.

Ireton, K., and Cossart, P. (1997). Host-Pathogen interactions during entry and actin based movement of *Listeria monocytogenes. Ann. Rev. Genet.,* **31**:113-138.

Jakes, K.S., Davis, G.D., and Zinder, N.D. (1988). A hybrid toxin from bacteriophage f1 attachment protein and colicin E3 has altered cell receptor specificity. *J. Bacteriol.*, **170**:4231-4238.

Janeway, C.A., Jr., Travers, P., Walport, M. and Capra, J. (1999). The recognition of antigen, p79-113, in *Immunobiology: the immune system in health and disease,* 4th edition, Austin, P. and Lawrence, E. (eds.). Garland Publishing, New York, USA.

Jensen, A., Frederiksen. W., and Gerner-Smidt, P. (1994). Risk factors for Listeriosis in Denmark, 1989-1990. *Scand. J. Infect. Dis.*, **26**:171-178.

Johns, M., George, A. J. T. and Ritter, M. A. (2000). *In vivo* selection of sFv from phage display libraries. *J. Immunol. Methods*, **239**:137-151.

Johnson, K. S. and Hawkins, R. E. (1996) Affinity maturation of antibodies using phage display, p41-58, in *Antibody Engineering*, McCafferty, J., Hoogenboom, H.R. and Chiswell, D.J. (eds.), A practical approach series, Oxford University Press, Oxford, UK.

Johnson, W.M, Tyler, S.D., Ewan, E.P., Ashton, F.E., Wang, G., Rozee, K.R. (1992). detection of genes coding for listeriolysin and *Listeria monocytogenes* antigen A (LmaA) in Listeria spp. by the polymerase chain reaction. *Microb. Pathogenesis,* **12**;79-86.

Jones, D., and Seeliger, H. (1992). The genus Listeria, p1595-1616, in *The Prokaryotes* 2nd Ed. Balows, C.A., Truper, H.G., Dworkin, M., Harder, W. and Schleur, K-H. (eds.), Springer Verlag, Heidelberg, Germany.

Jönsson, U., Fägerstam, L., Ivarsson, B., Lundh, K., Löfås, S., Persson, B., Roos, H., Rönnberg, I., Sjölander, S., Stenberg, E., Ståhlberg, R., Urbaniczky, C., Östlin, H., Malmqvist, M., (1991). Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *Biotechniques,* **11**:620-627.

Jonquieres, R., Bierne, H., Fiedler, F., Gounon, P. and Cossart, P. (1999). Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of gram-positive bacteria. *Mol. Microbiol.,* **34**:902-914.

Jonquieres, R., Pizarro-Cerda, J. and Cossart, P. (2001). Synergy between the N- and C-terminal domains of InlB for efficient invasion of non-phagocytic cells by *Listeria monocytogenes*. *Mol. Microbiol.*, **42**:955-965.

Jung, S., Arndt, K.M., Müller, K.M. and Plückthun, A. (1999). Selectively infective phage (SIP) technology: scope and limitations. *J. Immunol. Methods,* **231**:93-104.

Jung, Y.S., Frank, J.F., Brackett, R.E., and Chen, J. (2003). Polymerase chain reaction detection of *Listeria monocytogenes* on frankfurters using oligonucleotide primers targeting the genes encoding Internalin AB. *J. Food Protection,* **66**:237-241.

Kabat, E.A., Wu, T.T., Reid-Miller, M., Perry, H.M., Gottesman, K.S. and Foeller, C. (1991), in *Sequences of proteins of immunological interest*, 5th Ed., US Department of Health and Human Services, Public Services, NIH, Washington, USA.

Kaclikova, E., Pangallo, D., Drahovska, H., Oravcova, K. and Kuchta, T. (2003). Detection of *Listeria monocytogenes* in food, equivalent to EN ISO 11290 or ISO 10560, by a three-days polymerase chain reaction-based method. *Food Control,* **14**:175-179.

Karlsson, R., Michaelsson, A. and Mattsson, L. (1991). Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor-based analytical system. *J. Immunol. Methods,* **145**:229-240.

Kathariou, S., Metz, P., Hof, H., Goebel, W. (1987). Tn916 induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. *J. Bacteriol.*, **169**:1291-1297.

Kettleborough, C.A., Saldanha, J., Ansell, K.H. and Bendig, M.M. (1993). Optimisation of primer for cloning libraries of mouse immunoglobulin genes using the ploymerase chain reaction. *Eur. J. Immunol.*, **23**:206-211.

Knocks, C., Hellio, R., Gounon, P., Ohayon, H., and Cossart, P. (1993). Polarized distribution of *Listeria monocytogenes* surface protein ActA at site of directional actin assembly. *J. Cell Sci.*, **105**:521-531.

Kobe, B. and Kajava, A.V. (2001). The leucine-rich repeat as a protein recognition motif. *Curr. Opin. Struc. Biol.*, **11**:725-732.

Köhler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.),* **256**:495-497.

Köhler, G. and Milstein, C. (1976). Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.*, **6**:511-519.

Koide, A., Bailey, C.W., Huang, X. and Koide, S. (1998). The fibronectin type III domain as a scaffold for novel binding proteins. *J. Molec. Biol.*, **284**:1141-1151.

Kolb-Maurer, A., Pilgrim, S., Kampgen, E., McLellan, A.D., Brocker, E-B, Goebel, W. and Gentschev, I. (2001). Antibodies against Listerial protein 60 act as an opsonin for phagocytosis of *Listeria monocytogenes* by human dendritic cells. *Infect. Immunity,* **69**:3100-3109.

Koubova, V., Brynda, E., Karasova, L., Skvor, J., Homola, J., Dostalek, J., Tobiska, P. and Rosicky, J. (2001). Detection of foodborne pathogens using surface plasmon resonance biosensors. *Sens. Actuat. B,* **74**:100-105.

Krebber, A.; Bornhauser, S.; Burmester, J.; Honegger, A.; Willuda, J.; Bosshard, H.R.; Plückthun, A. (1997a). Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a re-engineered phage display system. *J. Immunol. Methods*, **201**:35-55.

Krebber, C., Spada, S., Desplancq, D., Krebber, A., Ge, L. and Plückthun, A. (1997b). Selectively-infective phage (SIP): a mechanistic dissection of a novel *in vivo* selection for protein-ligand interactions. *J. Molec. Biol.*, **268**:607-618.

Kreft, J., Vazquez-Boland, J.A., Altrock, S., Dominguez-Bernal, G. and Goebel, W. (2002). Pathogenicity islands and other virulence elements in *Listeria. Curr. Top. Microbiol. Immunol.*, **264**:109-125.

Kretschmann, E. (1971). The determination of the optical constants of metals by excitation of surface plasmons resonance. *Z. Phys.*, **241**:313-324.

Kuhn, M. and Goebel, W. (1989). Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. *Infect. Immunity,* **57**:55-61.

Larue, L., Ohsugi, M., Hirchenhain, J. and Kemler, R. (1994). E-cadherin null mutant embryos fail to form a trophoectoderm epithelium. *Proc. Natl. Acad. Sci. (USA),* **91**:8263-8267.

Lasa, I., Gouin, E., Goethal, S., Vancompernolle, K., David, V., Vandekerckhave, J., Cossart, P. (1997). Identification of two regions in the N-terminal domain of actA involved in the actin comet tail formation by *Listeria monocytogenes. EMBO J.,* **16**:1531-40.

Lathrop, A. A., Jaradat, Z. W., Haley, T. and Bhunia, A. K. (2003). Characterisation and application of a *Listeria monocytogenes* reactive monoclonal antibody C11E9 in a resonant mirror biosensor. *J. Immunol. Methods,* 281:119-128.

Laurent, V., Loisel, T.P., Harbeck, B, Wehman, A., Grobe, L., Jockusch, B.M., Wehland, J., gertler, F.B., and Carlier, M.F. (1999). Role of protein of the Ena/VASP family in actin-based motility of *Listeria monocytogenes. J. Cell Biol.*, **144**:1245-1258.

Lecuit, M., Dramsi, S., Gottardi, C., Fedor-Chaiken, M., Gumbiner, B. and Cossart, P. (1999). A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes. EMBO J.,* **18**:3956-3963.

Lecuit, M., Ohayon, H., Braun, L., Mengaud, J. and Cossart, P. (1997). Internalin of *Listeria monocytogenes* with an Intact leucine-rich repeat region is sufficient to promote internalisation. *Infect. Immunity,* **65**:5309-5319.

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., Babinet, C. and Cossart, P.. (2001). Transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science,* **292**:1722-1725.

Leonard, P., Hearty, S., Brennan, J., Dunne, L., Quinn, J., Chakraborty, T. and O'Kennedy, R. (2003). Advances in biosensors for the detection of pathogens in food and water. *Enzyme Microb. Tech.,* **32**:3-13.

Li, X., Boudjellab, N. and Zhao, X. (2000). Combined PCR and slot blot assay for the detection of *Salmonella* and *Listeria monocytogenes. Int. J. Food Microbiol.,* 56:167-177.

Liedberg, B., Lundström, I. and Stenberg, E. (1993). Principles of biosensing with an extended coupling matrix and surface plasmon resonance. *Sens. Actuat. B,* **11**:63-72.

Liedberg, B., Nylander, C. and Lundström, I. (1983). Surface plasmons resonance for gas detection and biosensing. *Sens. Actuat.,* **4**;299-304.

Liedberg, B., Nylander, C. and Lundström, I. (1995). Biosensing with surface plasmon resonance- how it all started. *Biosens. Bioelectron.,* **10**:i-ix.

Little, M., Kipriyanov, S.M., Le Gall, F. and Moldenhauer, G. (2000). Of mice and men: hybridoma and recombinant antibodies. *Immunol. Today,* **21**:364-370.

Liu, J., Lusha, L. and Jemmerson, R. (2000). Immunoglobulin gene joints compensate for reduced on-rates imposed by somatic mutation in a V_H gene. *Mol. Immunol.,* **37**:95-105.

Longhi, C., Maffeo, A., Penta, M., Petrone, G., Seganti, L. and Conte, M.P. (2003). Detection of *Listeria monocytogenes* in Italian-style soft cheeses. *J. App. Microbiol.,* **94**:879-885.

Machesky, L. and May, R.C. (2001). Phagocytosis and the actin cytoskeleton. *J. Cell Sci.,* **114**:1061-1077.

Mackaness, G.B. (1962). Cellular resistance to infection. *J. Exp. Med.,* **116**:380-406.

Maddock, J.R., Alley, M.R.K., and Shapiro, L. (1993). Polarised Cells, Polar Actions. *J. Bacteriol.,* **175**:7125-7129.

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

Marks, J., S., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D. and Winter, G. (1991). By-passing Immunisation: Human antibodies form V-gene libraries on phage. *J. Molec. Biol.*, **222**:581-597.

Matsuura, T. and Plückthun, A. (2003). Selection based on the folding properties of proteins with ribosome display. *FEBS Lett.*, **539**:24-28.

Mattheakis, L.C., Bhatt, R.R. and Dower, W.J. (1994). An *in vitro* polysome display system for identifying ligands from very large peptide libraries. *Proc. Natl. Acad. Sci. (U.S.A.),* **91**:9022-9026.

Mayforth, R.D. (1993). Antibody structure, p 4-23, in *Designing Antibodies*, Academic Press Ltd., London, UK.

Maynard, J. and Georgiou, G. (2000). Antibody engineering. *Ann. Rev. Biomed. Eng.*, **2**:339-376.

McLauchlin, J. Hall, S.M., Veloni, S.K, Gilbert, R.J. (1991). Human listeriosis and paté-a possible association. *Brit. Med. J.,* **303**:773-775.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R-M., Cossart, P. (1996a). E-cadherin is the receptor for internalin, a surface protein required for entry of *Listeria monocytogenes* into epithelial cells. *Infect. Immunity,* **65**:1615-1625.

Mengaud, J., Lecuit, M., Lebrun, M., Nato, F., Mazie, J-C. and Cossart, P. (1996b). Antibodies to the leucine-Rich Repeat Region of Internalin block entry of *Listeria monocytogenes* into cells expressing E-cadherin. *Infect. Immunity,* **64**:5430-5433.

Merz, A.J. and Higgs, H. N. (2003). *Listeria* motility: Biophysics pushes things forward. *Curr. Biol.,* **13**:302-304.

Minett, A.I., Barisci, J.N. and Wallace, G.G. (2003). Coupling conducting polymers and mediated electrochemical responses for the detection of *Listeria. Analytica Chimica Acta,* 475:37-45.

Minunni, M., Mascini, M, Carter, R.M., Jacobs, M.B., Lubrano, G.J. and Guilbault, G.G. (1996). A quartz crystal microbalance displacement assay for *Listeria monocytogenes. Anlaytica Chimica Acta,* 325:169-174.

Mitchell, BA, Milbury, J.A, Brookins, A.M, Jackson, B.J. (1994). Use of immunomagnetic capture on beads to recover Listeria from environmental samples. *J. Food Protection,* **57**:743-745.

Müller, K.M., Arndt, K.M., Bauer, K. and Plückthun, A. (1998a). Tandem Immobilised Metal-Ion Affinity Chromatography/Immunoaffinity Purification of His-tagged Proteins; Evaluation of two anti-His tag monoclonal antibodies. *Anal. Biochem.,* **259**:54-61.

Müller, S., Hain, T., Pashalidis, P., Lingnau, A., Domann, E., Chakraborty, T. and Wehland, J. (1998b). Purification of the InlB gene product of *Listeria monocytogenes* and demonstration of its biological activity. *Infect. Immunity*, **66**:3128-3133.

Murray, E.G.D., Webb, R.A. and Swann, H.B.R. (1926). A disease of rabbits characterized by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n. sp.). *J. Pathol. Bacteriol.*, **29**:407-439.

Murray, P.R., Rosenthal, K.S., Kobayashi, G. and Pfaller, M.A. (1998). Bacteriology, p152-371, in *Medical Microbiology*, 3rd Edition, Brown, M. (ed,), Elsevier, Mosby, St. Louis, USA.

Navarre, W.W. and Schneewind, O. (1999). Surface proteins of Gram positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol. Mol. Biol. Rev.*, **63**:174-229.

Nieba, L., Nieba-Axmann, S.E., Persson, A., Hamalainen, M., Edebratt, F., Hansson, A., Lidholm, J., Magnusson, K., Karlsson, A.F. and Plückthun, A. (1997). BIAcore analysis of histidine-tagged proteins using a chelating NTA sensor chip. *Anal. Biochem.*, **252**:217-228.

Nielsen, U.B. and Marks, J. D. (2001). Affinity Maturation by Chain Shuffling and Site Directed Mutagenesis, p515-539, in *Antibody Engineering*, Kontermann, R. and Dübel, S. (eds.), Springer Lab Manual, Springer-Verlag, Berlin/Heidelberg, Germany.

Nieman, R.E., and Lober, B. (1980). Listeriosis in adults: a changing pattern: report of eight cases and a review of literature, 1968-1978. *Rev. infect. Dis.*, **2**:207-227.

Nilsson, F., Tarli, L., Viti, F. and Neri, D. (2000) The use of phage display for the development of tumour targeting agents. *Adv. Drug Deliver. Rev.*, **43**:165-196.

Nolan, O. and O'Kennedy, R. (1990). Bifunctional antibodies: concept, production and applications. *Biochim. Biophys. Acta- Protein Struct. Molec. Enzymol.*, **1040**:1-11.

Norton, D-M. and Batt, C.A. (1999). Detection of viable *Listeria monocytogenes* with a 5' Nuclease PCR assay. *App. Environ. Microbiol.*, **65**:2122-2127.

Notermans, S.H.W., Dufrenne, J., Leimeister-Wachler, M., Domann, E., Chakraborty, T. (1991). Phosphatidylinositol-specific Phospholipase C activity as a marker to distinguish between pathogenic and non-pathogenic Listeria species. *Appl. Environ. Micro.*, 57;2666-2670.

Novy, R. and Morris, B. (2001). Use of glucose to control basal expression in the pET System, in *inNovations: advanced products and protocols for molecular biology research,* newsletter of Novagen, no 13, Novagen Inc., a brand of CN Biosciences Ltd, Nottingham, UK.

Nylander, C., Leidberg, B. and Lind, T. (1982). Gas detection by means of surface plasmons resonance. *Sens. Actuat.*, **3**;79-88.

O'Connor, L., Joy, J., Kane, M., Smith, T. and Maher, M. (2000). Rapid polymerase chain reaction/DNA probe membrane-based assay for the detection of Listeria and Listeria monocytogenes in food. *J. Food Prot.*, 63(3):337-342.

O'Shannessy, D.J., Brigham-Burke, M., Soneson, K.K., Hensley, P. and Brooks, I. (1993). Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: use of nonlinear least squares analysis methods. *Anal. Biochem.*, **212**:457-468.

Ohlin, M., Owman, H., Mach, M. and Borrebaeck, C.A.K. (1996). Light chain shuffling of a high affinity antibody results in a drift in epitope recognition. *Mol. Immunol.*, **33**:47-56.

Ørum, H., Andersen, P.S. Øster, A., Johansen, L.K., Riise, E., Bjørnvad, M., Svendsen, I. and Engberg, J. (1993). Efficient method for constructing comprehensive murine Fab antibody libraries displayed on phage. *Nucleic Acids Res.*, **21**:4491-4498.

Pangallo, D., Kaclikova, E., Kuchta, T. and Drahovska, H. (2001). Detection of *Listeria monocytogenes by polymerase chain reaction oriented to inlB Gene. Microbiologica,* 24:333-339.

Parida, S.K., Domann, E., Rohde, M., Muller, S., Darji, A., Hain, T., Wehland, J. and Chakraborty, T. (1998). Internalin B is essential for adhesion and mediates the invasion of *Listeria monocytogenes* into human endothelial cells. *Mol. Microbiol.,* **28**:81-93.

Park, S. G., Lee, J. S., Je, E. Y., Kim, I. J., Chung, J. H. and Choi, I. H. (2000a). Affinity maturation of natural antibody using a chain shuffling technique and the expression of recombinant antibodies in *Escherichia coli. Biochem. Biophs. Res. Communs.*, **275**:553-557.

Park, J.-H., Lee, Y.-S., Lim, Y.-K., Kwon, S.-H., Lee, C.-U. and Yoon, B.-S. (2000b). Specific binding of recombinant *Listeria monocytogenes* p60 protein to caco-2 cells. *FEMS Microbiol. Lett.,* **186**:35-40.

Peake, R.C., James, D.A., Susman, M., Kennedy, C.A., Singleton, M.J.D and Schuenke, S. (1996). Bacteriology, in *Medical Microbiology*, 4th Ed., Baron, S. (ed.), The University of Texas Medical Branch at Galveston, Galveston, USA.

Perkins, E.A. and Squirrell, D.J. (2000). Development of instrumentation to allow the detection of microorganisms using light scattering in combination with surface plasmon resonance. *Biosens. Bioelectron.*, **14**:853-859.

Pini, A., Spreafico, A., Botti, R., Neri, D. and Neri, P. (1997). Hierarchical affinity maturation of a phage library derived antibody for the selective removal of cytomegalovirus from plasma. *J. Immunol. Methods*, **206**:171-182.

Pistor, S., Chakraborty, T., Walter, O., Wehland, J., (1995). The bacterial nucleator protein ActA of *Listeria monocytogenes* contains multiply binding sites for host microfilament proteins. *Curr. Biol.*, **5**:517-25

Pizarro-Cerda, J., Jonquieres, R., Gouin, E., Vandekerckhove, J., Garin, J. and Cossart, P. (2002). Distinct protein patterns associated with *Listeria monocytogenes* InlA- or InlB-phagosomes. *Cellular Microbiol.*, **4**:101-115.

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

Plückthun, A., Krebber, A., Krebber, C., Horn, U., Knupfer, U., Wenderoth, R. Nieba, L., Proba, K. and Riesenberg, D. (1991). Producing antibodies in *Escherichia coli*: from PCR to fermentation, p203-249, in *Antibody Engineering-A practical approach*, edited by McCafferty, J., Hoogenboom, H.R. and Chiswell, D.J. The practical approach series, Oxford University Press, Oxford, UK.

QIAgen Ltd. (2000). In *The QIA Expressionist: A handbook for the high-level expression and purification of 6 His-tagged proteins*, QIAgen Ltd., West Sussex, UK.

Quinn, J.G. and O'Kennedy, R. (1999). Transduction platforms and biointerfacial design of biosensors for "real-time" biomolecular interaction analysis. *Anal. Lett.,* **32**:253-258.

Rasooly, A. (2001). Surface plasmon resonance analysis of Staphylococcal Enterotoxin B in food. *J. Food Protection,* **64**:37-43.

Rea, M.C., Cogan, T.M., Tobin, T. (1992). Incidence of pathogenic bacteria in raw milk in the Ireland. *J. Appl. Bacteriol.,* **73**:331-336.

Rich, R.L. and Myszka, D.G. (2000). Advances in surface plasmon resonance biosensor analysis. *Curr. Opin. Biotechnol.,* **11**:54-61.

Robbins, J.R., Barth, A.I., Marquis, H., deHostos, E.L., Nelson, W.J., and Theriot, J.A. (1999). *Listeria monocytogenes* exploits normal host cell processes to spread from cell to cell. *J. Cell Biol.,* **146**:1333-1349.

Roberts, P. (1994). An improved cultural/immunoassay for the detection of *Listeria* species in foods and environmental samples. *Microbiol. Eur.,* Sept/Oct:18-21.

Roberts, R.W. and Szostak, J.W. (1997). RNA-peptide fusions for the *in vitro* selection of peptides and proteins. *Proc. Natl. Acad. Sci. (U.S.A.),* **94**:12297-12302.

Rowan, N.J., Candlish, A.A.G., Bubert, A., Anderson, J.G., Kramer, K. and McLauchlin, J. (2000). Virulent rough filaments of *Listeria monocytogenes* from clinical and food

samples secreting wild-type levels of cell-free p60 protein. *J. Clin. Microbiol.*, **38**:2643-2648.

Sanger, J.M., Sanger, J.W., and Southwick, F.S. (1992). Host Cell actin assembly is necessary and likely to provide the propulsive force for intracellular movement of *Listeria monocytogenes. Infect. Immunity,* **60**:3609-3619.

Schier, R., Bye, J., Apell, G., McCall, A., Adams, G.P., Malmqvist, M., Weiner, L.M. and Marks, J.D. (1996a). Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. *J. Molec. Biol.*, **255**:28-43.

Schier, R., McCall, A., Adams, G.P., Marshall, K.W., Merritt, H., Yim, M., Crawford, R.S., Weiner, L.M. and Marks, J.D. (1996b). Isolation of picomolar affinity anti-c-erbB-2 single chain Fv by molecular evolution of the complementarity determining regions in the centre of the antibody binding site. *J. Molec. Biol.*, **263**:551-567.

Schlech, W.F. III (2000). Foodborne Listeriosis. *Clin. Infect. Dis.*, **31**:770-775.

Schlsch, W.F., III (1997). Listeria gastroenteritis-Old syndrome, New Pathogen. *New Engl. J. Med.*, **336**:130-131.

Schmid, M., Walcher, M., Burbert, A., Wagner, M., Wagner, M. and Schleifer, K-H. (2003). Nucleic acid-based, cultivation-independent detection of *Listeria* spp. and genotypes of *L. monocytogenes. FEMS Immunol. Med. Microbiol.,* **35**:215-225.

Schneewind, O., Fowler, A., and Faull, K.F. (1995). Structure of cell wall anchor of surface proteins in *Staphylococcus aureus. Science,* **268**:103-106.

Schubert, W-D., Gobel, G., Diepholz, M., Darji, A., Kloer, D., Hain, T., Chakraborty, T., Wehland, J., Domann, E. and Heinz, D.W. (2001). Internalins from the human pathogen *Listeria monocytogenes* combine three distinct folds into a contiguous Internalin domain. *J. Molec. Biol.*, **312**:783-794.

Scotter, S.L., Langton, S., Lombard, B., Schulten, S., Nagelkerke, N., In't Veld, P.H., Rollier, P. and Lahellec, C. (2001a). Validation of ISO method 11290 Part 1-Detection of *Listeria monocytogenes* in foods. *Int. J. Food Microbiol.*, **64**:295-306.

Scotter, S.L., Langton, S., Lombard, Lahellec, C., B., Schulten, S., Nagelkerke, N., In't Veld, P.H. and Rollier, P. (2001b). Validation of ISO method 11290 Part 2-Enumeration of *Listeria monocytogenes* in foods. *Int. J. Food Microbiol.,* **70**:121-129.

Sewell, A.M., Warburton, D.W., Boville, A., Daley, E.F. and Mullen, D.K. (2003). The development of an efficient and rapid enzyme-linked fluorescent assay method for the detection of *Listeria* spp. from foods. *Int. J. Food. Microbiol.,* **81**:123-129.

Seyhi, R.S. (1994). Transducer aspects of biosensors. *Biosens. Bioelectron.*, **9**:243-264.

Shantha Raju, T. (2003). Glycosylation variations with expression systems. *Bioprocess International,* **1**:44-53.

Shen, Y., Naujokas, M., Park, M. and Ireton, K. (2000). InlB-dependent internalisation of *Listeria* is mediated by the Met receptor tyrosine kinase. *Cell,* **102**:501-510.

Silva, I.M.M, Almeida, R.C.C., Alves, M.A.O. and Almeida, P.F. (2003). Occurrence of *Listeria* spp. in critical control points and the environment of Minas Frescal cheese processing. *Int. J. Food Microbiol.,* **81**:241-248.

Smith, G.P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, **228**:1315-1317.

Southwick, F.S., and Purich, D.L., (1996). Intracellular pathogenesis of listeriosis. *New Engl. J. Med.,* **334**:770-775.

Spada, S., Krebber, C. and Plückthun, A. (1997). Selectively Infective Phages (SIP). *J. Biol. Chem.,* **378**:445-456.

Stapleton, S., Tully, E. and O'Kennedy, R. (*In press*). Immunoassays: the production of antibodies, in *Encyclopedia of Analytical Science,* 2nd Edition, Townsend, A. (ed). Academic Press , New York, USA.

Stenberg, E., Persson, B., Roos, H. and Urbaniczky, C. (1991). Quantitative determination of surface concentration of protein with surface plasmon resonance using radiolabeled proteins. *J. Colloid. Interf. Sci.,* **143**:513-526.

Stengele, I., Bross, P., Garces, X., Giray, J., and Rasched, I. (1990). Dissection of functional domains in phage fd adsorption protein. *J. Molec. Biol.*, **212**:143-149.

Susmel, S., Guilbault, G.G. and O'Sullivan, C.K. (2003). Demonstration of labeless detection of food pathogens using electrochemical redox probe and screen printed gold electrodes. *Biosens. Bioelectron.*, 18:881-889.

Takeichi, M. (1990). Cadherins: a molecular family important in selective cell-cell adhesion. *Ann. Rev. Biochem.*, **59**:237-252.

Takkinen, K., Hemminki, A. and Söderlund, H. (2001) Affinity and specificity maturation by CDR walking, p540-546, in *Antibody Engineering*, Kontermann, R. and Dübel, S. (eds.), Springer Lab Manual, Springer-Verlag, Berlin/Heidelberg, Germany.

Theriot, J.A., Mitchison, T.J., Tilney, L.G., and Portnoy, D.A (1992). The rate of actin based motilty of intracellular *Listeria monocytogenes* equals the rate of actin polymerisation. *Nature (Lond.),* **357**:257-260.

Thompson, J.E., Vaughan, T.J., Williams, A.J., Wilton, J., Johnson, K.S., Bacon, L., Green, J.A., Field, R., Ruddock, S, Martins, S., Pope, A.R., Tempest, P.R., Jackson, R.H. (1999). A fully human antibody neutralising biologically active human TGFbeta2 for use in therapy. *J. Immunol. Methods*, **227**:17-29.

Todorovska, A., Roovers, R. C., Dolezal, O., Kortt, A. A., Hoogenboom, H. R. and Hudson, P. J. (2001). Design and applications of diabodies, triabodies and tetrabodies for cancer targeting. *J. Immunol. Methods*, **248**:47-66.

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

Ueda, E.K.M., Gout, P.W. and Morganti, L. (2003). Current and prospective applications of metal ion-protein binding. *J. Chromatogr. A*, **988**:1-23.

Vaughan, R.D., O'Sullivan, C.K. and Guilbault, G.G. (2001). Development of a quartz crystal microbalance (QCM) immunosensor for the detection of *Listeria monocytogenes. Enzyme Microbiol. Technol.*, 29:635-638.

Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J. and Johnson, K. S. (1996). Human antibodies with sub-nanomolar affinities isolated form a large non-immunised phage display library. *Nature Biotechnol.*, **14**:309-314.

Vázquez-Boland, J. A., Domínguez-Bernal, G., González-Zorn, B., Kreft, J. and Goebel, W. (2001). Pathogenicity islands and virulence evolution in *Listeria. Microbes Infect.*, **3**:571-584.

Vega-Mercado, H., Dekleva, M., Sharnez, R. and Baez, L. (2003). HACCP: A process validation tool for ensuring quality of Biotech and Pharmaceutical products. *Bioprocess International,* **1**(5):50-57.

Vlaemynck, G.M. and Moermans, R (1996). Comparison of EB and Fraser enrichment broths for the detection of *Listeria monocytogenes* in raw-milk dairy products and environmental samples. *J. Food, Protection,* **59**:1172-1175.

Volokhov, D., Rasooly, A., Chumakov, K and Chizhikov, V. (2002). Identification of *Listeria* species by microarray-based assay. *J. Clin. Microbiol.,* **40**:4720-4728.

Welch, M.D., Mallavarpu, A., Rosenblatt, J., Mitchison, T.J. (1997). Actin dynamics *in vivo*. *Curr. Opin. Cell Biol.,* **1**:54-61.

Welch, M.D., Rosenblatt, J., Skoble, J., Portnoy, D.A., Mitchison, T.J. (1998). Interaction of human Arp2/3 complex and the *Listeria monocytogenes* ActA protein in actin filament nucleation. *Science,* **281**:105-108.

Wilson, I.G, (1995). Occurrence of Listeria species in ready to eat foods. *Epidemiol Infect.*, **115**:519-26.

Wong, R.L., Mytych, D., Jacobs, S., Bordens, R. and Swanson, S. (1997). Validation parameters for a novel biosensor assay which simultaneously measures serum concentrations of a humanised monoclonal antibody and detects induced antibodies. *J. Immunol. Methods,* **209**:1-15.

Wörn, A. and Plückthun, A. (1999). Different equilibrium stability behaviour of scFv fragments: identification, classification, and improvement by protein engineering. *Biochemistry*, **38**:8739-8750.

Wörn, A. and Plückthun, A. (2001). Stability engineering of antibody single-chain Fv fragments. *J. Molec. Biol.*, **305**:989-1010.

Wyatt, G.M. (1999). Overview of Listeria. *Inco Copernicus News* issue 1;p2.

Xu, L., Aha, P., Gu, K., Kuimelis, R.G., Kurz, M., Lam, T., Lim, A.C., Liu, H., Lohse, P.A., Sun, L., Weng, S., Wagner, R.W. and Lipovsek, D. (2002). Directed evolution of high-affinity antibody mimics using mRNA display. *J. Chem. Biol.*, **9**:933-942.

Zhang, L.M. and Uttamchandani, D. (1988). Optical chemical sensing employing surface plasmon resonance. *Electron. Letts.*, **23**:1469-1470.

Zhou, H., Fisher, R.J. and Papas, T.S. (1994). Optimisation of primer sequences for mouse scFv repertoire display library construction. *Nucleic Acids Res.*, **22**:888-889.

Zhu, Z., Presta, L.G., Zapata, G. and Carter, P. (1997). Remodelling domain interfaces to enhance heterodimer formation. *Protein Sci.*, **6**:781-788.