Antibody-Based Biosensor Assays For The Detection of Zilpaterol and Markers for Prostate Cancer

A thesis submitted for the degree of M.Sc.

By

Mary Dillon B.Sc.

School of Biotechnology, Dublin City University

Research Supervisor

Professor Richard O'Kennedy

School of Biotechnology

February 2008

Dedication

This thesis is dedicated to Mum, to Dad who is no longer with us, and to my family.

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Masters of Science is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Mary Dillon ID No.: 53154533 Date: 11/02/2008

Acknowledgements

I wish to thank Professor Richard O'Kennedy for his constant support and guidance and for his genuine interest in my development. I would also like to thank the lab group with whom I worked, with special thanks going to Dr. Paul Leonard for his direction and help. To my family, I would like to thank them all for their support both financial and emotional during my study.

Table of Contents

Antibody-Based Biosensor Assays for the Detection of Zilpaterol and Markers for Prostate Cancer

Mary Dillon

Abstract

The research presented in this thesis describes the production and application of antibodies against the drug of abuse zilpaterol, and the application of antibodies against prostate-specific antigen (PSA), a cancer marker.

Polyclonal antibodies were used in the development of immunoassays in a competitive ELISA format and on the Biacore (a surface plasmon resonance-based optical biosensor capable of monitoring biomolecular interactions in 'real-time').

A zilpaterol-HSA conjugate was used to generate and characterise single chain antibody fragments. A combinatorial single chain (scFv) antibody phage display library was generated to zilpaterol. Splenomic mRNA from mice pre-immunised with a zilpaterol-HSA conjugate was used in the amplification of antibody genes followed by cloning into vectors from a well-established phage display system. Four positive clones were isolated during panning. One clone (B1) was selected and re-cloned into a plasmid from soluble scFv antibody expression. The soluble scFv antibody was purified and used in the development of a competitive ELISA-based assay. Further analysis of the B1 clone was carried out during the development of an inhibition assay for zilpaterol on Biacore. Affinity determinations of the scFv antibody for zilpaterol were carried out using 'real-time' biomolecular interaction analysis.

A recombinant form of PSA was also produced and characterised. Commercial anti-PSA antibodies were used to generate a competitive ELISA.

Abbreviations

A/A_0	absorbance detected excluding background
Ab	antibody
Abs	absorbance
Ag	antigen
APC	antigen-presenting cell
BIA	biomolecular interaction analysis
Bp	base pairs
BSA	bovine serum albumin
Cam	chloramphenicol
cDNA	complementary DNA
CDR	complementarity determining regions of antibody
cfu	colony forming units
CM	carboxymethylated
conc (c)	concentration
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidyl triphosphates
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	binding region of antibody above the hinge region
Fc	constant region of antibody molecule
FLAG	Hydrophilic tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys)
Fv	variable binding region of antibody
HAT	Hypoxanthine aminopterine thymidine
HT	Hypoxanthine thymidine
HBS	Hepes buffered saline
HPLC	High Performance Liquid Chromatography
HRP	horse radish peroxidase
IgG	Immunoglobulin class G
IgA	Immunoglobulin class A

IgD	Immunoglobulin class D
IgE	Immunoglobulin class E
IgM	Immunoglobulin class M
IPTG	Isopropyl-b-thiogalactoside
IMAC	immobilised metal affinity chromatography
IR	infra-red
Ka	association affinity constant
Ka	association rate constant
LED	light emitting diode
LOD	limit of detection
Log	logarithmic
MAb	monoclonal antibody
MHC	Major histocompatibility complex
MRL	maximum residue level
mRNA	messenger RNA
MW	molecular weight
n	refractive index
NK	natural killer cell
OD	optical density
OVA	ovalbunin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PEG	polyethylene glycol
pH	log of the hydrogen ion concentration
R	regression coefficient
RI	refractive index
RIA	radioimmunoassay
R/R_0	response units detected excluding background
RT	room temperature
RU	response units
scFv	single chain Fv antibody derivative

SDS	sodium dodecyl sulphate
SPR	surface plasmon resonance
T_C	T-cells
Tet	tetracycline
TCR	T cell receptor
THY	Thyroglobulin
TIR	total internal reflection
UV	ultraviolet
VH	variable region of heavy chain
VL	variable region of light chain

Units

µg	microgram
(k)Da	(kilo) Daltons
µl	microlitre
µM	micromoles
^{o} C	degrees celsius
AU	arbitrary units
cm	centimetres
g	grams
h	hours
kg	kilogram
l	litre
m	metre
M	molar
mg	milligram
min	minute
ml	millimetres
nM	nanomolar
mol	moles

pg	picograms
rpm	revolutions per minute
RU	response units
sec, s	seconds
v/v	volume per unit volume
w/v	weight per unit volume

Chapter 1

Introduction

1.1 The Immune System

The mammalian immune system consists of a range of mechanisms aimed at removing foreign entities such as bacteria, viruses, fungi and parasites that might prove harmful to the body. The immune system consists of two distinct mechanisms of defence, innate immunity and acquired immunity. Innate immunity consists of a series of physical and chemical barriers and non-specific cell-mediated responses. It has an immediate effect on foreign materials, and is not enhanced through repeated exposure to the same foreign body. Innate immunity functions on two levels; primary and secondary.

The primary defence mechanism includes the skin and mucous membranes; the former acting as a barrier against microbial invasion, the latter allowing for the entrapment and elimination of foreign bodies. The secondary line of defence involved in innate immunity consists of cell-mediated non-specific responses to foreign cells and includes such internal systems as phagocytosis by macrophages and cell lysis by natural killer (NK) cells. Phagocytes are derived from bone marrow stem cells. They engulf and ingest foreign bodies, and are present in areas most likely to encounter foreign bodies. Natural killer cells have the ability to recognise infected somatic cells based on cell-surface changes, i.e. viral infection. They then bind the infected cells and destroy them by inducing apoptosis.

1.1.1 Acquired immunity

Acquired immunity refers to the mechanisms of the immune response that are capable of adapting themselves to previously unseen molecules, producing specific memory cells that can act specifically to prevent further infection. There are two types of acquired immunity, humoral and cell-mediated. Humoral immunity involves B-lymphocytes producing antigen-specific molecules called antibodies. The coating of an antigen with antibodies results in the attraction of proteins, such as those of the complement system, that rupture the bacterial cell and, ultimately, engulfing of the antigen by phagocytes that digests the foreign cell. Cell-mediated immunity targets intracellular pathogens through recognition and elimination of infected somatic cells displaying surface antigen associated with the pathogen. The cells involved in this

response are mainly cytotoxic T-cells (T_C) derived from activated T-lymphocytes in the thymus (Elliott and Elliott, 2001). In general, while humoral and cell-mediated immunity function separately, elements of both types of acquired immunity also interact to facilitate for complete activation of the immune response.

All nucleated cells of the body that have been infected by a virus display antigen on their surfaces associated with proteins of the Major Histocompatability Complex class I (MHC I). These proteins are recognised by T_C cells. T_C cell recognition of the antigens results in signalling that causes the cell to become apoptotic, killing both the cell and any intracellular pathogens. Each T_C cell contains granules consisting of perforin and a set of serine proteases called granzymes, which when released, also result in death of the infected cell. Perforin causes lysis of the infected cell, facilitating the entry of granzymes and causes eventual cell death. T_C cells also release cytokines, such as γ-interferon that reduce the spread of the virus to adjacent cells (Male *et al.*, 1996).

Following exposure to an antigen, macrophages engulf, process and present the antigen on the surface of the cell via the Major Histocompatability Complex class II (MHC II) proteins. Such cells are known as antigen presenting cells (APC), and it is the antigens displayed on the surface of these cells to which the helper T-cell receptors bind. There are two subsets of T_H cells, T_H1 and T_H2, which are primarily different due to the cytokines they produce. T_H1 cells recognise antigens presented on the surface of macrophages and subsequently produce γ-interferon and tumour necrosis factor beta (TNF-β), which are mainly responsible for activation of T_C cells, natural killer cells and macrophages. T_H2 cells secrete cytokines such as interleukins 4, 5, 10 and 13 and their main function is the activation of B-lymphocytes (Roitt *et al.*, 1998).

1.1.2. Humoral immunity

Antibodies produced by B-lymphocytes mediate the humoral immune response. The interaction between B-lymphocytes and T_H2 cells is of critical importance for the production of antigen-specific antibodies. Immature B-lymphocytes are formed in the

bone marrow and have the potential to produce antibodies to attack virtually every macromolecule present in the body. Those B-lymphocytes that produce antibodies against a bodily protein (a self-protein) are eliminated during primary maturation. In response to other foreign antigens present, each B-lymphocyte displays a membrane-bound immunoglobulin with a single specificity, on its surface, which acts as an antigen receptor. Binding of the antigen to the cells displaying antibodies with the highest specificity for the antigen, results in internalisation and surface display of that antigen. It is at this stage that T_H2 cells, already activated though contact with an APC, recognise and bind specifically to B-lymphocytes displaying the same antigen. Their combination causes cytokine production, which results in B-cell multiplication and differentiation into antibody-secreting plasma cells. The types of cytokines secreted by the T_H cell determine the class of immunoglobulin produced.

Upon B-lymphocyte activation and proliferation, a proportion of the resulting clones mature into long-lived memory cells that can circulate for years. These cells form the basis of long-term immunity in the event of repeated infection. If the same antigen is encountered again, a much more rapid immune response is generated.

1.2 Antibody structure

Antibodies are a group of glycoproteins present in the serum of mammals, and are involved in immunity. Upon infection of the body by a foreign antigen, an immune response in initiated. Through contact of the antigen with B-cells and subsequent differentiation of the B-cells into plasma cells, antibodies specific for the antigen are secreted to aid in its elimination from the body. This differentiation of B-cells leads to the production of multiple different antibodies, all with varying affinity and specificity to the antigen (De Franco, 1999). Antibody affinity can be further improved by the process of affinity maturation within the producer cells, involving somatic hypermutation of the amino acid sequence of the CDR (complementarity determining region) of the variable heavy and light chains. This results in increased production of antibodies demonstrating new binding affinities, a minority of which will show affinities higher than that of the pre-maturation antibodies. These are selectively amplified. The growth of these clones is encouraged, while the growth of cells producing antibodies of low affinity ceases. The continuation of this process within the body's immune system results in the active production, selection and amplification of highly specific antibodies towards the target antigen (Berek, 1999).

Antibodies (immunoglobulins) are highly soluble glycoproteins. The basic structure of an antibody e.g. IgG (Figure 1.1) consists of four polypeptide chains with two identical heavy chains (H) and two identical light chains (L) linked by disulphide bonds. This structure can vary depending on the isotype. The amino terminal ends of both heavy and light antibody chains are characterised by variability of the sequence. These areas are known as V_H and V_L regions. Constant regions (C_H and C_L) are located further downstream. Disulphide bonds link the two heavy chains by the constant regions, as shown in Figure 1.1. The hinge region consists of an area containing a higher number of proline residues, which allows movement of the two Fab regions between 60^0 and 180^0. This aspect confers many desirable characteristics upon the activity of the antibody. It allows the 2 binding sites to work independently of each other, and facilitates binding of the antibody to a wide range of sites of varying size and conformation on the antigen surface (Frazer and Capra, 1999).

Several highly variable areas are present in each chain within the variable regions of the antibody light and heavy chains. It is these regions that confer most of the antigenic recognition, specificity and affinity. These hypervariable regions are collectively known as complementary determining regions (CDRs). They consist of approximately 110 amino acids, interspersed by constant areas called framework regions (FR). The FRs provide a backbone structure for the antibody, and can also have an influence on antigenic specificity. The conformation of the light and heavy chain CDRs result in six hypervariable loop structures which structurally form antigen-binding surfaces or pockets (Branden and Tooze, 1998) (Figure 1.1).

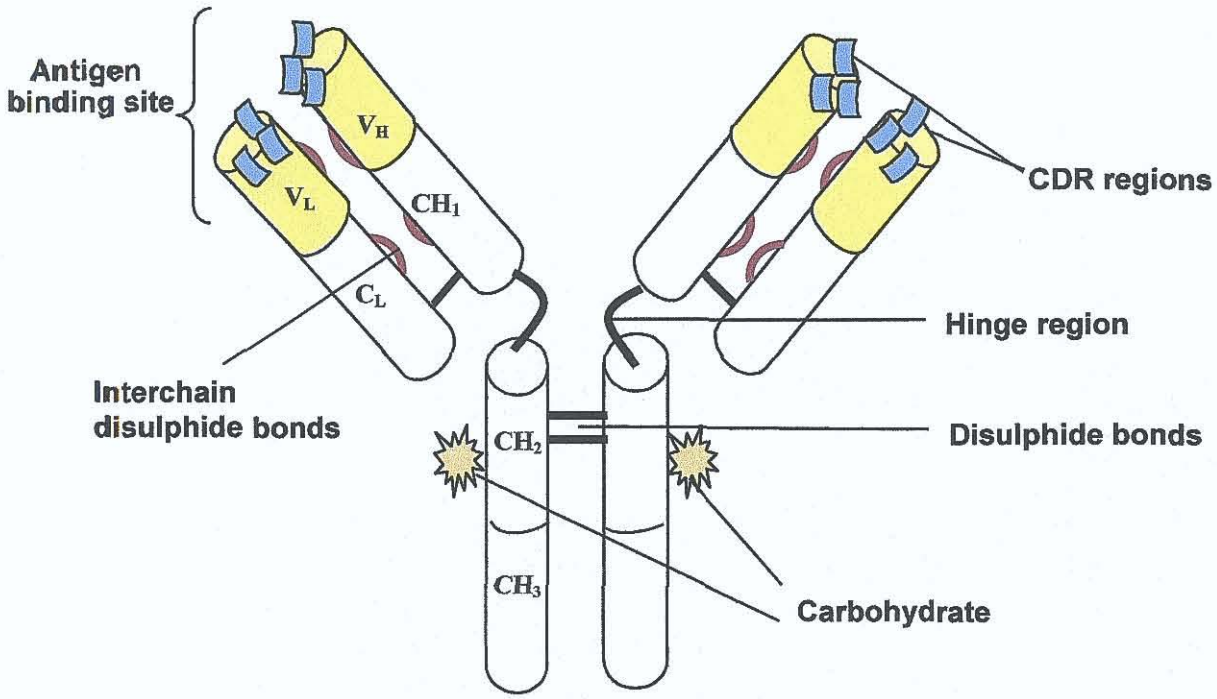

Figure 1.1 *Diagrammatic representation of an immunoglobulin G (IgG) molecule. The IgG antibody consists of two identical heavy chains and two identical light chains. Each chain consists of a variable region at the amino terminal end, referred to as variable heavy (V_H) and variable light (V_L), respectively. The remainder of the light chain is called the constant region (C_L). The constant region of the heavy chain is longer and is divided into three separate regions, C_H1, C_H2 and C_H3. Interchain and intrachain disulphide bonds link the heavy and light chains. The variable regions of the heavy and light chains contain the CDR regions, which confer antigen specificity on the antibody. A hinge region also exists between the C_H1 and C_H2 domains that allow conformational changes to occur in the antibody resulting in flexibility of between 60^0 and 180^0.*

1.2.1 Antibody diversity

The capability of the body to generate an enormous diversity of antibodies accounts for the remarkably adaptive nature of the immune system. It is because of the unique genetic mechanisms governing antibody synthesis that the body is capable of producing these highly specific molecules to previously unknown agents. This diversity is mainly confined to the heavy and light chain variable regions and, more specifically, the CDRs, and can be attributed to mechanisms such as the specialised replication of the antibody light and heavy chain germline genes, clonal selection and somatic mutation (Tizard, 1995).

Variable light chain genes are composed of two loci, the V (variable) and J (joining) loci, while heavy chain gene sequences are composed of three loci; the V and J loci with an additional D (diversity) locus. During B-cell maturation, these different regions combine randomly to produce the diversity observed. Recombination of the various VDJ sequences on the heavy chain and the VJ sequences on the light chain results in the formation of different structural conformations in the binding sites of the resulting antibodies (Max, 1999).

Binding site diversity can be further enhanced through various adaptations in the bases between gene segments. Mutations including the insertion, deletion and copying of bases within the genes, result in additional variation in the amino acid sequence of the antibody binding sites. This added diversity, called junctional diversity, further increases the variety of antibodies generated. Point mutations occur frequently in this region and also increase antibody diversity. The combined result of all these mechanisms is the ability of the body to produce antibodies with potential specificity for almost any antigen (Max, 1999).

1.3 Antibody technology

1.3.1 Monoclonal antibody technology

Initially, all antibodies available for analysis originated from the serum of immunised animals and were polyclonal in nature. Polyclonal antibodies consist of a mixture of antibodies to both the antigen of interest and to unrelated antigens naturally found in the host. Such antibodies may have a range of affinities and specificities (Kane and Banks, 2000). There are several advantages associated with the use of polyclonal antibodies in immunoassays. Polyclonal antibodies can demonstrate high specificity, despite the fact that their affinities and specificities may widely vary. This is because polyclonal antibodies may potentially include antibodies that can bind to multiple different sites on the antigen surface, and may be particularly useful in the initial detection of a previously unknown molecule (Mayforth, 1993). In addition, their production requires a minimum of technical input relative to that necessary for monoclonal or recombinant antibody generation (Dean and Shepherd, 2000).

Monoclonal antibodies, unlike polyclonal, are a homogenous population of antibodies derived from a single antibody-producing cell. Hence, all antibodies are identical and of the same precise specificity for a defined epitope (Köhler and Milstein, 1975; Delves, 1997; Kane and Banks, 2000). Since the antibody is the product of a single cell line, it is possible to produce unlimited amounts, as long as the source cell line is maintained. This contrasts with polyclonal antibody production whereby antibody quantity is limited by the animal. In addition, the use of a homogenous antibody preparation results in elimination of assay variation and leads to significant increases in assay reliability (Nelson *et al.*, 2000).

Monoclonal antibody production begins with immunisation of a host animal with the required antigen (Figure 1.2). Once a sufficient antibody response is generated, the animal is sacrificed and the spleen removed. Primed B-cells are then fused to myeloma cells (tumorigenic B-lymphocytes) in order to confer immortality upon the antibody-producing B-cell. Fusion of the cells is facilitated by the use of

polyethylene glycol (PEG) and other methods, which promote membrane bridging and transfer of nuclei (Hurrell, 1985).

Fusion of the myeloma and B-cells is carried out in HAT medium (media containing Hypoxanthine, Aminopterin and Thymidine). Aminopterin prevents the formation of tetrahydrofolate, which is necessary for thymidine synthesis. Therefore, the formation of dGTP, dATP and dTTP is inhibited and, subsequently, *de novo* synthesis of DNA is blocked. A salvage pathway capable of using hypoxanthine and the enzyme, Hypoxanthine Guanidine Phosophoribosyl Transferase (HGPRT), is present. However, the myeloma cells are selected that specifically lack HGPRT and are, therefore, incapable of using this pathway. These myeloma cells cannot reproduce since they cannot use either pathway and subsequently die in culture. However, since the spleen cells do not lack the enzyme, they are capable of DNA synthesis via the salvage pathway. Hence, when the myeloma and B-cells fuse, the resulting hybridoma is capable of DNA synthesis and is immortal. Any remaining B-cells that did not form hybridomas are unable to proliferate, since somatic cells cannot grow continuously outside the body, and die. The unfused myeloma cells cannot synthesise DNA in HAT medium. Therefore, only the hybridoma cells will grow (Figure 1.2), resulting in an immortal cell line with the potential for monoclonal antibody production (Kohler and Milstein, 1975; King, 1998; Dean and Shepherd, 2000; Stapleton *et al.*, 2004). The antibody-producing clones are then screened by enzyme-linked immunoassay (ELISA) and antibodies with the required characteristics selected.

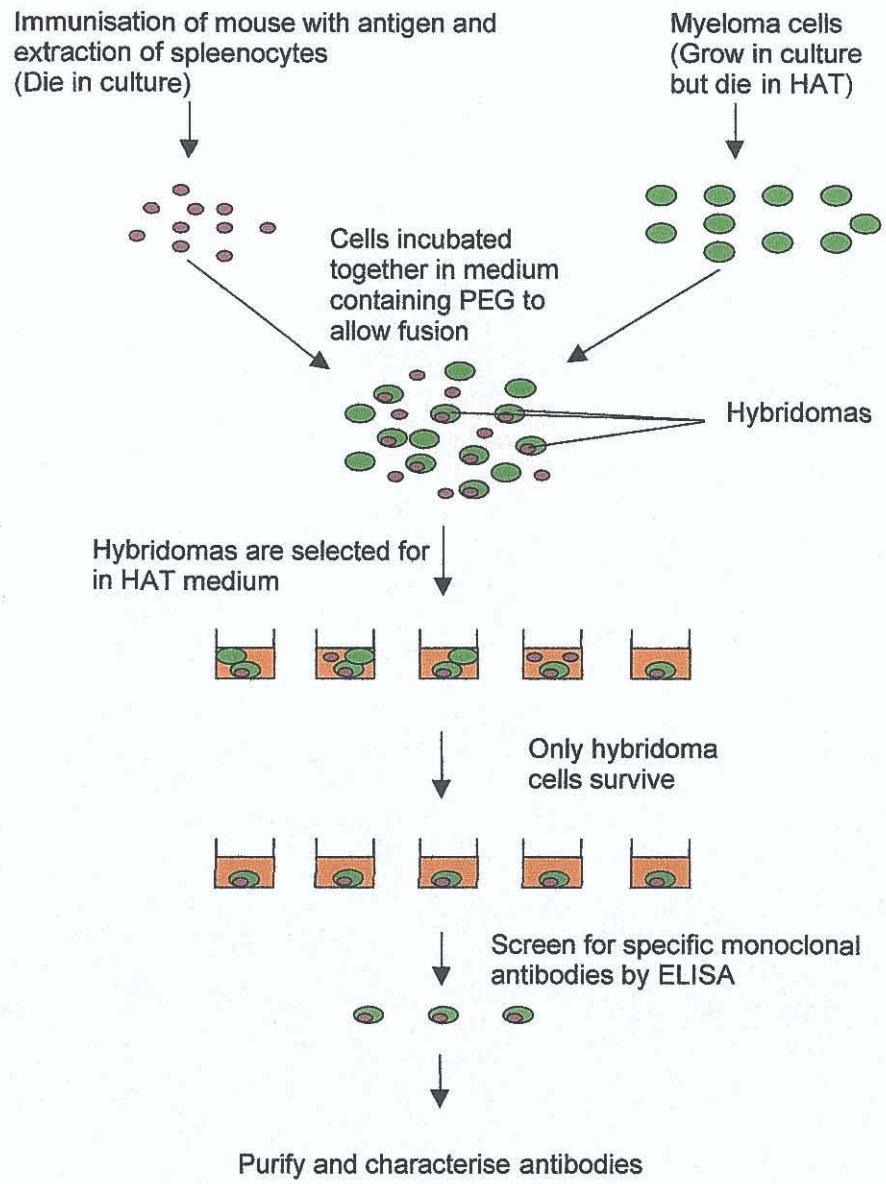

Figure 1.2 *Principles of monoclonal antibody production.*
Specialised myeloma cells and spleenocyte cells extracted from an immunised mouse are incubated with polyethylene glycol (PEG) to facilitate their fusion. HAT media contains Hypoxanthine, Aminopterin and Thymidine and blocks de novo DNA synthesis. Since the myeloma cells chosen specifically lack the salvage pathway of DNA synthesis, they cannot grow in this media. Subsequent addition of HAT to the media eliminates unfused myeloma cells from the culture. Since somatic cells cannot survive for long, in culture, fused hybridoma cells are selected. The remaining cells are screened by ELISA for the production of monoclonal antibodies specific for the required antigen. The selected antibodies are then characterised and, subsequently, purified.

1.3.2 Recombinant antibodies

Recombinant techniques have facilitated the generation of specifically engineered antibody fragments that have been successfully employed in immunoassays (Dillon *et al.*, 2002; Brennan *et al.*, 2003; Blazek *et al.*, 2004). Functional antibody fragments consist of various chains of the antibody, i.e. the whole antibody minus some of the structural regions that still display the specificity and affinity properties of the whole antibody molecule.

Figure 1.3 (b, c and d) illustrates some of the recombinant antibody fragments used in immunoassay development. These fragments are named after the specific section of the antibody from which they are derived (Smith, 1985). The Fc region of the antibody consists of the two constant chains linked with inter-chain disulphide bonds. Very useful smaller fragments can also be engineered. The Fab fragment consists of the light and the corresponding heavy chains of one arm of the antibody (Figure 1.3 b), and as with a complete antibody, the specificity of this fragment is dictated by the variable region. Stability is maintained as a result of the intrachain disulphide bond which links the heavy and light chains. The Fv (variable fragment) is smaller and is comprised of only the variable light and heavy chains of one arm of the antibody (Figure 1.3 c). The advantages of this fragment are due to its small size, and its retention of almost 100% of the specificity of the whole antibody from which it was derived, since the binding sites remain intact. However, this fragment lacks stability, due to the absence of a disulphide bond linking the two chains (Killard *et al.*, 1995; Liddell, 2001). A new variation on this fragment has emerged and has become a significant alternative. The scFv, or single chain variable fragment, consists of the heavy and light chain variable regions joined by a 15-20 amino-acid linker peptide (usually a glycine-serine repeat linker) molecule (Figure 1.3 d). This linker does not interfere with the binding capacity of the scFv and confers stability on the molecule (Hudson, 1998; Gosling, 2000). The versatility of scFv antibody fragments has led to use of methods such as phage display for easier antibody selection.

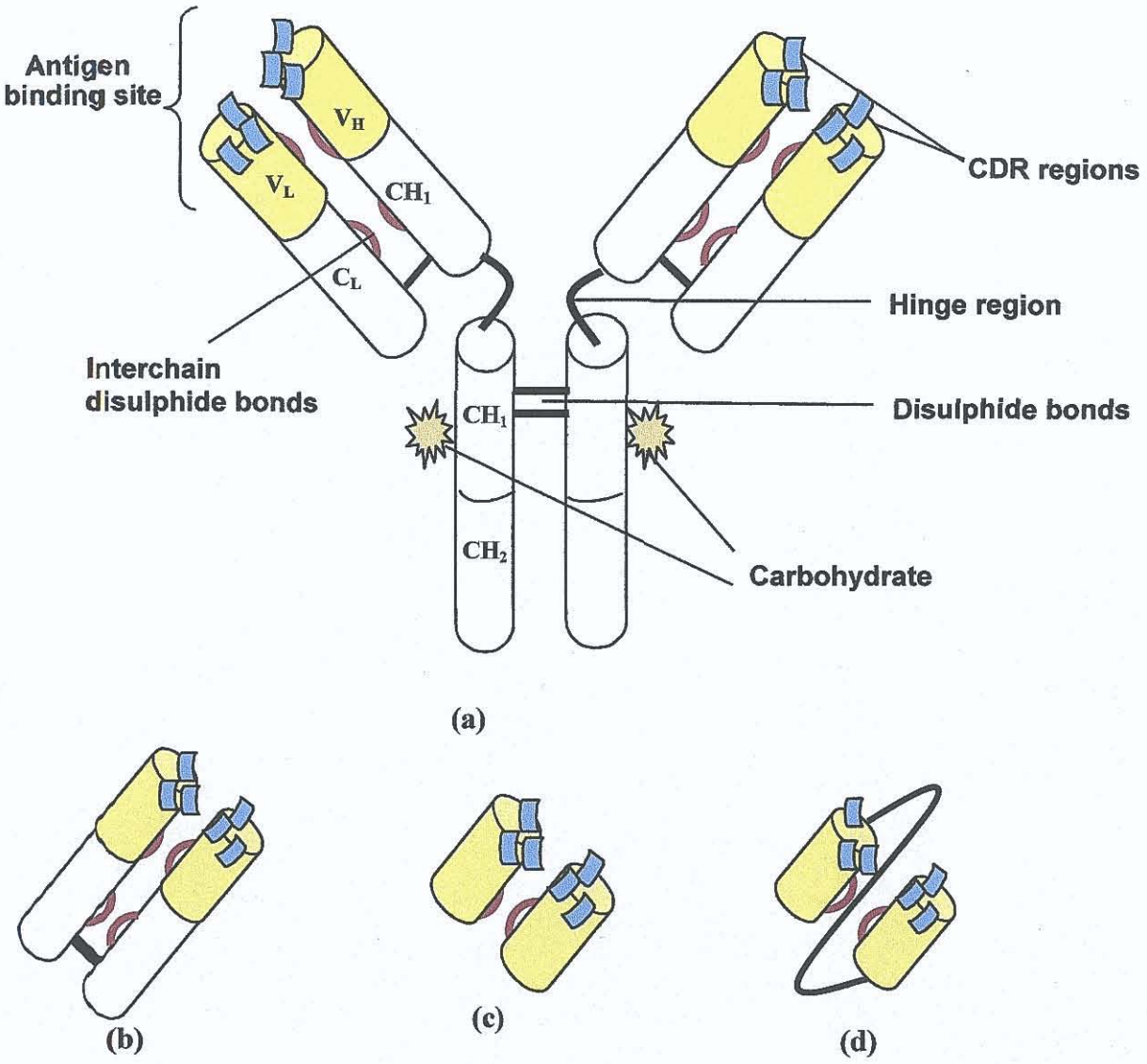

Figure 1.3 *Illustration of an IgG antibody and antibody fragments.*

(a) *Whole IgG antibody*

(b) *Fab is a complete arm of the antibody including the variable regions of the heavy and light chains, the constant region of the light chain and the corresponding heavy chain region. The disulphide bond of the antibody holds the chains together.*

(c) *The Fv, or variable fragment, consists of the variable regions of both heavy and light chains.*

(d) *ScFv, or single-chain variable fragment, consists of the Fv fragment made stable by the addition of a linker molecule between the V_H and V_L regions.*

1.3.3 Phage display technology

Phage display refers to the display of functional foreign peptides or small proteins on the surface of bacteriophage (Smith, 1985). Phage display is recognised as a powerful way in which to screen and select peptides on the basis of binding or molecular recognition. For diagnostic immunologists, the most important advantage of this technique is the ability to directly select the genetic information coding the most highly-specific antibodies. It also allows optimal antibody selection from unbiased libraries with more speed and accuracy than other methods (Osborn, 2000).

Viruses are extremely small parasites that require a host cell for replication. Virus particles essentially comprise of a nucleic acid genome and protein coat. Bacteriophage (or phage) refer to viruses that infect bacteria. The replication of bacteriophage can be roughly divided into two types; lytic, involving lysis of the host cell upon particle release, and non-lytic, where release of the phage particles does not result in cell death.

Various functional peptides can be displayed on the surfaces of viruses such as bacteriophage λ, the tobacco mosaic virus, *herpes simplex* virus, baculovirus and *hepatitis B* virus (Yau *et al.*, 2003). For affinity selection, however, the most commonly used virus is the *Escherichia coli*-specific filamentous phage, including M13, fd and f1 (Hoogenboom *et al.*, 1992). Filamentous phage contain a circular single-stranded DNA genome contained within a protein capsid cylinder. Filamentous phage do not reproduce by the traditional lytic cycle, but propagate within the bacterial cell and are secreted without lysis of the cell. Each infected cell is capable of producing over a thousand phage particles.

Filamentous phage are covered by approximately 3000 coat proteins present on the filament surface. The major coat protein pVIII (approximately 2700 copies per phage particle) covers most of the surface. The minor coat proteins, pIII, pVI, pVII and pIX are displayed at either extremity of the filament. All of these coat proteins have been used for displaying peptides on the phage surface. However, pIII is most frequently used for antibody fragment display. Each pIII consists of 406 amino acids (excluding

the leader sequence) hooked to the virion surface at the carboxy terminus. The region of pIII between this and the N-terminal domain is required for attachment and penetration of the bacterial cell during infection. Foreign peptides are covalently linked to the free amino ends of this coat protein, as shown in Figure 1.4. This allows the coat protein to remain functional and infection capabilities of the particle are not significantly impaired (Smith, 1985; Orum *et al.*, 1993).

The principle of phage display for antibody selection, therefore, is to successfully display the antibody or antibody fragment on the surface of the phage by integrating its genetic information into the sequence coding for the pIII protein. This is done using either a phage vector or a phagemid vector. Several different types of these vectors are available. Complete phage vectors or bacteriophage carry all the genetic information required for the phage life cycle (Hoogenboom *et al.*, 1998). However, more recently, phagemid systems that have been developed for phage display are used. Phagemids were originally derived from an expression vector engineered to express the foreign peptide as a fusion of the N-terminus of the phage coat protein. They contain the origins of replication for both the M13 phage and *E. coli* bacteria in addition to gene III. They also contain multiple cloning sites and an antibiotic-resistance gene, but no other gene products required for generating a complete phage. When a bacterial cell harbouring the phagemid vector is super-infected with a helper phage such as M13K07, the protein synthesis machinery of the host is used to produce all the wild type phage proteins (from the helper phage genome) plus the antibody-phage coat fusion protein (from the phagemid vector). When new phage particles are assembled, single stranded DNA from the phagemid bearing the fusion peptide gene will be packaged, while the encoded fusion protein will be displayed on the surface of the phage particle (Osbourn, 2000; Liddell, 2001). The advantages of the use of a phagemid vector in comparison to phage vectors include the smaller size and the higher bacterial transformation efficiency observed with the phagemid vector. In addition, phagemid display promotes the monovalent display of antibody particles, resulting in selection based on affinity of the antibody rather than cumulative avidity (MacKenzie and To, 1998; Tout *et al.*, 2001).

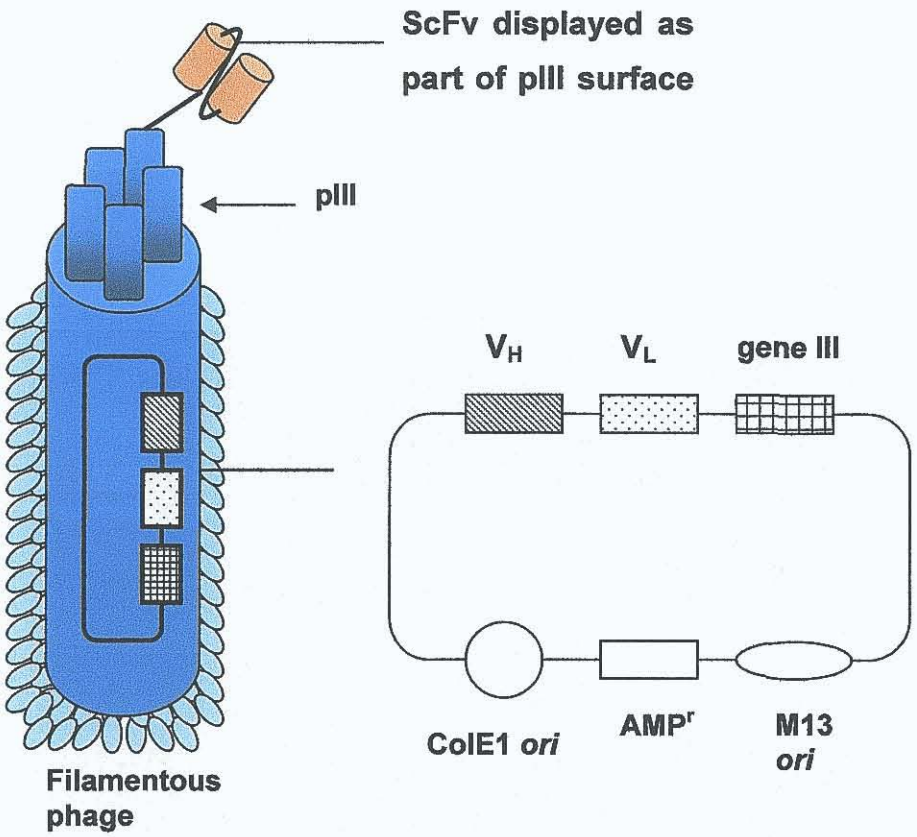

Figure 1.4 *A filamentous phage particle. A scFv fragment is displayed as part of the pIII coat protein present on the filamentous phage surface. The phagemid vector within the phage particle contains the variable heavy and light chain genes fused with the pIII gene, an ampicillin resistance gene and origins of replication for both M13 helper phage* and *E. coli.*

Col E1 ori: E. coli origin of replication gene

AMP^r: ampicillin resistance gene

M13 ori: M13 phagemid origin of replication gene

1.3.4 Phage antibody libraries and combinatorial libraries

A number of antibody-producing cells can be used as the starting material for the construction of a phage display library. These include hybridoma cells, splenocytes or lymphocytes from bone marrow or blood. Affinity selection of antibody fragments displayed on the surface of phage can replace the time-consuming and labour-intensive procedure of hybridoma production. The initial step involves mRNA extraction and the use of PCR to amplify the antibody genes *in vitro*. Specific heavy and light chain primers are used to separately synthesise the variable regions of the heavy (V_H) and light (V_L) chain genes of the antibody using the template produced from the cells. Once obtained, these heavy and light genes can be artificially paired through splice by overlap extention (SOE) PCR, a technique in which the genes are joined through the use of each heavy and light chain gene as a primer for the other, and then amplification of the product. The antibody fragment genes are then ligated into the digested vector and transformed into *E. coli*. In this way, a random combinatorial library is created (Krebber *et al.*, 1997) (Figure 1.5). If a library size of 10^8 members or more is obtained, all possible V_H/V_L pairings, including the pairs originating from B cells selected by the immune system, are present. Subsequently, the library of recombinant antibody fragments may be displayed on phage and functionally selected against the target antigen.

Affinity selection of the scFv by panning and subsequent reinfection into *E. coli* greatly enhances the number of strong binding scFvs. Panning of the phage library involves the passing of the fragment-displaying phage over a surface coated with the antigen in question. Unbound phage i.e. particles not displaying antigen-specific fragments, are washed away. Remaining phage are then eluted and used to reinfect *E. coli* cells. After several rounds of panning are completed, only the most high affinity antigen-specific fragments remain (Liddell, 2001). Phage display scFvs and Fabs with comparable or better affinities and specificities to monoclonal antibodies can be isolated (Figure 1.6).

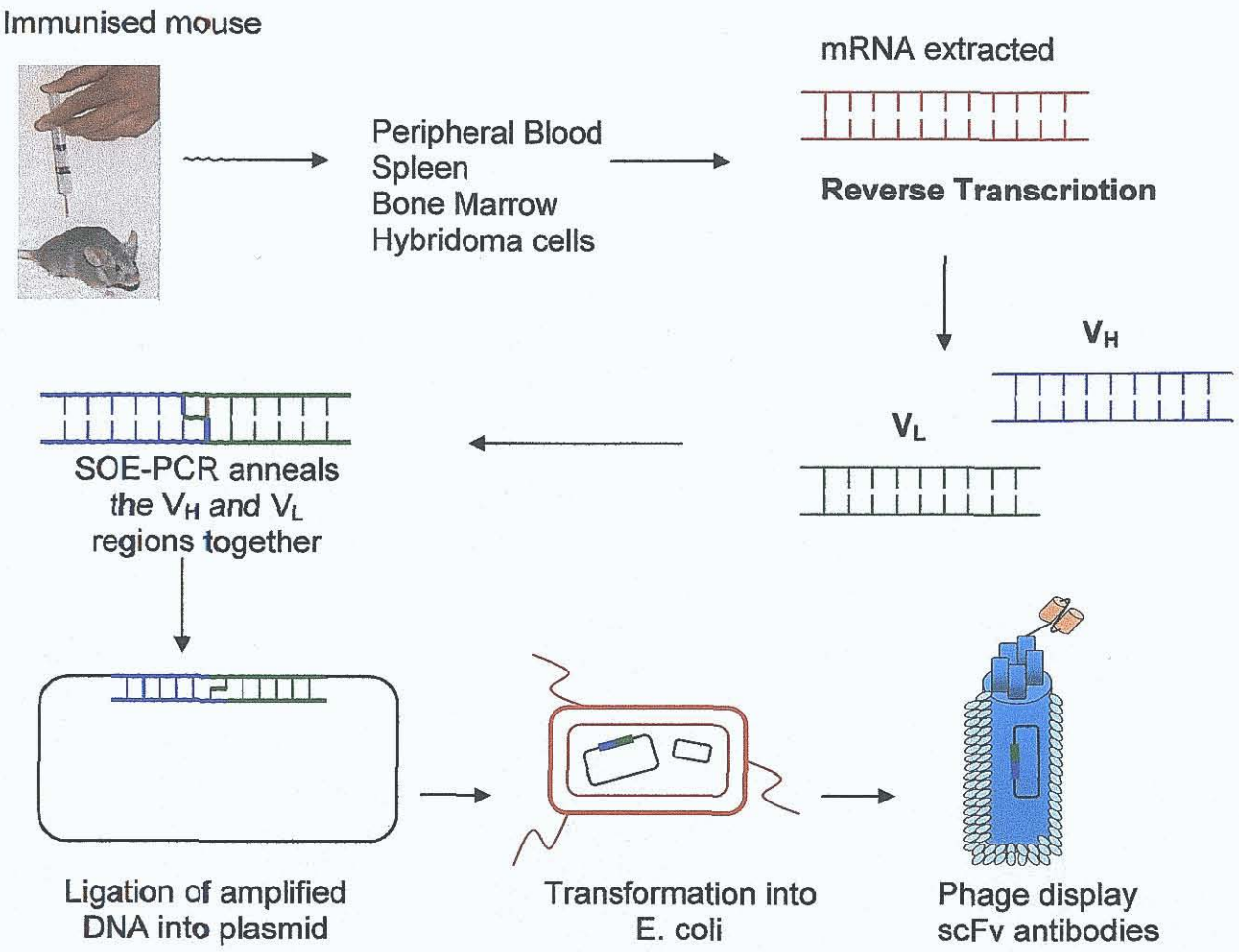

Figure 1.5 *Construction of a phage display library. mRNA is extracted from an immunised animal and PCR used to amplify the antibody genes in vitro. The variable regions of the heavy (V_H) and light (V_L) chain genes of the antibody are amplified using the template produced from the cells, then artificially paired through splice by overlap extension (SOE) PCR. The antibody fragment genes are then ligated into the digested vector and transformed into E. coli. In this way, a random combinatorial library is created.*

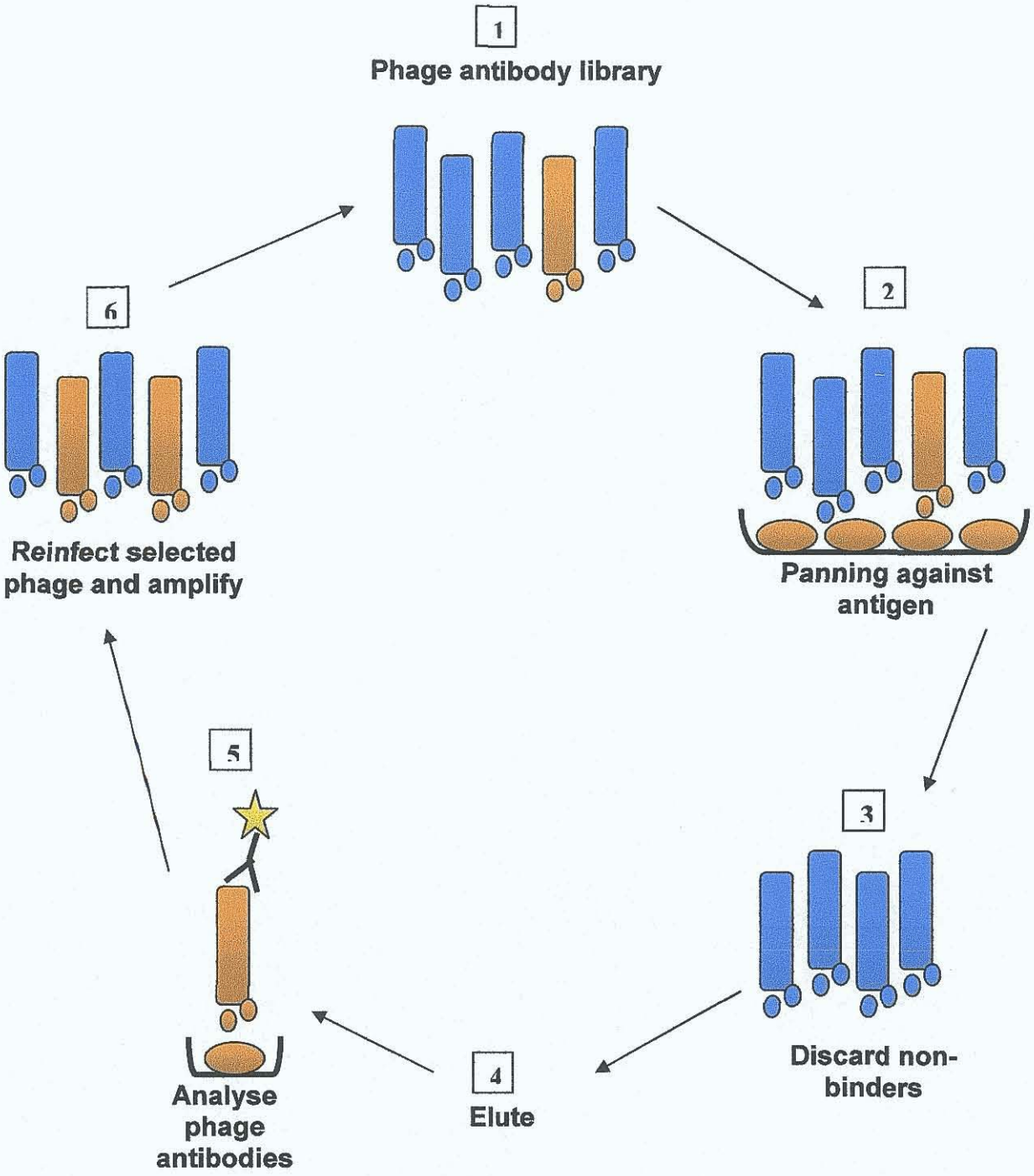

Figure 1.6 *Procedure for selection of specific scFv–displaying phage from phage libraries. A phage library [1] is passed over an antigen-coated surface and phage displaying antigen-specific scFvs bind to the surface [2]. Any unbound phage are washed away [3]. Specific library phage are then eluted [4] and subsequently analysed [5] and reinfected into E. coli for amplification and rescue [6]. This panning process is repeated for several further rounds. Enrichment of antigen-specific phage is achieved after each round of panning.*

1.3.5 Expression of recombinant scFv antibodies

Bacterial expression systems are commonly used for the production of all functional antibody fragments, due to the rapid and inexpensive fermentation of bacteria, ease of genetic manipulation and the fact that antibody fragments do not require post-translational modification.

Antibody fragments that have been selected while displayed on the surface of a phage particle can be produced in a soluble form. The gene sequence coding for the fragment can be ligated into a phagemid that contains an amber stop codon and a leader sequence. The amber stop codon is located on the phagemid between the scFv gene and the gIII gene, encoding the pIII phage coat protein. In non-supressor strains of *E. coli*, such as HB2151 and JM83, this amber codon is recognised as a stop codon. This halts transcription and results in secretion of soluble antibody.

The leader sequence, which has also been translated, directs the expressed scFv into the periplasmic space of *E. coli*. This sequence is cleaved during transport through the membrane allowing the correct folding of the antibody for expression.

1.3.6 Affinity maturation of scFvs

Once an antibody or antibody fragment specific for a given antigen has been isolated, improvement of the affinity of that antibody is often desirable. Antibody affinity can be improved *in vitro* using a variety of techniques including site-directed mutagenesis, CDR shuffling, chain shuffling or error-prone PCR. The effects of these mutations is then analysed by the process of selection and screening.

1.3.6.1 Site-directed mutagenesis

Site-directed mutagenesis involves the specific mutation of one or more amino acids present in the antibody sequence (mainly the CDR regions), and subsequent selection of antibodies demonstrating higher affinities.

1.3.6.2 CDR and chain shuffling

Chain shuffling mutagenesis is the isolation and pairing of the optimal V_H and V_L chains from an antibody library in order to select the most specific antibody possible from that library. The process involves recloning of one gene for one chain of the scFv (e.g. V_H), with a repertoire of genes for the other chain (V_L) (Marks *et al.*, 1992). The resulting antibody library consists of phage encoding an scFv containing V_H chains specific for the target antigen and random V_L chains. This library is screened for specific binders and the clones that produce scFvs displaying improved binding properties are identified. These V_L chain genes are then fixed, and cloned along with the repertoire of genes for the V_H chain. In many cases, in order to maintain the specificity of the parent scFv, the CDR H3 may be kept constant to retain specificity, and only frameworks 1 to 3 of the variable chain segment are replaced.

1.3.6.3 Error prone PCR

Error prone PCR involves the introduction of mutations during PCR amplification of the antibody gene fragment in an attempt to increase antibody affinity. One method involves the use of DNA polymerases that lack proof-reading capabilities, resulting in random insertions, deletions and substitutions of the gene sequence. Panning of the resulting library is conducted and the affinity of the positive clones isolated is compared to that of the wild type.

1.3.7 Ribosomal *in vitro* display

Ribosomal and mRNA *in vitro* display technologies are established as the alternatives to phage display for the isolation of specific antibody fragments with direct linkage to the DNA encoding that fragment. Phage display is a cell-dependent system, a fact that limits cellular transformation efficiencies and subsequent library size. *In vitro* systems have no such limits and can therefore yield much larger libraries. Selection disadvantages of host environments (i.e. host bacterial cells used in phage display) against specific scFvs also do not occur in such "cell free" systems. Ribosomal and mRNA antibody display also facilitate direct protein evolution (Figure 1.7).

Ribosomal display was first developed in 1994 (Mattheakis *et al.*, 1994), and involves the *in vitro* transcription of the scFv fragment of interest, followed by *in vitro* translation, whereby the mRNA is retained on the ribosome by the absence of a stop codon. With no stop codon, protein elongation stops. However, the ribosome remains complexed to the mRNA. In addition, certain polypeptide releasing factors are not present and the nascent antibody remains attached to the ribosome. Therefore, through the ribosome, the antibody fragment remains attached to its encoding nucleotide sequence (Hanes and Plückthun, 1997; He and Taussig, 1997). The complex is stabilised at low temperature (4^0C) and in buffer containing magnesium, then affinity selected from the translation mixture through binding of the native scFv to the specific immobilised antigen. Non-specific binders are eliminated following stringent wash steps and specific binders are eluted by dissociation with EDTA or upon addition of free antigen (Hanes and Plückthun, 1997). In this way the mRNA coding for the most specific antibody fragment can be isolated and reverse transcribed into cDNA for use in the next enrichment cycle.

Plückthun and colleagues have studied complexes of mRNA-ribosome-protein systhesised by *E. coli* extract. They demonstrated that ribosomal display could select and enrich high affinity binders from a library and could also provide *in vitro* hypermutation to evolve the selected clones (Hanes *et al.*, 2000). In their experiments, insulin-specific scFv fragments were selected from the synthetic naïve human combinatorial antibody library (HuCal). After six rounds of selection, strong binders with dissociation constants in low nanomolar to picomolar ranges were isolated, with the lowest K_d being 82 pM. Sequence analysis indicated that certain specific mutations were identified in some of the sequences that contributed to the high affinities seen in these binders.

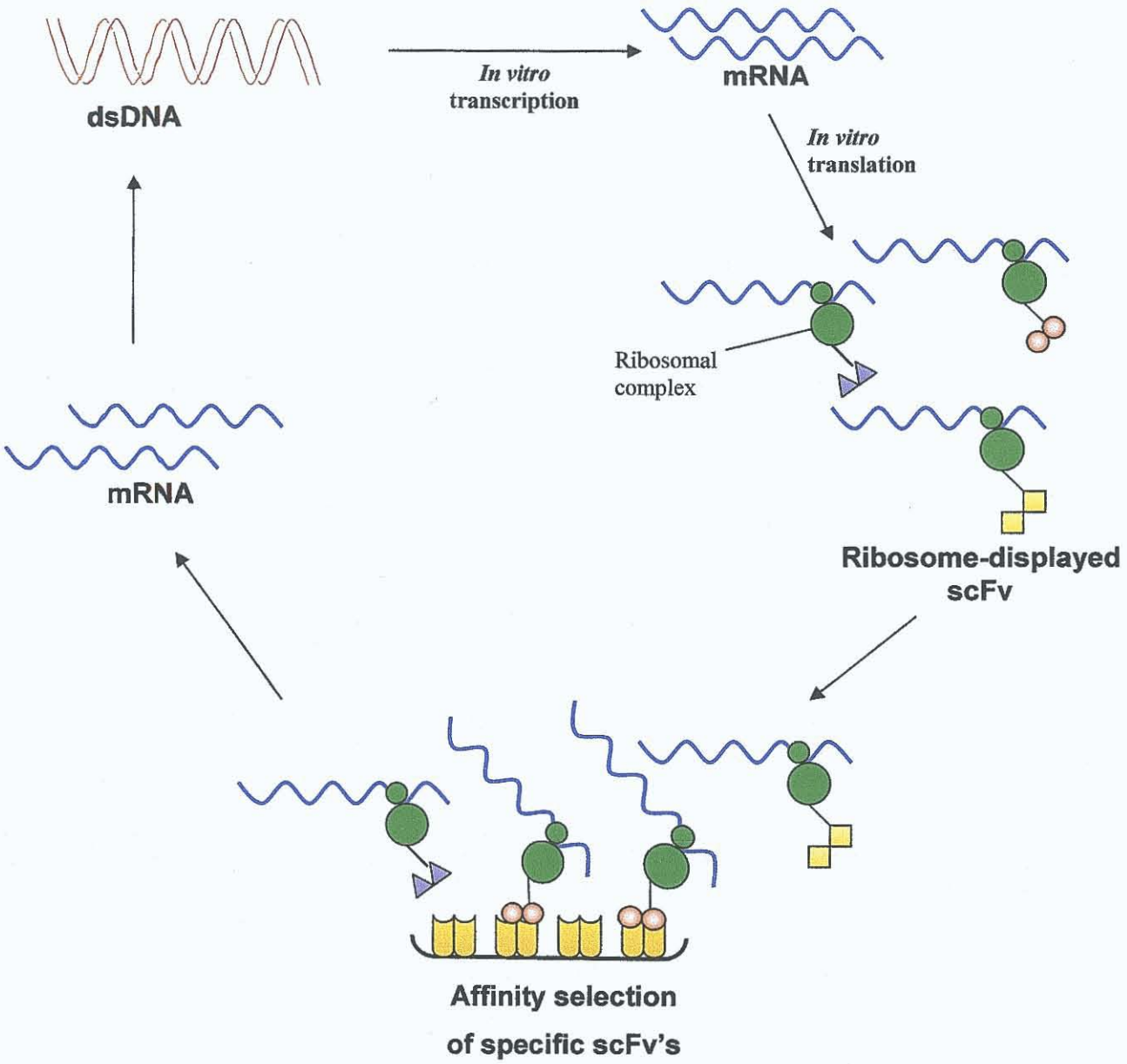

Figure 1.7 *Selection of scFv antibody fragments using ribosomal display. Double-stranded DNA constructs are transcribed and translated in vitro. The ribosome stalls at the end of each transcript due to the absence of a stop codon, forming a ribosome-displayed scFv complex consisting of the mRNA, the nascent scFv or the antibody molecule outside of the ribosome tunnel. Under optimised conditions, these properly folded ribosome-displayed antibodies can be subjected to affinity selection. The mRNA in the affinity-selected ribosome-displayed scFv complex can be extracted for use in RT-PCR to generate the double-stranded cDNA that can be subjected to another cycle of selection.*

1.4 Immunoassays

Immunoassays are analytical techniques that rely on the use of antibodies specific for a certain compound for its detection in a complex sample or matrix. Immunoassays are extensively used to quantify a large range of molecules due to their accuracy and specificity, both of which relate to the affinity of the antibody for the target analyte. In addition, immunoassays are inexpensive, fast and convenient.

Immunoassays can be divided into several categories depending upon the label used to detect the antibody-antigen interaction. Radioimmunoassays (RIA) use radioisotopes for detection, fluorescent immunoassays (FIA) involve fluorescently-labelled antibodies, and enzyme-labelled antibodies are involved in enzyme-linked immunosorbent assay (ELISA). In addition, immunoassays can be defined as fluid phase or solid phase depending on whether the assay is carried out in solution, i.e. antibody / antigen interaction and detection mechanism occurs in solution (Brennan *et al.,* 2003) or immobilised onto the surface of plastic or other support matrices i.e. antibody / antigen interaction is immobilised and detection is based on presence on the matrix surface (Dillon *et al.,* 2003), and whether or not a separation step is necessary (Gosling, 2000).

1.4.1 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a frequently used immunoassay format for the detection of a wide range of molecules, and its reliability is well established. It is the most common assay format used for initial characterisation and evaluation of antibodies destined for other immunoassays. The assay can be conducted using polyclonal, monoclonal or recombinant antibodies. In an ELISA, an enzyme that catalyses a chromogenic or chemiluminescent reaction is used to label an antibody or antigen. This is then used to detect the presence of the corresponding antibody or antigen in the sample being assayed. The intensity of the colour or the light produced by the enzyme upon addition of the substrate can be accurately measured to allow quantitative analysis (Liddell, 2001). The enzyme horseradish peroxidase (HRP) is often used in conjunction with chromogenic substrates such as diammonium-2,2'-azino-bis (3-ethylbenzothiazole-6-sulfonate) or *o*-PD (*o*-phenylenediamine). The end product

from this reaction is measured spectrophotometrically. These substrates are selected due to the fact that they are easy to prepare and are available at a reasonable price (Yau *et al.*, 2003).

A direct, non-competitive ELISA involves direct detection of the analyte present in a sample. Antigen is added to the wells of a solid support matrix, and upon incubation becomes immobilised through hydrophobic interactions. A protein solution (e.g. a 5% (w/v) solution of milk protein) is then added and incubated in order to block any remaining sites on the surface of the support matrix and avoid subsequent non-specific interactions. Any unbound protein is then washed from the surface using phosphate-buffered saline (PBS) and PBS – 0.04% (v/v) Tween (detergent). Antibody specific for the antigen is then added and incubated to allow binding to the immobilised antigen. The wells are again washed to remove any unbound protein. A secondary, enzyme-labelled antibody specific to the primary antibody is then added and the surface incubated. Excess antibody is then washed away. The presence of this secondary antibody can then be measured following the addition of a suitable substrate for the enzyme label. This is detected spectrophotometrically with suitable controls and standards for the use for the construction of a calibration curve. From this information, the quantity of antigen present in the sample can be measured (Figure 1.8).

A competitive ELISA involved determination of analyte concentration by competition with a known concentration of analyte for specific binding to the antibody. In this case, antibody is immobilised to the surface and the remaining sites blocked with protein solution, as described before. The plate is washed, and a known concentration of enzyme-labelled antigen, plus an equal volume of the sample, containing an unknown quantity of the antigen, is added simultaneously to the surface and incubated. During incubation, both the labelled and unlabelled antigen compete for specific binding to the immobilised antibody. The surface is then washed to remove any unbound antigen, substrate is added and the absorbance read spectrophotometrically. Since only the labelled antigen that has been captured by the antibody is detected, the signal measured in inversely proportional to the quantity of antigen present in the original sample (Figure 1.9).

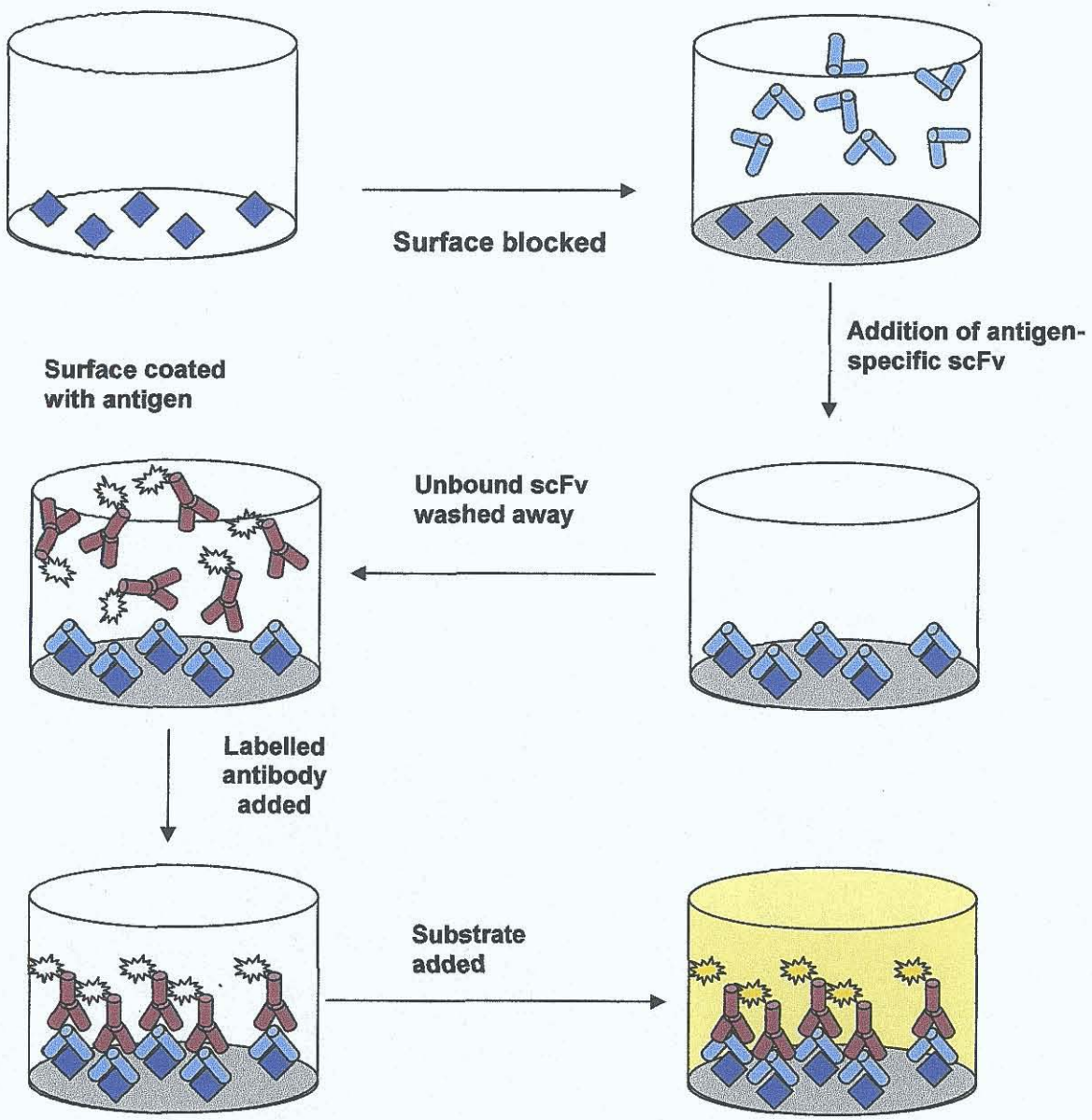

Figure 1.8 *Schematic diagram of a typical direct ELISA. Each step requires an appropriate incubation period (usually 37^{0}C for 1 hour). Antigen is coated onto a well of a microtitre plate. The remaining sites on the plate are blocked with a protein solution to prevent non-specific binding to the plate. ScFv is added and binds to the immobilised antigen. Unbound scFv is washed away and a secondary, enzyme-labelled antibody is added to the well. This is allowed to bind the scFv and unbound antibody is washed away. Finally, substrate specific for the enzyme is added, and the absorbance is measured spectrophotometrically.*

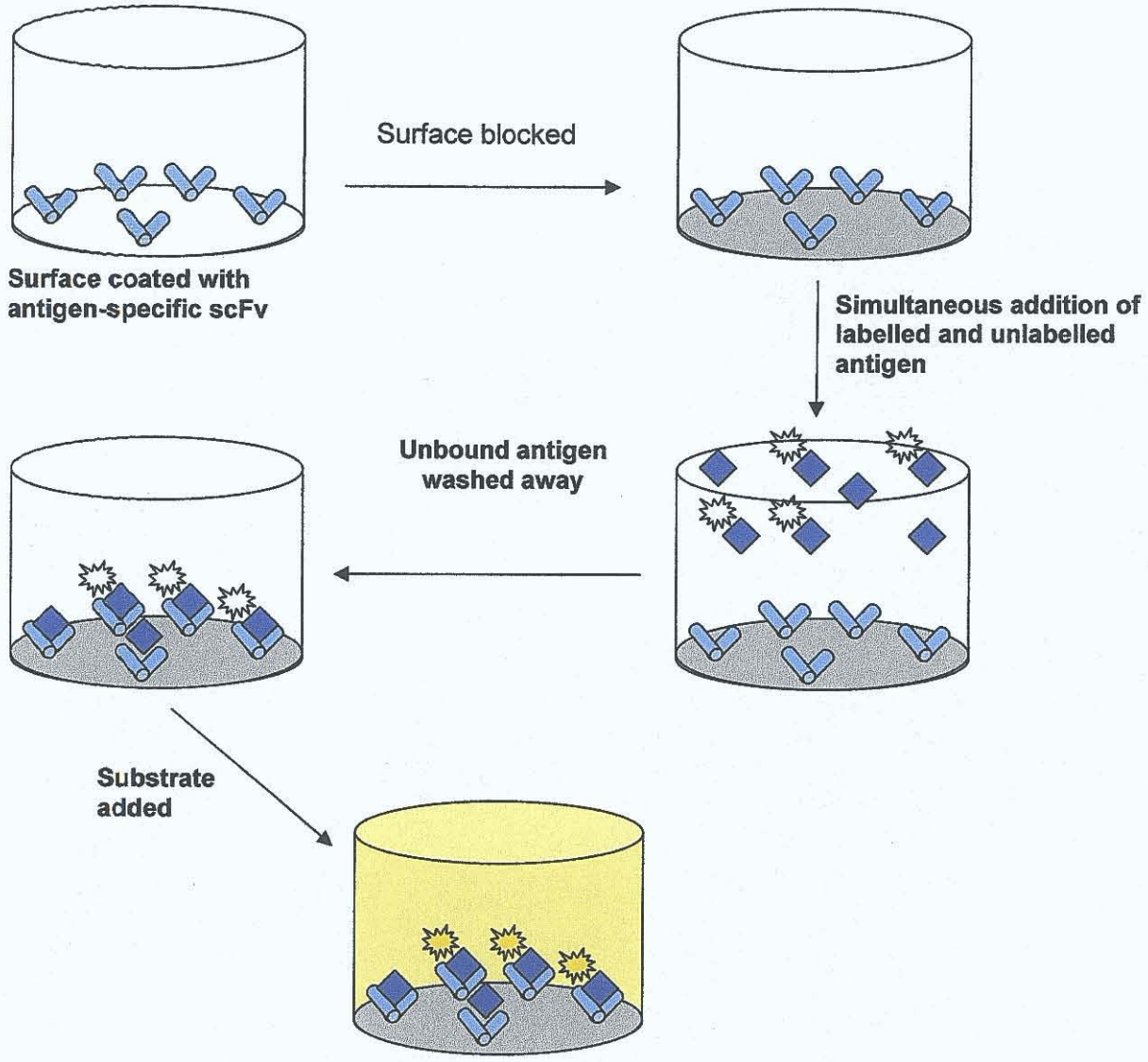

Figure 1.9 *Schematic diagram of a typical competitive ELISA. ScFv specific for the antigen are coated onto a well of a microtitre plate. The remaining sites on the plate are blocked to prevent non-specific binding to the plate. Labelled antigen is added simultaneously with the sample containing the unknown antigen concentration. Labelled and unlabelled antigen compete for the immobilised scFv. Unbound antigen is washed away and substrate specific for the enzyme is added. The absorbance is measured spectrophotometrically. The result obtained is taken as inversely proportional to the free antibody present in the sample.*

1.4.2 Biosensors

A Biacore biosensor was used in the research described to develop inhibitive immunoasssays. The Biacore works on the principle of surface plasmon resonance, as discussed in Section 3.1.3, and can, therefore, be used to develop immunoassays to various compounds. With this system, either hapten or antibody may be immobilised onto the surface of the chip. However, immobilisation of the hapten may prove difficult depending on the functional groups available. Therefore, some assay formats require the immobilisation of protein-hapten conjugates onto the surface. Standards containing varying concentrations of free hapten are prepared and premixed with antibody and, after a suitable incubation time, passed over the surface of the chip with protein-hapten conjugate immobilised. A proportion of the antibody has already bound to the free hapten, inhibiting further binding to the surface. Therefore, the amount of antibody that binds to the surface is inversely proportional to the amount of free hapten in solution (Figure 1.10). The results of this immunoassay may be used in the construction of a standard curve and unknown target analyte concentrations determined from such a curve.

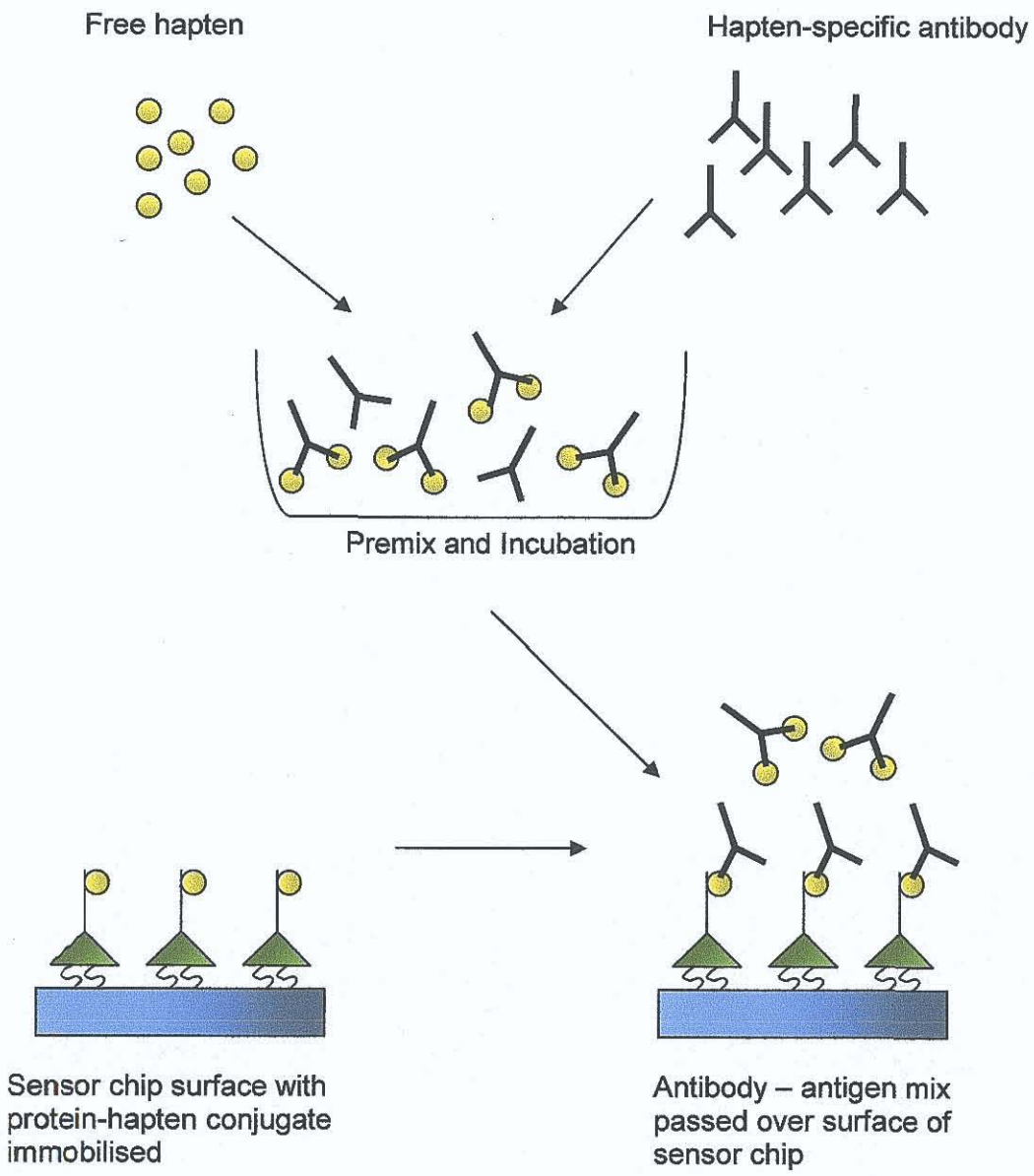

Figure 1.10 *Schematic representation of a Biacore inhibitive immunoassay format for quantatitive determination of a hapten. Standard of free hapten and antibody were premixed, incubated, and passed over the surface of a chip with protein-hapten conjugate immobilised on its surface. Any unbound antibody binds to the immobilised hapten. Antibody already bound to free hapten is subsequently washed away. The amount of immobilised antibody is, therefore, inversely proportional to the amount of free hapten in solution.*

1.5 Aims of Research

The aim of this project was to develop antibodies to Zilpaterol for use in competitive ELISA immunoassays and rapid and sensitive Biacore immunoassays for the analysis of these compounds. The aim was also to produce a recombinant form of Prostate-Specific Antigen (PSA) and to develop a competitive ELISA using commercial anti-PSA antibodies.

Chapter 3 describes the characterisation of anti-zilpaterol rabbit polyclonal antibodies and the subsequent development of a competitive ELISA immunoassay for the detection of free zilpaterol. Cross reactivity studies were then carried out with four β-agonists, and a number of other potential cross-reactants. The polyclonal antibodies were then used in the development of a Biacore-based inhibition immunoassay for the detection of free zilpaterol. The chapter also describes the development of a combinatorial phage display library using the system devised by Krebber *et al.* for the production of single chain Fv (scFv) antibodies that detect zilpaterol. Competitive ELISA and Biacore inhibition immunoassays were developed for the detection of zilpaterol using an affinity selected scFv antibody. Cross reactivity studies were also conducted using the same potential cross-reactants.

Chapter 4 describes the cloning, expression and purification of a recombinant form of prostate-specific antigen protein, and its use in the development of a competitive ELISA.

Chapter 2

Materials and Methods

2.1 Materials and equipment

2.1.1 Materials

All reagents and chemicals were supplied by Sigma-Aldrich Co. (Poole, Dorset, U.K.), unless listed below (Table 2.1).

Table 2.1

Reagent	Supplier
Acetic acid Hydrochloric acid Sodium chloride Tween 20 Parathion	Riedel de-Haen AG, Wunstorfer, Strabe 40, D-30926, Hannover, Germany.
Bacteriological Agar Tryptone Yeast Extract PBS tablets	Oxoid, Basingstoke, Hampshire, RG24 8PW, England.
Bicinchoninic acid assay (BCA) kit	Pierce and Warriner (UK) Ltd., Chester, England.
DNA ligase	Boehringer-Mannheim Ltd., East Sussex, England.
CM-Dextran	Fluka Chemicals, Gillingham, Dorset, England.
Hepes	Gibro BRL, Renfew Rd, Paisley PA49RF, Scotland.
HPLC-grade solvents	Lab-Scan, Stillorgan, Co. Dublin, Ireland.
Hydrogen peroxide	BDH Chemicals Ltd., Poole, Dorset, England.
PCR Optimizer Kit ProBond™ Resin	Invitrogen, 9704-CH-Groningen, Netherlands.
PCR Primers	Sigma-Genosys Ltd, Cambridge CB2, England.
Sfi1 enzyme	New England Biolabs, Hitchin, Herts., England.
VCSM13 Helper Phage	Stratagene, North Torrey Pines Rd. La Jolla, USA.
Wizard Plus Mini-prep kit and PCR prep DNA purification kit PCR-related Reverse-Transcription System	Promega Corporation, 2800 Wood Hollow Rd, Madison, WI 53711-5399, USA.

2.1.2 Equipment

Equipment	Supplier
Atto dual minislab AE-6450 Atto AE-6100	Atto Corp, Bunhyo-Kui, Tokyo 113, Japan.
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 Boelevard, South Plainfield, New Jersey, USA.
Titretek Twinreader Plus	Medical Supply Company, Damastown, Mulhuddart, Dublin 12, Ireland.
Image Master VDS	Pharmacia Biotech, San Francisco, CA., USA.
IR Spectrophotometer	The Nicolet Instrument Corp., Madison, USA.
NMR Spectrometer, 400MHz AC400	Brucker, Coventry, CV4 9GH, England.
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 clothing	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 Composition of culture media

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 broth (LB) medium	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
	Bacteriological agar	15 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
Expression Medium (EM) for soluble scFv antibodies	2 x TY medium	
	Chloramphenicol	25 μg/ml
Super optimal catabolites (SOC) medium	Tryptone	20 g/l
	Yeast Extract	5 g/l
	NaCl	0.5 g/l
	KCl	2.5 mM
	$MgCl_2$	20 mM
	Glucose	20 mM
	pH	7.0
Tryptone and yeast extract (TYE) medium	2 x TY medium	
	Bacteriological agar	15 g/l

2.1.4 Buffers

Phosphate buffered saline (PBS)

One tablet (Oxoid) was dissolved per 100 mls of distilled water according to manufacturer's instructions. When dissolved, the tablets prepare Dulbecco's A PBS which contains 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.

Tris buffered saline (TBS)

Tris buffered saline containing 1 mM $CaCl_2$, 0.05 M Tris, 0.15 M NaCl, pH 7.4, was prepared and used as the dilution buffer for the monoclonal anti-FLAG antibody (FLAG is a small hydrophilic tag which improves detection and purification of recombinant proteins). The anti-FLAG antibody is used for western blotting and ELISA analysis of soluble scFv's.

Tris-acetic acid-EDTA buffer (TAE)

A stock solution of 50 X TAE buffer was prepared 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 in a final volume of 1 litre. All gels were run in 1 X TAE.

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 2 M 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 (Sartorius) and degassed prior to use.

2.2 Characterisation of polyclonal antibodies

2.2.1 Licensing for drug use

The possession and use of zilpaterol for analytical purposes was licensed by the Department of Health.

2.2.2 Production of zilpaterol-specific polyclonal antibodies

Immunisation of New Zealand White female rabbits with covalently-linked zilpaterol-HSA conjugate and isolation of serum containing the zilpaterol-specific polyclonal antibodies was conducted by the Department of Agriculture and Rural Development for Northern Ireland, Queens University, Belfast. This antibody was designated as R699.

2.2.3 Competitive checkerboard ELISA to determine optimal zilpaterol and R699 polyclonal antibody concentrations

An ELISA plate (Nunc) was coated with varying dilutions of R699 polyclonal antibody (1/1600 to 1/25600) in 10 mM sodium acetate buffer, pH 7.2, with 100 µl of each dilution added to a different row on the ELISA plate and incubated for 1 hour at 37^0C. The plate was blocked with PBS containing 5% (w/v) milk marvel, incubated at 37^0C for 1 hour and then washed 3 times with PBS. A 1/4000 dilution (final dilution) of HRP-labelled zilpaterol was mixed with varying concentrations of free zilpaterol 0.48 to 500 ng/ml (final concentrations) and 100 µl of each was added to a different column on the ELISA plate. The plate was then incubated for 1 hour at 37^0C and washed 3 times with PBS containing 0.05% (v/v) tween (PBST) and 3 times with PBS. 100 µl OPD substrate (0.4 mg/ml *o*-phenylenediamine in 0.05 M phosphate citrate buffer, pH 5.0) was added per well and the plate was incubated for 30 minutes at 37^0C. Absorbance readings were taken at 450 nm.

2.2.4 Buffer optimisation for R699 polyclonal antibody ELISA

The ELISA was conducted using several different buffers in order to determine the optimal buffer for this assay.

2.2.4.1 Competitive ELISA to determine optimal R699 polyclonal antibody coating buffer

An ELISA plate (Nunc) was coated with a 1/1600 dilution of polyclonal antibody diluted in 4 different buffers; 10mM sodium acetate buffer, pH 7.2, PBS, pH 7.4, 0.05M carbonate buffer, pH 9.6 and 25mM TBS, pH 7.4. The plate was blocked with PBS containing 5% (w/v) milk marvel, incubated at 37^0C for 1 hour and then washed 3 times with PBS. 50 µl zilpaterol-HRP, to a final dilution of 1/4000, were added to each well of the plate, followed by 50 µl of free zilpaterol with concentrations ranging from 0.06 to 1000 ng/ml. The assay was completed as described in Section 2.2.3.

2.2.4.2 Competitive ELISA to determine optimal zilpaterol dilution buffer

An ELISA plate (Nunc) was coated with a 1/1600 dilution of polyclonal antibody diluted in 10mM sodium acetate buffer, pH 7.2, and incubated for 1 hour at 37^0C. The plate was blocked with PBS containing 5% (w/v) milk marvel, incubated at 37^0C for 1 hour and then washed 3 times with PBS. Zilpaterol-HRP, to a final dilution of 1/4000, and free zilpaterol, with dilutions ranging from 1000 ng/ml to 0.06 ng/ml (final dilutions), were added to the plate. The dilutions were made up in 4 different buffers; 10 mM sodium acetate buffer, pH 7.2, PBS, pH 7.4, 0.05M carbonate buffer, pH 9.6 and 25mM TBS, pH 7.4. The assay was completed as described in Section 2.2.3.

2.2.5 Competitive ELISA with R699 polyclonal antibody

An ELISA plate (Nunc) was coated with a 1/1600 dilution of polyclonal antibody in 10 mM sodium acetate buffer, pH 7.2, and incubated for 1 hour at 37^0C. The plate

was blocked with PBS containing 5% (w/v) milk marvel, incubated at 37^0C for 1 hour and then washed 3 times with PBS. Zilpaterol-HRP, to a final dilution of 1/4000, and free zilpaterol, with concentrations ranging from 0.06 to 1000 ng/ml were added to the plate. All dilutions were made in 10 mM sodium acetate buffer, pH 7.2. The assay was described in Section 2.2.3.

2.3 Production of a murine scFv antibody library to zilpaterol

2.3.1 Immunisation and total RNA extraction

Immunisation of BALB/c mice with zilpaterol-HSA conjugate and total RNA extraction from the spleen was conducted by Dr. Paul Dillon, Dublin City University.

2.3.2 Reverse transcription of mouse spleen mRNA

Complementary DNA (cDNA) was synthesised from the total RNA using a Promega cDNA synthesis kit containing random hexamer primers. 4 µl of mRNA was used per 20 µl total reaction volume, into which was also added $MgCl_2$, 2 µl 5x buffer, 2 µl dNTPs, 0.5 µl random primers, and brought to a final volume of 20 µl with RNAse free H_2O. The reaction was mixed and left at room temperature for 10 minutes to allow primer annealing, then incubated at 42°C for 1 hour to allow for cDNA synthesis. The presence of cDNA was then observed by gel electrophoresis.

2.3.3 Amplification of antibody light and heavy chain genes

2.3.3.1 PCR primers

The listed PCR primers were obtained from Sigma-Genosys Ltd and designed to be 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'gccatggcggactacaaaGAYGCTGTTGTGACTCAGGAATC3'

Variable light chain forward primers

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

LF2 5'ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTATTTCCAGCTTGG3'

LF3 5'ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTTATTTCCAACTTTG3'

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'

2.3.3.2 PCR amplification of light and heavy chain genes

Component	Volume added
cDNA	2 µl
dNTP	1 µl
5 X buffer	10 µl
Forward primers	1 µl
Reverse primers	1 µl
Ultrapure water	35 µl
Taq polymerase (5U/µl)*	5 µl

2.3.3.3 PCR conditions for amplification of light and heavy chain genes

Typical thermal cycling conditions were as follows:
95°C X 5 min 'Hot Start'
* *Taq* polymerase added to the reaction mix at this point

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 secs
72°C X 1 min
repeat X 24 cycles

72°C X 10 min

All ramping rates were at 4^0C/sec

2.3.3.4 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 400bp fragment was cut from the gel with a sterile scalpel and the DNA purified from the low melt agarose as follows. The excised band was placed into an eppendorf tube and weighed. Three volumes of buffer were added for every one volume of the gel fragment, and the eppendorf tube was incubated in a water bath at 50°C for 5 to 10 minutes until the agarose had melted. One volume of isopropanol equal to the original gel slice volume was added and the solution was mixed. The solution was added to a spin column and placed into a 2 ml collection tube. The column was then

centrifuged at room temperature for 1 minute at 14,000 rpm, and the filtrate discarded. 750 µl of wash buffer was added and the column centrifuged as before. The filtrate was discarded and the column added to a new 2 ml collection tube. 30 µl of ultra pure H_2O was added and the column centrifuged as before. The eluted DNA fragment was stored at – 20°C until required.

2.3.3.5 Quantification of purified PCR products

After purification the fragments were re-electrophoresed on an agarose gel with a quantatitive Molecular Weight Marker (Promega, 100 bp (100 – 4,000 range) Molecular Weight Markers), as the concentration must be determined prior to splice by overlap extention PCR (SOE-PCR). The 500 bp fragment of this marker was used as a reference for densiometric quantitation as 5 µl contains 150ng of the 500 bp fragment and 50ng of the other molecular weight fragments.

2.3.4 Components of splice by overlap extention (SOE) PCR

The purified light and heavy chain genes (approximately 400 bp each) were joined and amplified together to produce an 800 bp fragment by SOE-PCR.

SOE Primers

Single chain back	5′ttactcgcggcccagccggccatggcggactaccccg3′
Single chain forward	5′ggaattcggcccccgag3′

The components for amplification of joined antibody light and heavy chain genes by SOE-PCR are as follows:

Component	Stock concentration	Working concentration
10 X Buffer	10 X	1 X
dNTPs	20mM	0.4 mM
V_H	as determined	10 ng/reaction

V_L	as determined	10 ng/reaction
scfor	varied	0.05 nmol/reaction
scback	varied	0.05 nmol/reaction
upH_2O	to 50 µl including *Taq*, DNA and scfor, scback	
Taq polymerase	5 U/µl	5 U/reaction

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

94°C X 1 min
45°C X 5 min
72°C X 1 min
repeat X 5 cycles
*0.05 mM scfor and scback primers and 5U *Taq* polymerase added per reaction at this point

94°C for 1 min
63°C X 30 sec
72°C X 1 min
repeat X 25 cycles

All ramping rates were at 4^0C/sec

2.3.5 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 2 x TY supplemented with 30 µg/ml tetracycline. Purification of the plasmid was carried out using the Wizard miniprep system as follows:

The 5 ml culture was centrifuged for 5 minutes at 4000 rpm, the supernatant decanted and the 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 a sterile 1.5 ml microcentrifuge tube, 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 14000 rpm for 10 minutes at room temperature. The cleared lysate was transferred (approx. 850 µl) to the prepared spin column by decanting. The supernatant was again centrifuged at 14000 rpm for 1 minute at room temperature, the spin column removed from the tube and the flow through discarded. 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 spin column. The supernatant centrifuged at 14000 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 14000 rpm for 2 minutes at room temperature, and the spin column subsequently transferred to a new sterile 1.5 ml microcentrifuge. 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 14000 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.

2.3.6 Digestion of pAK100 and antibody light and heavy chain genes SOE-PCR products

Digestion of pAK100 and SOE-PCR products were performed using the *Sfi*1 restriction enzyme. pAK100 requires digestion with *Sfi1* restriction enzyme prior to ligation with light and heavy chain genes. Antibody light and heavy chain gene SOE-PCR products are also digested with *Sfi1* in order to facilitate ligation.

Components

5 µl reaction buffer (10X)

0.5 µl BSA

0.5 µl *Sfi 1* (10 U)

150-250 ng/reaction SOE-PCR product or 250-500 ng/reaction pAK100 vector

Sterile ultrapure water was added to a final volume of 50 µl.

The mixture was incubated at 50°C overnight. The fragments were purified using a Promega purification kit, as described previously in Section 2.2.3.4.

2.3.7 Ligation of antibody light and heavy chain genes into the pAK 100 vector

Purified SOE-PCR products were ligated into the pAK100 plasmid vector at a ratio of 1.5:1. The reaction was carried out under the following conditions:

Components

300 ng pAK100 vector

200 ng purified SOE light and heavy chain genes

4 µl ligase (4 U)

5 µl reaction buffer (10 X)

Sterile ultrapure water was added to give a final volume of 50 µl

The reaction mixture was incubated at 16°C overnight and transformed into *E. coli* XL1-blue competent cells.

2.3.8 Preparation of high efficiency competent cells

High efficiency competent cells were prepared as previously described by Inoue *et al.* (1990). A stock of *E. coli* XL1-Blue was streaked on 2xTY agar containing 25 μg/ml chloramphenicol and incubated at 37^0C overnight. Approximately 10-12 large colonies were removed from the plate and inoculated into 250 ml of SOB medium containing 25 μl/ml chloramphenicol in a 2 litre baffled flask. The culture was grown at 18°C with vigorous shaking (200-250 rpm) until an OD_{600} of 0.6 is reached. The culture was then placed on ice for 10 minutes before being transferred to two 250 ml centrifuge tubes and spun at 4000 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 55mM $MnCl_2$, sterile filtered and stored at 4°C), placed on ice for 15 minutes, pooled and centrifuged as before. The cell pellet was resuspended in 20 ml of ice-cold TB buffer and DMSO added slowly with gentle swirling to a final concentration of 7% (v/v). After incubation in an ice bath for 10 minutes the cell suspension was dispensed in 0.4 ml aliquots into microcentrifuge tubes. The cells were immediately flash frozen in liquid nitrogen and stored at –80°C.

2.3.9 Transformation of *E. coli* XL1-Blue competent cells with pAK 100 vector containing light and heavy chain genes and measurement of transformation efficiencies

Competent *E. coli* XL1-Blue (Stratagene) cells were thawed gently on ice. The cells were gently mixed by hand and 200 μl transferred into two 20 ml sterilin tubes (one tube for the experimental transformation and the other tube for the control transformation). 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 in the same manner. Both tubes were then incubated on ice for 30 minutes. The cells were then heat pulsed for 30 seconds at 42^0C followed by incubation on ice for 2 minutes. 0.8 ml of SOC medium, preincubated at 37°C, was then added and the tubes incubated at 37°C for 1 hour with shaking at 225-250 rpm. 100 μl of the resulting pAK100 transformants were plated

out onto LB media supplemented with 25 μg/ml chloramphenicol and 1% (w/v) glucose. The control transformants were plated out on LB agar supplemented with 25 μl/ml 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 Production of scFv antibodies to zilpaterol

2.4.1 Rescue of scFv displaying phage

50 ml of non-expression (NE) media (2 x TY containing 1% (w/v) glucose and 25 μl/ml chloramphenicol) was inoculated with 100 μl cells from the stock antibody library. The culture was grown with shaking at 37°C until the $O.D._{550}$ = 0.5, then 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 low expression (LE) medium (NE medium containing 0.5 mM IPTG) and shaken for 2 hours at 26°C to allow phage production. 30 μg/ml kanamycin was added to the culture and it was incubated as before for a further 8 hours. A phage particle precipitation was then done using PEG/NaCl to purify and concentrate the phage.

2.4.2 PEG/NaCl precipitation

150 ml of the *E. coli* XL1-blue/library was transferred to a sterile Sorvall 250 ml centrifuge tube and spun at 10,000 rpm for 10 minutes at 4°C. The supernatent was collected and 20% of the volume of PEG/NaCl (36.525g NaCl and 50g PEG 10,000 in 250 ml H_2O) was added, mixed and incubated on ice for 1 hour. The mixture was then centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatent was removed and the pellet was centrifuged again at 7000 rpm for a further 10 minutes at 4°C. Any remaining supernatent was removed. The pellet was then resuspended in 2 ml sterile filtered PBS, then micro-centrifuged at 4000 rpm for 10 minutes at 4°C to remove any

bacterial debris. The phage-containing supernatant was retained and stored at 4°C for the short term, or at –80°C in a 15% (v/v) glycerol solution.

2.4.3 Phage titre

5 ml of 2 x TY containing 30 μg/ml tetracycline was inoculated with a single colony of XL1-blue and grown overnight at 37°C. The culture was then diluted 1/100 in 5 ml of 2 x TY containing 30 μg/ml tetracycline and left to grow shaking at 37°C until $O.D._{550}$ = 0.5-0.6. It was allowed to stand at 37°C for 15 minutes without shaking. Serial dilutions (10^{-1} – 10^{-11}) of phage were prepared using the bacterial culture (180 μl *E. coli* XL1-blue + 20 μl phage) and incubated for 30 minutes at 37°C without shaking. 100 μl of each dilution was spread on 2 x TY agar plates containing 25 μg/ml chloramphenicol and 1% (v/v) glucose. Colonies were allowed to grow overnight at 37°C.

2.4.4 Affinity selection of antigen binders by biopanning

A NUNC immunotube was coated with 10 μg/ml of zilpaterol-HSA conjugate. The tube was washed 5 times with PBS containing 0.05% (v/v) tween (PBST) followed by 5 washes with PBS. The surface 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 containing 2% (w/v) milk marvel. This was incubated with gentle shaking for 2 hours at room temperature. The tube was then washed as before. The bound phage were eluted from the tube by adding 1 ml of 1 mM triethylamine to the tube. The tube was mixed on a rotating wheel for 8-10 minutes, then neutralised using 500 μl of 1 M Tris-HCl, pH 7.4. A phage titre was then carried out, as described in Section 2.3.3, to determine the amount of phage recovered from the panning step.

2.4.5 Reinfection of phage displaying scFv antibodies

5 ml of 2xTY containing 30 μg/ml tetracycline was inoculated with 50 μl XL1-blue stock and grown with shaking overnight at 37°C. The culture was then diluted 1/100

in 5 ml 2xTY containing 30 μg/ml tetracycline and grown shaking until the $O.D._{550}$ = 0.4-0.5. It was then placed at 37°C without shaking for 10 minutes. 750 μl of phage rescued from the panning step was added and the culture was allowed to stand at 37°C for 30 minutes. It was then shaken at 37°C for 1 hour before being spread on 2xTY agar plates containing 25 μg/ml chloramphenicol and colonies allowed to grow overnight at 37°C.

2.4.6 Preparation of clones for phage ELISA

Single colonies were selected from plates and used to inoculate individual wells of a 96 well cell culture plate, each containing 200 μl 2xTY containing 1% (v/v) glucose, 25 μg/ml chloramphenicol and 30 μg/ml tetracycline. Two wells were left uninoculated as controls. The plate was shaken at 150 rpm overnight at 37°C to allow growth of the colonies. This master plate was used to inoculate an identical plate, before addition of 15% (v/v) glycerol to each well and storage of the plate at -80°C. The second plate consisted of 180 μl/well 2xTY containing 1% (v/v) glucose, 25 μg/ml chloramphenicol and 30 μg/ml tetracycline. 20 μl from each well on the master plate was transferred to the corresponding well on the second plate, and incubated shaking at 37°C for approximately 6-8 hours. 25 μl of 2xTY containing 1% (v/v) glucose, 25 μg/ml chloramphenicol, 1.5 mM IPTG and 5 x 10^9 phage/ml were added to each well after 5 hours. The culture was then allowed to stand for 30 minutes at 37°C followed by shaking at 26°C at 150 rpm for 2 hours. The plate was then centrifuged at 4000 rpm for 10 minutes. The supernatant was removed and the pellets were resuspended in 200 μl 2xTY containing 1% (v/v) glucose, 25 μg/ml chloramphenicol, 1.5 mM IPTG and 30 μg/ml kanamycin. The plate was incubated overnight at 26°C with shaking at 150 rpm, then centrifuged at 4000 rpm for 10 minutes. 75 μl of the supernatant were used for analysis of the phage by ELISA.

2.4.7 ELISA of phage-displayed scFv antibodies

An ELISA plate was coated with 5 μg/ml zilpaterol-HSA conjugate and incubated overnight at 4°C. The plate was then blocked with PBS containing 5% (w/v) milk marvel for 1 hour at 37°C, then washed 3 times with PBS. 75 μl of supernatant from

the culture plate was added to the corresponding wells on the ELISA plate. 25 µl of 4% (w/v) milk marvel-PBS was also added into each well and mixed gently. The plate was left for 2 hours at room temperature, then washed 3 times with PBST and 3 times with PBS. 100 µl of a 1/1000 dilution of an anti-M13 bacterophage rabbit antibody was added to each well and the plate was incubated for 1 hour at 37°C, then washed as before. 100 µl of a 1/2000 dilution of HRP-labelled anti-rabbit antibody per well was added and incubated and washed as before. 100 µl OPD substrate (0.4 mg/ml *o*-phenylenediamine in 0.05 M phosphate citrate buffer, pH 5.0) was added per well and the plate was incubated for 30 minutes at 37°C. Absorbance readings were taken at 450 nm.

2.4.8 Preparation of bacterial glycerol stocks

Bacteria cells were inoculated into 2xTY media containing chloramphenicol and grown at 37°C until the $O.D._{600}$ was 0.5-0.8. The cultures were then centrifuged at 4000 rpm for 10 minutes and the pellet resuspended in 2xTY with 20% (v/v) sterile glycerol to 1/5 the original volume. Cell/glycerol cultures were aliquoted into 0.5 ml aliquts in 1.5 ml sterile microcentrifuge tubes and flash frozen with liquid nitrogen. Cell stocks were stored at –80°C.

2.4.9 Isolation of the scFv gene from the pAK100 vector within the positive clones

10 µl of cells from each positive clone identified was taken from the master plate and used to inoculate 10 ml NE medium. This was grown overnight at 37°C. A plasmid prep was then prepared on the culture using the Wizard miniprep system as described in Section 2.3.5 to isolate the pAK100 vector containing the scFv gene. The purified pAK100 vector was digested with the *SfiI* digestion enzyme, as described in Section 2.3.6, and the product was run on a 0.5% (w/v) low-melt agarose gel containing 0.5 µg/ml ethidium bromide, for 2 hours at 50 V until the 800 bp fragment containing the light and heavy chain genes has separated from the digested pAK100 vector. The 800 bp fragment was cut from the gel with a sterile scalpel and the DNA purified from the

gel as described in Section 2.3.3.4. The fragment was then ready for ligation into the pAK400 vector.

2.4.10 Ligation of scFv gene into pAK400 for soluble scFv antibody expression

Ligation of the scFv gene into pAK400 was performed using the same protocol as described in Section 2.3.7 for ligation into pAK100.

2.4.11 Preparation of competent *E. coli* JM83 bacterial cells

Preparation of competent *E. coli* JM83 cells was performed using the same protocol as described in Section 2.3.8 for the preparation of XL-1 Blue competent cells. 25 μg/ml streptomycin-sulphate was the required antibiotic in this case.

2.4.12 Transformation of *E. coli* JM83 competent cells with pAK400 vector

E. coli JM83 competent cells were transformed with the pAK400 vector containing the scFv gene insert. The transformation was carried out as described in Section 2.3.9.

2.4.13 Soluble expression of scFv antibodies from JM83 cells containing the pAK400 vector with scFv gene insert

The transformed *E. coli* JM83 cells were grown in 2xTY media containing 25 μg/ml chloramphenicol with shaking at 37°C until an $O.D._{600}$ = 0.5-0.8 was reached. Soluble scFv production was induced by addition of 1 mM IPTG and the culture was incubated with shaking for a further 4-5 hours at 25°C. The culture was then centrifuged at 4000 rpm for 15 minutes and the supernatant stored for analysis. The cell pellets were resuspended in 1/10 the original volume of PBS and sonicated for 30 seconds/1 ml aliquot or 3 minutes/10 ml aliquot at an amplitude of 40 using a microtip Vibra Cell™ sonicator. The cell debris was removed by centrifugation at room temperature, at 14000 rpm for 5 minutes. This supernatant was also retained for analysis.

2.4.14 Non-competitive ELISA for the determination of soluble scFv

A 96 well ELISA plate (Nunc) was coated with 5 μg/ml of zilpaterol-HSA conjugate and incubated for 1 hour at 37^{0}C or overnight at 4°C. The plate was then blocked with 200 μl of PBS containing 5% (w/v) milk marvel and incubated at 37°C for 1 hour. The plate was washed 3 times with PBS. Dilutions of the supernatant containing the scFv antibody were prepared and 100 μl of each dilution were added to the wells of the plate in triplicate. The plate was again incubated at 37°C for 1 hour and washed with 3 times with PBS and 3 times with PBST. 100 μl of a 1/400 dilution of anti-flag antibody in PBS was added to each well, and the plate incubated and washed as before. 100 μl of a 1/2000 dilution of an HRP-labelled anti-mouse antibody in PBS was then added to each well and the plate incubated and washed as before. 100 μl of OPD substrate was then added to each well, and the absorbance was read at 450 nm after 30 minutes using a Titretek Plate Reader Plus.

2.4.15 Checkerboard ELISA for determination of the optimal concentrations of immobilised zilpaterol-HSA conjugate and scFv antibody dilution for generation of a competitive ELISA

A 96-well ELISA plate (Nunc) was coated with varying zilpaterol-HSA conjugate concentrations (5 μg/ml, 2.5 μg/ml, 1.25 μg/ml), with 100 μl of each concentration added to a different row on the ELISA plate and incubated for 1 hour at 37°C. The plate was then washed 3 times with PBS. The plate was blocked with PBS containing 5% (w/v) milk marvel, incubated at 37°C for 1 hour and washed 3 times with PBS. Serial dilutions of the supernatant containing the scFv antibody were prepared in PBS and 100 μl of each dilution were added to a different column on the ELISA plate. The plate was again incubated at 37°C for 1 hour and washed with 3 times with PBST and 3 times with PBS. 100 μl of a 1/400 dilution of anti-FLAG antibody in PBS was added to each well, and the plate incubated and washed as before. 100 μl of a 1/2000 dilution of an HRP-labelled anti-mouse antibody in PBS was then added to each well and the plate incubated and washed as before. 100 μl of OPD substrate was then added to each well, and the absorbance was read at 450 nm after 30 minutes using a Titretek Plate Reader Plus.

2.4.16 Competitive ELISA for soluble scFv antibody characterisation

A 96-well ELISA plate (Nunc) was coated with the optimal concentration of the zilpaterol-HSA conjugate, and incubated for 1 hour at 37°C. The plate was blocked with PBS containing 5% (w/v) milk marvel, incubated at 37°C for 1 hour and washed 3 times with PBS and 3 times with PBST. The optimised dilution of scFv-containing supernatant was mixed with varying concentrations of free zilpaterol (10 µg/ml to 4.9 ng/ml, final concentrations) and incubated at 37°C for 30 minutes. 100 µl of each dilution was added to the ELISA plate in duplicate. The plate was then incubated for 1 hour at 37°C and the assay completed as described in Section 2.4.13.

2.5 Cloning of the gene sequences encoding PSA into *E. coli*

2.5.1 Preparation of PSA gene

The gene encoding the PSA protein was donated by Dr. Evaggelia Emmanouilidou from the Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Greece (Emmanouilidou *et al.*, 2002). The gene insert was provided as an insert in the psa.pcDNA3 vector.

2.5.2 Transformation of psa.pcDNA3 vector into *E. coli* JM83 cells

E. coli JM83-competent cells were transformed with the psa.pcDNA3 vector. The transformation was carried out as described in Section 2.3.9.

2.5.3 Isolation of PSA gene

Single colonies were selected from the transformation plates and used to inoculate 10 ml NE medium. These were grown overnight at 37°C. A plasmid prep was then prepared on the culture using the Wizard miniprep system (as described in Section 2.3.5) to isolate sufficient quantities of the psa.pcDNA3 vector containing the PSA gene. The purified psa.pcDNA3 vector could then be used as the template from which the PSA gene was amplified.

2.5.4 PCR amplification of PSA gene

Component	Volume added
Purified psa.pcDNA3 vector	2 µl
dNTP (10 mM)	4 µl
10 X buffer	10 µl
Forward primers (100pmol/µl)	1 µl
Reverse primers (100pmol/µl)	1 µl
Ultrapure water	77 µl
Taq polymerase (5U/ µl)	5 µl

The PCR conditions for amplification of the PSA gene were as follows:

Stage 1:	95°C for 10 minutes
Stage 2:	95°C for 1 minute
	50°C for 30 seconds
	72°C for 1 minute

(Stage 2 was repeated for 30 cycles)

Stage 3:	72°C for 10 minutes

2.5.5 Purification of PCR reaction products

PCR purification was performed using the Wizard PCR prep DNA purification kit (Promega) as described in Section 2.3.3.4

2.5.6 Quantification of purified PCR products

After purification the fragment was 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 ligation. The 500 bp fragment of this marker was used as a reference for densiometric quantitation as 5 µl contains 150ng of the 500 bp fragment and 50ng of the other molecular weight fragments.

2.6 Inhibition ELISA for the detection of recombinant PSA

All the wells of a microtitre plate were coated with 100 µl neat recombinant PSA and the plate was then incubated at 37°C for 1 hour. The plate was washed 3 times with PBS and blocked with 200 µl of 5% milk marvel for 1 hour at 37°C. The plate was then washed 3 times with PBS. Doubling dilutions of the recombinant PSA (i.e. 1/2, 1/4, 1/6 etc) were made in PBS and pre-incubated with a fixed concentration of commercial rabbit-anti-PSA polyclonal antibody (at a final dilution of 1/4000). Samples were incubated at room temperature for 30 minutes. 100 µl of each sample were added to the wells of the plate in triplicate. To the last three wells, a 1/4000

dilution of antibody alone was added. The plate was incubated at 37°C for 1 hour, washed 3 times with PBS-tween and 3 times with PBS. 100 µl of a 1/2000 dilution of anti-rabbit AP-labelled antibody was added to each well, was incubated and washed as before. 100 µl of pNPP substrate was added to each well. Readings were taken at 405 nm after 30 minutes.

2.7 Development of SPR-based immunoassay using a Biacore 3000 instrument

Analysis was carried out on Biacore 3000 instrument using CM5 research grade 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.

2.7.1 Preconcentration studies

Immobilisation of proteins on the gold surface was achieved by means of N-hydroxysuccinimide esterification. Primary amine groups present allow covalent attachment of biomolecules. It is necessary to carry out 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 to take place. Preconcentration of a desired protein on the sensor surface can be facilitated by adjusting the pH below the isoelectric 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.

2.7.2 Immobilisation of protein G

The carboxymethylated dextran matrix was activated by mixing equal volumes of 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (N-ethyl-N-(dimethylaminopropyl) carbodiimide hydrochloride) and injecting the mixture over the sensor chip surface for 7 minutes at a flowrate of 5 μl/minute. The protein G was diluted in 10 mM sodium acetate at the optimised pH, at a typical concentration of 50-100 μg/ml. This solution was then passed over the derivatised chip surface for 30-45

minutes. Unreacted NHS groups were capped, and non-covalently bound protein removed by injection of 1 M ethanolamine hydrochloride, pH 8.5, for 7 minutes.

2.7.3 Inhibition immunoassay for the detection of zilpaterol using R699 anti-zilpaterol polyclonal antibody

Standards of free zilpaterol were prepared at a range of concentrations of between 0.02 and 500 ng/ml (final concentrations). Each sample was mixed with a 1/1600 dilution of polyclonal antibody, and allowed to equilibrate for 30 minutes at 37°C. The samples were then passed over the sensor surface in random order. The surface was regenerated between cycles by pulses 5 µl of 15 mM NaOH being passed over the surface. A calibration curve was constructed by plotting the change in response (RU) for each standard against the log of concentration.

2.7.4 Inhibition immunoassay for the detection of zilpaterol using the anti-zilpaterol scFv antibody

Standards of free zilpaterol were prepared at a range of concentrations between 0.98 ng/ml and 1 µg/ml (final concentration). Each sample was diluted 1/10 with cellular extract obtained after sonication. (An induced culture was centrifuged at 4000rpm for 20 minutes and the cell pellets were resuspended in 1/10 the original volume of PBS. The suspension was sonicated until cell lysis was achieved and the cell debris removed by centrifuging at room temperature at 14,000 rpm for 15 minutes. The supernatant, or cellular extract, contained the scFv). These solutions were allowed to equilibrate for 30 minutes at 37°C. The assay was then carried out as described in Section 2.6.4.

Chapter 3

Production and Application of Polyclonal Antibodies and scFv antibody fragments to Zilpaterol

3.1 Introduction

3.1.1 The Beta-agonists

Beta-agonists belong to a group of compounds called the catecholamines. They can be catalogued into three chemical classes according to their structure: anilines, resorcinols and phenols. The beta-agonists enhance growth efficiency by stimulation of beta-adrenergic receptors on cell surfaces. Stimulation of these receptors results in relaxation of smooth muscles. Beta-agonists are, therefore, frequently used as bronchodilators for the treatment of pulmonary disease in humans and animals. However, these compounds are also used as growth promoters in livestock production due to the anabolic effects exerted when higher doses are administered to the animals. β-adrenergic agonists have therefore been called 'repartitioning agents' and cause changes, such as increased live weight gain, increased carcass leanness and alterations in the ratio of muscle to fat tissue, and animal growth. Treated animals show these effects when the applied dose is five to ten times higher than necessary for therapeutic treatment (Courtheyn *et al.*, 2005).

Meat products obtained from treated animals may pose a potential risk for consumer health, mainly in persons with asthma or cardiac lesions. The danger that beta-agonists pose was demonstrated by several human poisoning incidences (Brambilla *et al.*, 2000). On the basis of such epidemiological evidence, the administration of beta-agonists to food-producing animals as growth promoters has been completely banned by Council Directive 96/22/EC. Their use has also been prohibited in the USA and Asia.

While clenbuterol is the most effective beta-agonist as a growth-promoting agent, in recent years other synthetic beta-agonists such as mabuterol or salbutamol have also been used, although their efficiency is lower than clenbuterol. Ractopamine is a beta-agonist belonging to the phenolic group. It was commercially developed in the USA (Eli Lilly), where its use is authorised, but in the EU the use of ractopamine is completely banned.

3.1.2 Zilpaterol

Zilpaterol has appeared as a new beta-agonist developed in South Africa and was approved for use in cattle as a growth promoter in South Africa and Mexico. However, its use in the EU, USA and Asia remains illegal. Zilpaterol is a powerful β-agonist, which is more effective than Ratopamine, but only 1/10 as effective as Clenbuterol. Structurally, Zilpaterol belongs neither to the group of anilinic (clenbuterol-like) nor the phenolic (salbutamol-like) beta-agonists (See Figure 3.1). Zilpaterol is capable of redirecting cellular metabolism in favour of protein synthesis. In muscle tissue, zilpaterol promotes protein synthesis and cell hypertrophy by inhibition of proteolysis (rate of protein systhesis is not affected). In adipose tissue, zilpaterol promotes lipolysis. In this way, zilpaterol functions as a repartitioning agent.

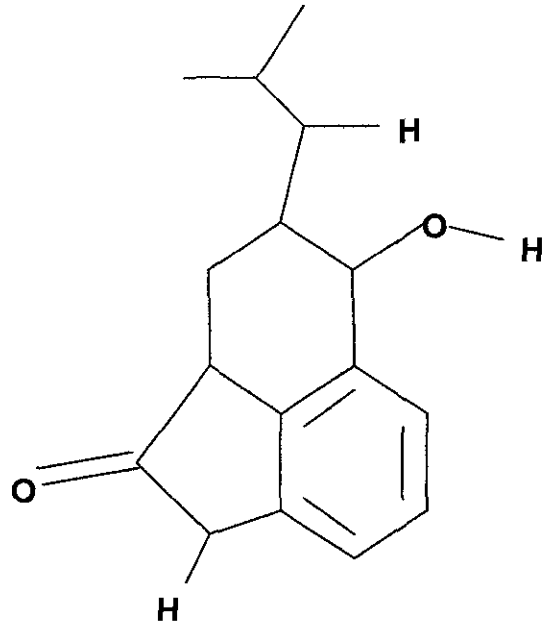

Figure 3.1 *Structure of zilpaterol.*

3.1.3 Detection of Zilpaterol

Regulatory agencies generally have relied on immunologically based rapid screening assays to screen large quantities of samples for the presence of illegal beta-agonist

residues. However, due to its chemical structure, zilpaterol was, until recently, not detected with commonly applied screening and confirmatory methods.

Bocca *et al.*, (2003) developed a method to identify and simultaneously quantify clenbuterol-like substances with anilinic moieties and drugs with phenolic and catecholic moieties, such as Ractopamine and Zilpaterol, in retinal tissue. After extraction in 0.1 N HCl, the samples were cleaned up on C18 non-endcapped solid-phase extraction columns and analysed as trimethylchlorosilane derivatives by gas chromatography/tandem mass spectrometry, in electron impact mode. At concentrations of agonists between 62.5 and 250 ng/g in bovine retina, mean recoveries ranged from 85.3 to 94.8%, repeatability was less than 9.6%, and within-laboratory reproducibility was less than 10.5%. The detection limits for this assay were within the range of 66.3 - 70.4 ng/g.

Shelver and Smith (2004) reported the development of an ELISA specific to zilpaterol. Zilpaterol was reacted with ethyl 4-bromobutyrate followed by refluxing in 0.1 M potassium hydroxide. The resulting hapten was reacted with two carrier proteins, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), using 1-ethyl-3-(3-dimethylaminopropyl)-carodiimide (EDC) as an activating agent. Immunisation of goats with the zilpaterol-butyrate-KLH resulted in an antibody useful for ELISA. Zilpaterol-butyrate-BSA was utilised as a coating antigen for ELISA development. The average IC_{50} derived from the developed zilpaterol immunoassay was 3.94 $\pm$ 0.48 ng/ml (n=25). The antibody did not cross react with other beta-agonists. The assay was tolerant of up to 10% (v/v) acetone, ethanol or methanol and 15% (v/v) of acetonitrile or DMSO. Salt concentrations ranging from 0.05 to 1.0M minimally affected B_0 (the absorbance reading obtained in the presence of zero antigen concentration) or IC_{50} values. When buffer pH was less than 7 or greater than 8.8, the IC_{50} values increased in comparison to those measured at pH 7.4.

A method was developed for the determination of three beta-agonists (one of which was zilpaterol) in liver and urine, by liquid chromatography / tandem mass spectrometry (Blanca *et al.*, 2005). The beta-agonists were identified after clean-up with mixed-mode SPE Bond Elut Certify cartridges (6ml, 300mg). This method of detection was developed and validated according to the Commission Decision

2002/657/EC. Decision limit (CCalpha) ranged from 40 to 110 pg/ml and 270 to 520 pg g^{-1} (ppt) for urine and liver, respectively.

A monoclonal antibody-based ELISA for zilpaterol was developed by Shelver *et al.*, in 2005. Mice immunised with zilpaterol-butyrate-keyhole limpet hemocyanin, were utilised for monoclonal antibody generation whereas zilpaterol-butyrate-bovine serum albumin was used as a coating antigen for ELISA. The resulting zilpaterol-specific antibodies underwent sensitivity and isotyping experiments, the best of which show high sensitivity to zilpaterol and some interaction with clenbuterol and terbutaline at high concentrations but not with other beta-agonist tested.

Shelver *et al.*, 2005 developed a biosensor method to measure zilpaterol in sheep urine. A CM-5 sensor chip, which had been previously reacted with ethylenediamine to produce an aminoethyl group, was coupled with 4-carboxybutyl zilpaterol activated using EDC/NHS. Five polyclonal and four monoclonal antibodies were screened for their suitability to detect low levels of zilpaterol using the biosensor technology. Total binding was greater for polyclonal than monoclonal antibodies, but a more concentrated antibody solution was required for polyclonal antibodies. A fixed antibody concentration and various concentrations of zilpaterol were injected to obtain a standard curve for each antibody to allow B_0 (the absorbance reading obtained in the presence of zero antigen concentration) in repeated experiments extending at least 6 hours. A measure of non-specific binding allowed the assessment of the specificity of the antibody-immobilised ligand interaction. The effect of varying concentrations of urine on B_0 and IC_{50} (50% of the mean absorbance at each antigen concentration / absorbance in the presence of zero antigen concentration) was evaluated to assess the degree of matrix effect that would be present in an assay. Based on these criteria the most promising antibody was selected for further evaluation. This antibody had good sensitivity with $IC_{50} = 4.47 \pm 0.41$ ng/ml (n=11). Both intra- and inter-assay variation studies showed excellent recovery and reproducibility for concentrations between 2 ng/ml and 8 ng/ml. A comparison of the biosensor method with a previously developed ELISA demonstrated that both methods give equivalent results (slope of the correlation plot = 1.02) with a high correlation ($r^2 = 0.91$).

Affinity-based sensors monitor the binding between two molecules. Such interactions may include enzymes, antibodies, whole cells, receptors nucleic acids and antigens (McCormack *et al.*, 1998). Recently, several commercial biosensors have been developed using surface plasmon resonance (SPR) technology as their detection method. In particular, the Biacore SPR-based biosensor has become a very important tool in the detection of a number of analytes, i.e. "real-time" observation of a specific biological event e.g. antibody–antigen interaction (Leonard *et al.*, 2003).

The principle behind SPR is described in the context of Biacore for convenience. At an interface between two media of different refractive indices, (e.g. glass and water), light being emitted from the side of the higher refractive index is partly reflected and refracted. Above a certain critical angle of incidence, the light is totally internally reflected and no light is refracted across the interface between the two surfaces of the different refractive indices.

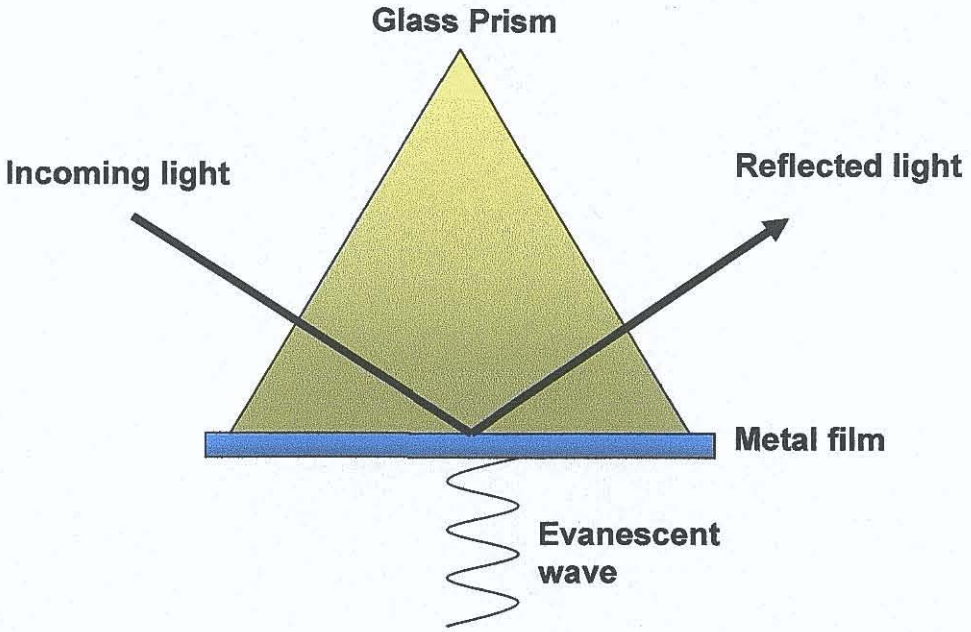

Figure 3.2 *Under conditions of total internal reflection at a metal-coated interface, and evanescent wave propagates into the medium of lower refractive index on the non-illuminated side.*

Under total internal reflection (TIR) conditions, an electromagnetic field component called the evanescent wave penetrates into the medium of lower refractive index a

short distance in the order of one wavelength (Figure 3.2). As the evanescent wave moves further away from the interface into the lower dense medium, the wave decays exponentially. The interface between the media is coated with a thin layer of metal (in the case of Biacore, this metal layer is gold), containing electron clouds at the surface. The passage of the evanescent wave through this metal layer causes the plasmons to resonate which results in a quantum mechanical wave known as a surface plasmon. Some of the energy of the reflected light (incident light) is taken up by the surface plasmon wave, resulting in a dip in the intensity of reflected light at a certain angle being observed (Panayotou *et al.,* 1993). The incident light angle at which this dip is observed is known as the SPR angle. The SPR angle is dependent on a number of factors i.e. properties of the metal film, the wavelength of the polarisation of incident light and the refractive index of the media on either side of the metal film. In real-time BIA, these properties are kept constant. The SPR signal can then be used to monitor the refractive index of the aqueous layer immediately adjacent to the gold metal layer. Changes in the refractive index are a direct result of changes in the mass or concentration on the surface of the chip and this characteristic of SPR has been used to monitor biological interactions. The change in reflected light is interpolated as a sensorgram.

The Biacore biosensor has a number of major advantages compared with conventional detection techniques. The absence of a need for labelling is of major significance as it minimises interference with the binding interaction being studied and also eliminates expensive and time-consuming purifications in many situations. Real-time analysis of interactants is also one of the main advantages of the Biacore instrument as interactions may now be monitored as they occur, providing valuable diagnostic information as well as kinetic data. Further advantages include the ability of the instrument to allow rapid and automated analysis.

Research has now focused on more mechanised, rapid and reliable assays. Biosensor technology has now emerged as a useful tool in immunodetection strategies for a variety of analytes. Biosensors use a combination of biological receptor compounds (antibody, enzyme, nucleic acid, etc.) and the physical or physico-chemical transducer directing, in most cases, "real-time" observation of a specific biological event e.g. antibody–antigen interaction (Leonard *et al.,* 2003).

3.2 Results

3.2.1 *Development of a competitive enzyme-linked immunosorbent assay (ELISA) for zilpaterol*

Serum containing anti-zilpaterol polyclonal antibodies was produced by Dr. Lisa Connolly of Queens University, Belfast. This polyclonal antibody was designated R699. A competitive ELISA was developed for the detection of free zilpaterol in solution using this antiserum.

3.2.1.1 Competitive checkerboard ELISA of R699 polyclonal antibody to determine optimal antibody and zilpaterol concentrations

The optimal working dilutions of serum and of free zilpaterol were determined using the protocol described in Section 2.3.3. The results were plotted as shown in Figure 3.3. The optimal serum coating dilution was found to be 1/1600. (Serum dilutions between 1/1600 and 1/102400 were made due to the limited amount of serum available). The optimal range of free zilpaterol concentrations for use in the development of a competitive ELISA was determined to be between 0.48 to 500 ng/ml.

3.2.1.2 Polyclonal antibody ELISA buffer optimisation

3.2.1.2.1 Competitive ELISA to determine optimal coating buffer

The format of the competitive ELISA performed to determine the optimal coating buffer was described in Section 2.2.4.1. The results were plotted as shown in Figure 3.3. The optimal serum coating dilution buffer was found to be sodium acetate.

3.2.1.2.2 Competitive ELISA to determine optimal zilpaterol dilution buffer

The ELISA protocol for the determination of the optimal zilpaterol dilution buffer is described in Section 2.2.4.2. The results were plotted as shown in Figure 3.4. It was determined that sodium acetate was the optimal buffer.

3.2.1.3 Development of a competitive ELISA for the detection of zilpaterol

The immunoassay format described in Section 2.2.5 was used to determine the optimal range of detection of free zilpaterol. In order to determine the inter-day assay variation, the ELISA was then repeated three times over three separate days. The normalised mean values, which were calculated as the mean absorbance at each antigen concentration / absorbance in the presence of zero antigen concentration (A/A0) over the three days were used to construct a calibration curve for the interday assay. Using BIAevaluation 3.1 software the calibration curve was plotted using the four-parameter equation. Comparisons were made between each zilpaterol concentration and its back-calculated counterpart concentration, as calculated from the calibration curve. The percentage accuracy for each concentration was calculated as the difference between the two (Table 3.1). Using these accuracy percentages to calculate the *limit of quantitation*, the range of detection for free zilpaterol was defined as 0.24 to 31.25 ng/ml (Figure 3.5). The coefficients of variation (CVs) between the three replicates of each cell concentration were determined by expressing the standard deviation as a percentage function of the mean. The CVs are shown in Table 3.1. The interday CVs ranged from 2.75 to 12.5%.

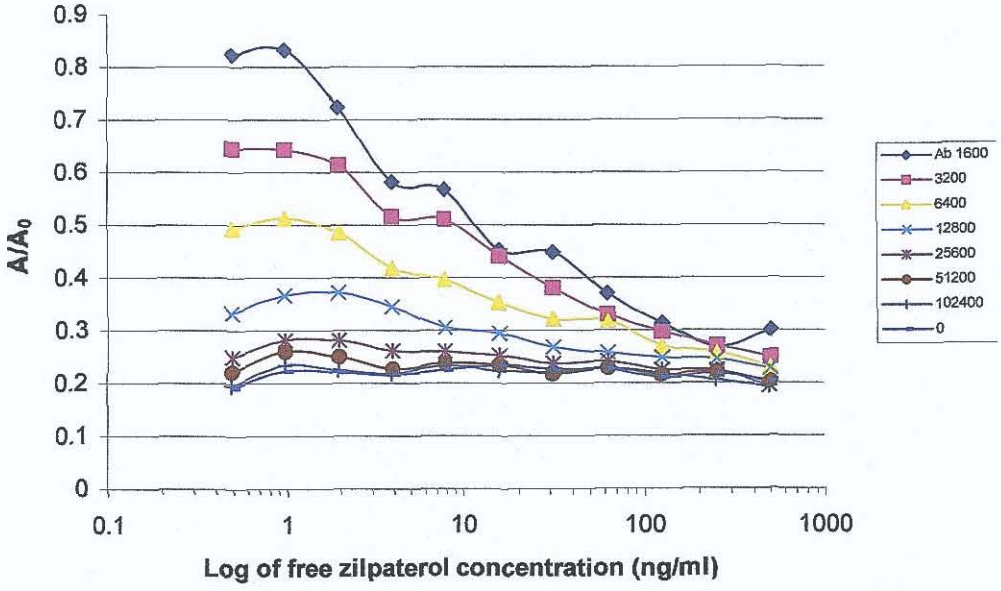

Figure 3.2 *Competitive checkerboard ELISA for free zilpaterol. Dilutions of R699 polyclonal antibody ranging from 1/1600 to 1/102400 were used to coat the wells of a microtitre plate. Free zilpaterol was added to the plate in concentrations ranging from 0.48 to 500 ng/ml. Zilpaterol-HRP, at a 1/4000 dilution (final concentration) was added simultaneously and allowed to compete with the free zilpaterol for binding to the antibody. The normalised mean values are the mean absorbance at each antigen concentration / absorbance in the presence of zero antigen concentration (A/A_0) over the three days. An antibody dilution of 1/1600 provided the best competitive results, while free zilpaterol dilutions of 0.48 to 500 ng/ml were deemed optimal to test the range of sensitivity of the antibody (Serum dilutions between 1/1600 and 1/102400 were made due to the limited amount of serum available).*

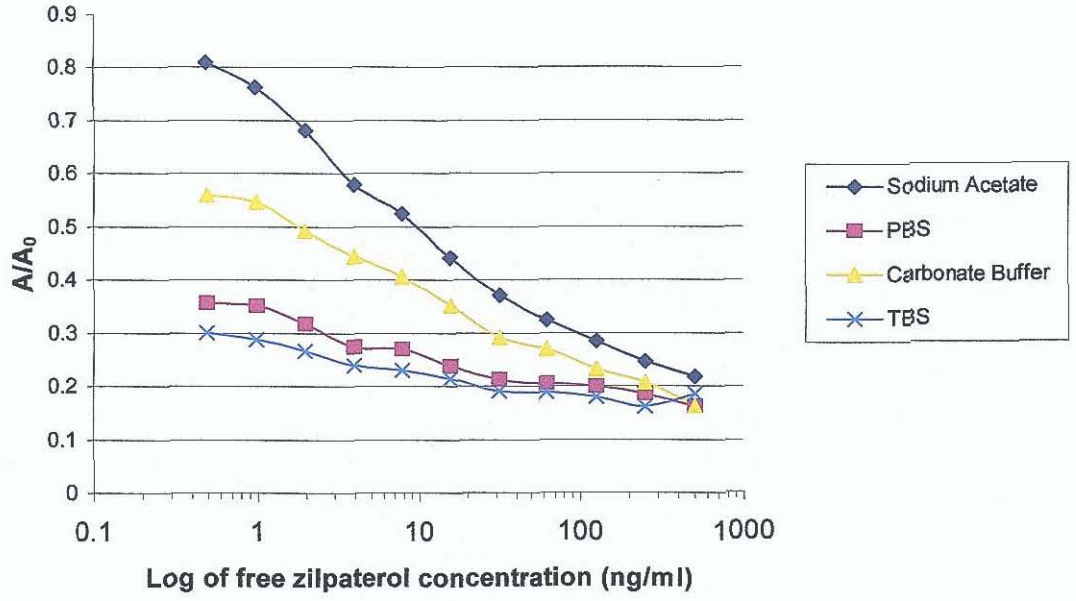

Figure 3.3 *Competitive ELISA with a 1/4000 dilution of zilpaterol-HRP conjugate, showing the comparison of assay sensitivity between four different coating buffers when R699 polyclonal antibody was diluted 1/1600 in each buffer. Sodium acetate proved to be the optimal coating buffer (A/A_0 represents the mean absorbance at each antigen concentration / absorbance in the presence of zero antigen concentration.*

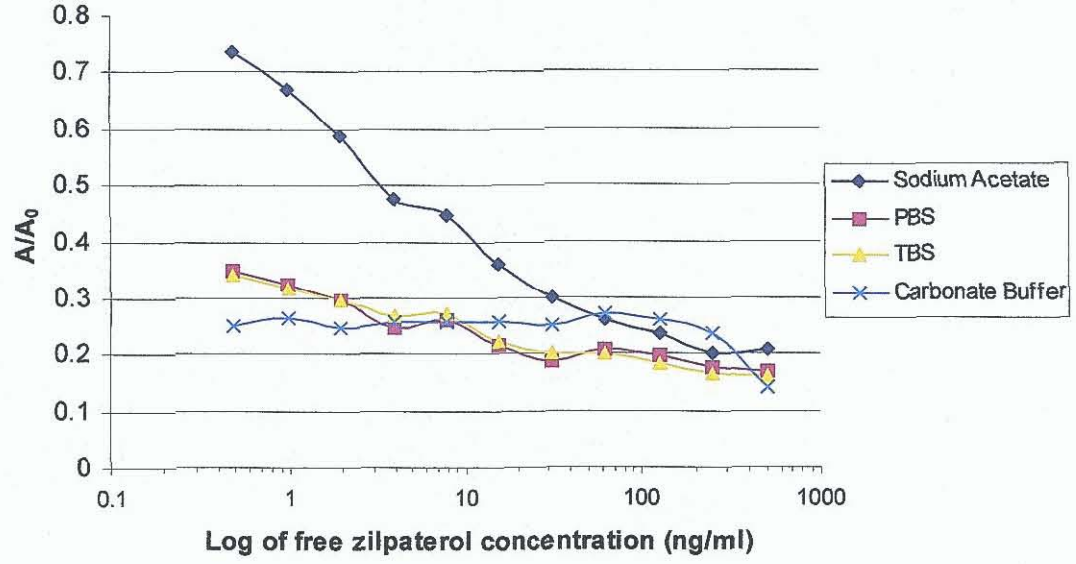

Figure 3.4 *Competitive ELISA with zilpaterol-HRP conjugate, showing the comparison of assay sensitivity between four different zilpaterol diluting buffers. Sodium acetate proved to be the optimal dilution buffer.*

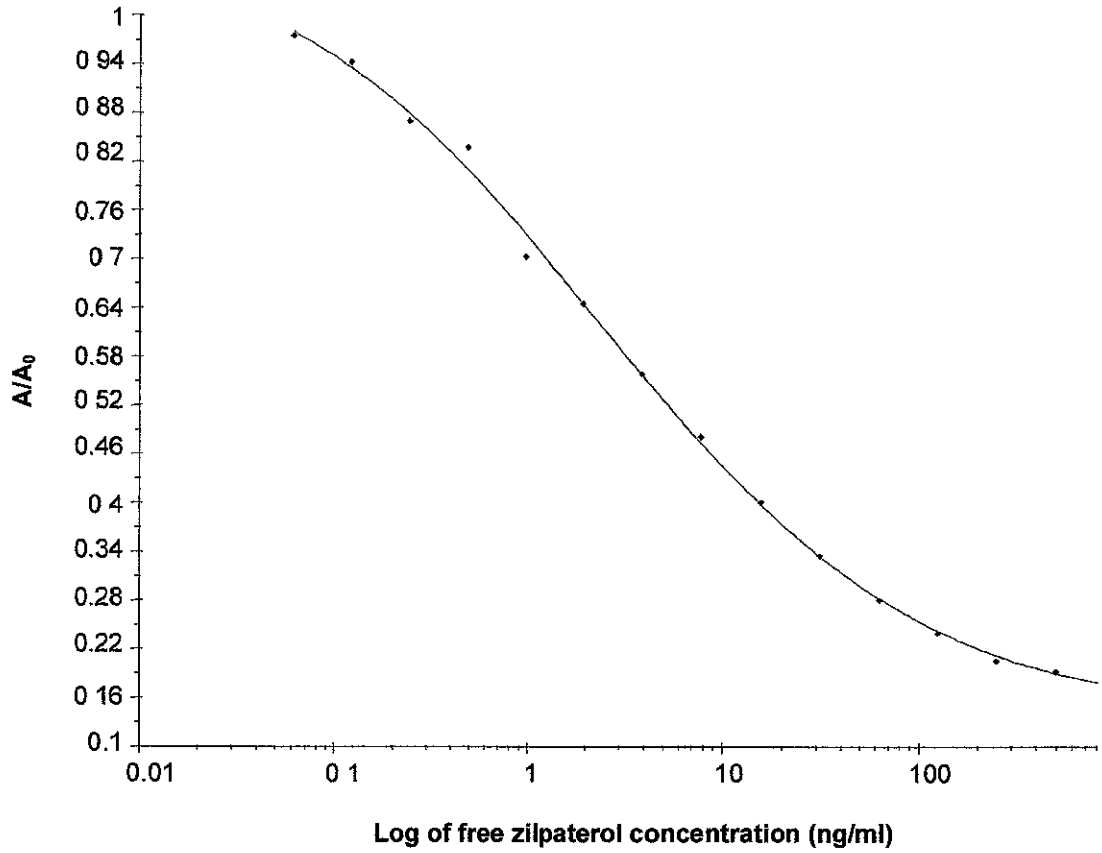

Figure 3.5 *Interday assay calibration curve for the R699 anti-zilpaterol polyclonal antibody in sodium acetate buffer A 4-parameter equation was fitted to the data set using BIAevaluation 3 1 software. The inter-assay coefficients of variance are tabulated in Table 1. Each point on the curve is the mean of three replicate measurements analysed over three days. The range of detection for free zilpaterol was calculated to be 0.24 to 31.25 ng/ml.*

Table 3.1 *Interday assay coefficients of variation (CV's) and percentage accuracies for R699 anti-zilpaterol polyclonal antibody in sodium acetate buffer. Interday studies were conducted and coefficients of variance (a quantitative measure of precision) calculated using the equation % CV = (S.D /Mean) x 100 where for intermediate precision (interday), the S.D is computed from replicate (three replicates) analyses over three validation runs on three separate days. The percentage accuracy for each concentration was calculated as the difference between each zilpaterol concentration and its back-calculated counterpart concentration, as determined from the calibration curve.*

Conc. (ng/ml)	**Back calculated conc. (ng/ml)**	**Calculated mean ± S.D**	**Coefficient of variation (%)**	**Accuracy (%)**
1000	802.94	0.02 ± 0.02	8.75	119.06
500	458.53	0.19 ± 0.02	12.59	108.29
250	303.63	0.21 ± 0.02	8.39	78.55
125	132.28	0.24 ± 0.03	11.9	94.18
62.5	64.86	0.28 ± 0.04	13.24	96.22
31.25	30.99	0.34 ± 0.04	11.18	100.83
15.63	15.25	0.40 ± 0.05	11.24	102.43
7.81	7.29	0.48 ± 0.03	6.71	106.66
3.91	3.86	0.56 ± 0.05	8.72	102.28
1.95	1.95	0.65 ± 0.05	7.34	100.00
0.98	1.23	0.70 ± 0.05	7.37	125.51
0.49	0.38	0.84 ± 0.05	6.19	122.45
0.24	0.27	0.87 ± 0.07	7.66	87.5
0.12	0.11	0.94 ± 0.03	3.32	108.33
0.06	0.06	0.97 ± 0.03	2.75	100.00

3.2.2 Cross-reactivity studies

The specificity of the polyclonal antibodies to zilpaterol is critical for the performance of the immunoassay. Cross reactivity studies were carried out with four β-agonists, and a number of other potential cross-reactants. Several related steroid compounds were chosen on the basis of their possible presence in real samples for analysis. These were zeranol, α-estradiol, β-estradiol, 19-nortestosterone and norethisterone. The ELISA was performed as described in Section 2.3, with equal concentrations of the drug to be tested added in place of zilpaterol. The displacement method described by Miller and Valdes (1993) was used to accurately estimate the levels of cross-reactivity, with the IC_{50} value defined as the analyte concentration that results in 50% displacement, or inhibition of the antibody, and the IC_{90} value defined as the analyte concentration that results in 90% displacement, or inhibition. Levels of cross-reactivity at IC_{50} (CR_{50}) were 100-fold the ratio between the IC_{50} values of zilpaterol and of the cross-reactant. IC_{90} (CR_{90}) levels of cross-reactivity were 100-fold the ratio between the IC_{90} values of zilpaterol and of the cross-reactant. The competitive ELISA for zilpaterol was repeated and plotted for comparison. The levels of cross-reactivity at the IC_{50} (CR_{50}) and IC_{90} (CR_{90}) are shown in Tables 3.2 and 3.3.

Table 3.2 *Cross-reactivity and specificity studies on R699 polyclonal anti-zilpaterol antibody against four β-agonists. The cross-reactivity potential was approximated at the IC_{90} value, which is the concentration of drug detectable at 90% A/A_0, and the IC_{50} value, which is estimated at 50% A/A_0. The CR_{90} and CR_{50} were then expressed as 100-fold the ratio of zilpaterol and of the cross-reactant.*

Drug	IC_{90}[a] (ng/ml)	IC_{50}[b] (ng/ml)	CR_{90}[c] (%)	CR_{50}[d] (%)
Zilpaterol	0.2	1	100	100
Mabuterol	200	-	0.1	-
Ractopamine	>1000	-	-	-
Clenbuterol	7	-	3	-
Salbutamol	200	-	0.1	-

[a] Least detectable dose calculated at 90% A/A_0

[b] 50% inhibition concentration (50% A/A_0)

[c] Percentage cross-reactivity determined at IC_{50}

[d] Percentage cross-reactivity determined at IC_{90}

Table 3.3 *Cross-reactivity and specificity studies on R699 polyclonal anti-zilpaterol antibody against several other potential cross-reactants The cross-reactivity potential was approximated at the IC_{90} value, which is the concentration of drug detectable at 90% A/A_0, and the IC_{50} value, which is estimated at 50% A/A_0. The CR_{90} and CR_{50} were then expressed as 100-fold the ratio of zilpaterol and of the cross-reactant.*

Drug	IC_{90}[a] (ng/ml)	IC_{50}[b] (ng/ml)	CR_{90}[c] (%)	CR_{50}[d] (%)
Zilpaterol	0.2	1	100	100
α-estradiol	>1000	-	-	-
19-nortestosterone	10	-	2	-
Zeranol	>1000	-	-	-
β-estradiol	>1000	-	-	-
Norethisterone	>1000	-	-	-

[a] Least detectable dose calculated at 90% A/A_0

[b] 50% inhibition concentration (50% A/A_0)

[c] Percentage cross-reactivity determined at IC_{50}

[d] Percentage cross-reactivity determined at IC_{90}

3.2.3 Development of a Biacore-based inhibition immunoassay for zilpaterol

3.2.3.1 CM5 Biacore chip directly immobilised with zilpaterol

A CM5 Biacore chip, which had been directly immobilised with zilpaterol, was obtained from Dr. Lisa Connolly of Queens University, Belfast.

3.2.3.2 Inhibition immunoassay for the detection of zilpaterol using R699 anti-zilpaterol polyclonal antibody

An inhibition assay was performed for the detection of free zilpaterol using the protocol described in Section 2.6.4. All dilutions were made in HBS buffer. Once equilibrated, the samples were passed over the sensor surface in random order, followed by regeneration with 15 mM NaOH. Interday variability studies were then carried out to estimate the reproducibility of the assay. To estimate the interday assay variation, the inhibition assay was conducted three times over three different days and the mean value of the response units of bound antibody for each zilpaterol concentration was plotted against the free zilpaterol concentration. Comparisons were made between each zilpaterol concentration and its back-calculated counterpart concentration, as determined from the calibration curve. The percentage accuracy for each concentration was calculated as the difference between the two (Table 3.4). These accuracy percentages were used to calculate the limit of quantitation. This study showed that the R699 polyclonal anti-zilpaterol antibody had a range of detection of 490 pg/ml to 62.5 ng/ml with CVs of 2.24 to 7.89 %. Figure 3.6 shows the interday assay curve. The CVs obtained are shown in Table 3.4.

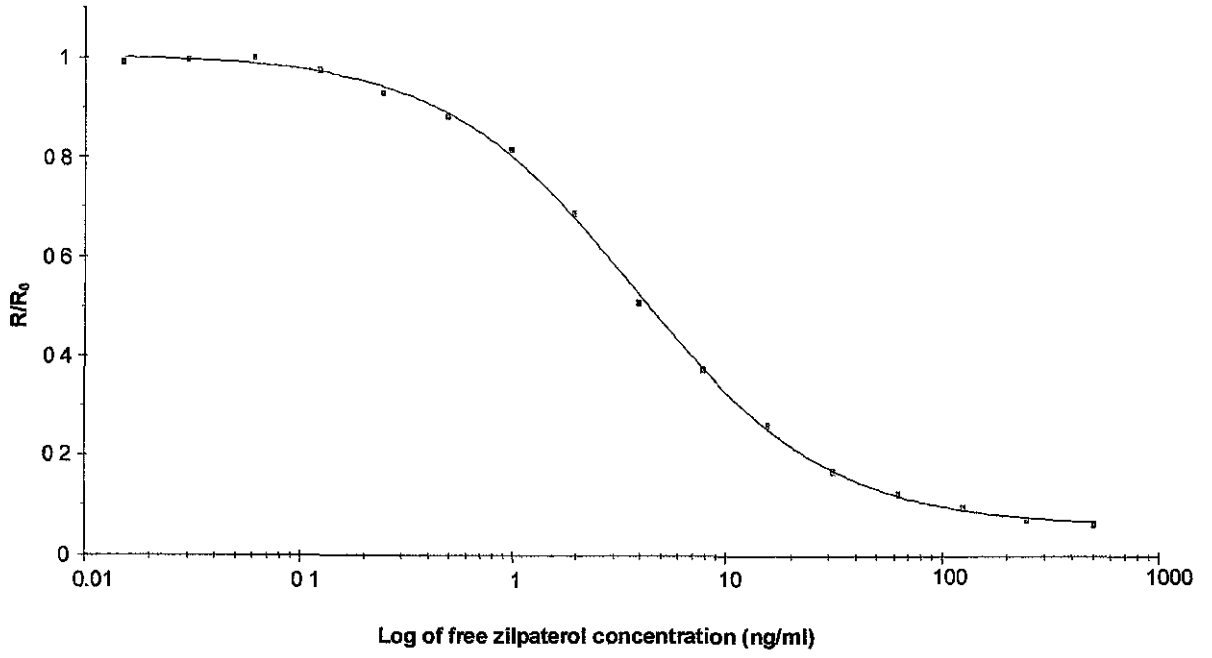

Figure 3.6 *Inter-day Biacore inhibition assay to determine the range of detection of free zilpaterol using R699 anti-zilpaterol polyclonal antibody. The range of detection was found to be from 490 pg/ml to 62.5 ng/ml.*

Table 3.4 *Interday assay coefficients of variation (CV's) and percentage accuracies for the inhibition assay developed on Biacore 3000 using R699 anti-zilpaterol polyclonal antibody. CV's were measured using the equation % CV = (S.D./Mean) x 100 where for intermediate precision (interday), the S.D. is computed from replicate (three replicates) analyses over three validation runs on three separate days The percentage accuracy for each concentration was calculated as the difference between each zilpaterol concentration and its back-calculated counterpart concentration, as determined from the calibration curve*

Conc. (ng/ml)	**Back calculated conc. (ng/ml)**	**Mean ± S.D. R/R_0**	**Coefficient of variation (%)**	**Accuracy (%)**
500	n/a	0.06 ± 0.0	0.00	0.00
250	336.49	0.07 ± 0.01	5.33	65.40
125	106.16	0.10 ± 0.01	7.89	115.07
62.5	58.23	0.12 ± 0.01	6.40	106.83
31.25	31.65	0.17 ± 0.02	5.15	98.72
15.63	14.71	0.26 ± 0.01	2.75	105.89
7.81	7.86	0.37 ± 0.01	3.33	99.36
3.91	4.23	0.51 ± 0.02	3.74	91.82
1.95	1.86	0.69 ± 0.03	4.46	104.62
0.98	0.9	0.82 ± 0.03	4.62	108.16
0.49	0.51	0.88 ± 0.02	3.69	95.92
0.24	0.30	0.93 ± 0.04	2.24	75.00
0.12	0.11	0.97 ± 0.05	3.73	108.33

3.2.4 The production of a murine scFv antibody library to zilpaterol

3.2.4.1 Immunisation of mice with zilpaterol-HSA and isolation of spleenocyte mRNA

Spleen cells isolated from mice immunised with zilpaterol-HSA were used to generate mRNA. This was carried out and the mRNA provided by Dr. Paul Dillon, DCU.

3.2.4.2 Reverse transcription of mouse spleen mRNA

Reverse transcription to synthesise cDNA was carried out on the mRNA provided as described in Section 2.3.2.

3.2.4.3 Amplification of antibody light and heavy chain genes

Amplification of the variable regions of the light and heavy chain genes was carried out using the protocol described in Section 2.3.3. A single band of approximately 400 bp for both the heavy and light chain genes was amplified from the cDNA. To amplify light chain genes the V_L back primer mix was paired with five V_L forward primers and to amplify heavy chain genes the V_H back mix was paired with four V_H forward primers. In all cases, the first PCR amplification yielded sufficient amounts for cloning, with a sharp band produced at the predicted band of approximately 400 bps for both the V_L and V_H genes (Figure 3.7). These fragments were purified from the gel, as described in Section 2.3.3.4, and their concentration determined (Table 3.5).

3.2.4.4 Splice by overlap extension (SOE) PCR

The amplified V_L and V_H domains were linked together by SOE-PCR after purification to produce a combined molecular weight fragment of approximately 800 bp (Figure 3.8).

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

The pAK 100 vector and SOE-PCR products formed by linking antibody light and heavy chain genes were digested with *SfiI* restriction enzyme, as described for both in Section 2.3.6. Figure 3.9 shows the digested pAK100 product. The digested pAK 100 and SOE-product were purified from the gel, as described in Section 2.3.3.4, and the concentrations determined (Table 3.5). After purification, the SOE-PCR fragment was ligated into the pAK 100 vector at a molar ratio of vector to insert of 1.5:1, as described in Section 2.3.7.

3.2.4.7 Transformation of *E. coli* XL1-Blue supercompetent cells with pAK 100 vector containing light and heavy chain gene

Supercompetent *E. coli* XL1-Blue cells were used for the production of a recombinant antibody library to zilpaterol using the procedure described in Section 2.3.9. Ligation reactions of pAK100 vector and SOE-PCR products were transformed resulting in the production of a zilpaterol recombinant library consisting of approximately 5 x 10^3 transformants.

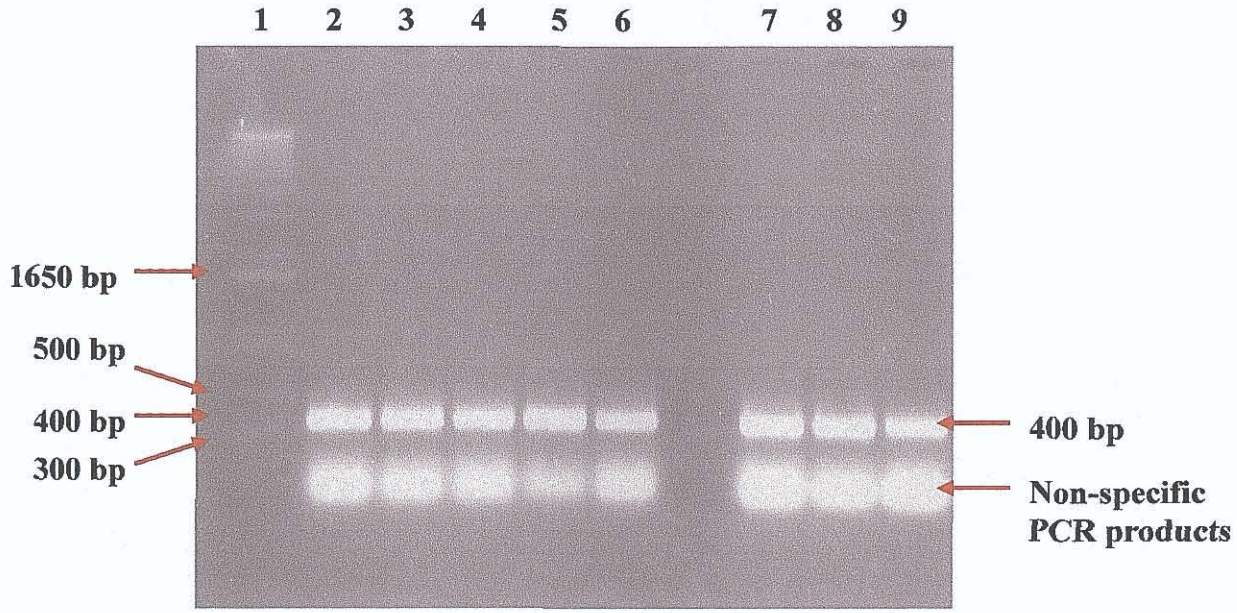

Figure 3.7 *Gel picture showing the amplification of murine light and heavy chain specifically amplified. Lanes: (1) Promega 1kB DNA ladder; lanes (2-6) Amplified 400 bp V_H fragment; lanes (7-9) Amplified 400 bp V_L fragment.*

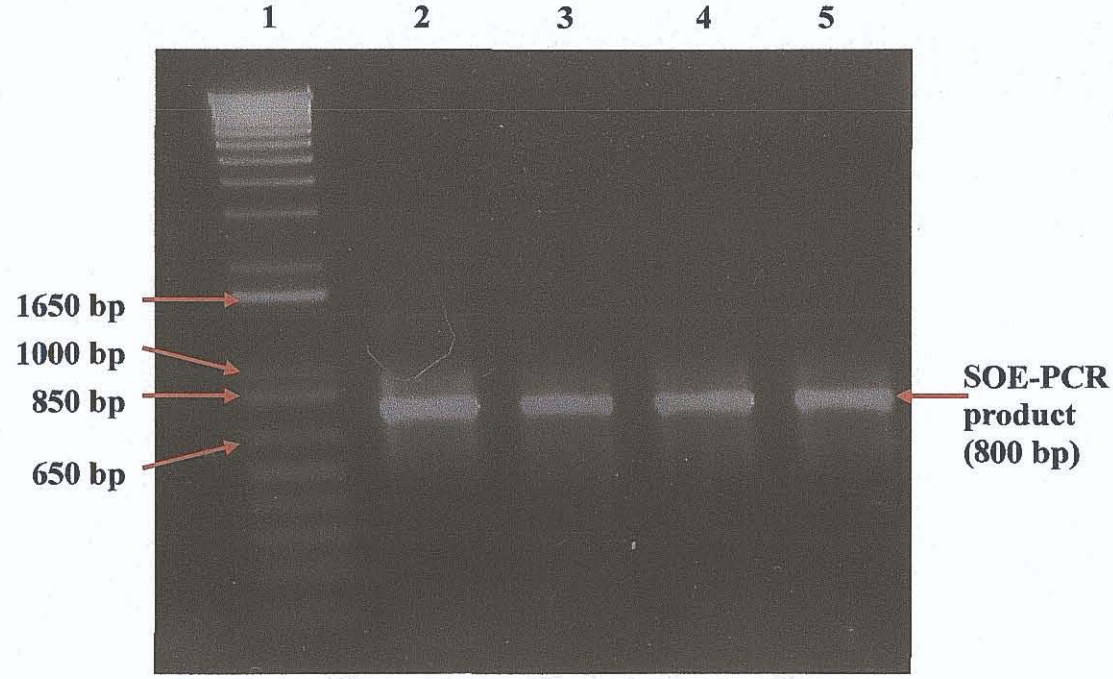

Figure 3.8 *Gel picture showing undigested SOE-PCR product. Lanes: (1) Promega 1kB DNA ladder; lanes (2-5) Amplified V_L and V_H domains linked by SOE-PCR..*

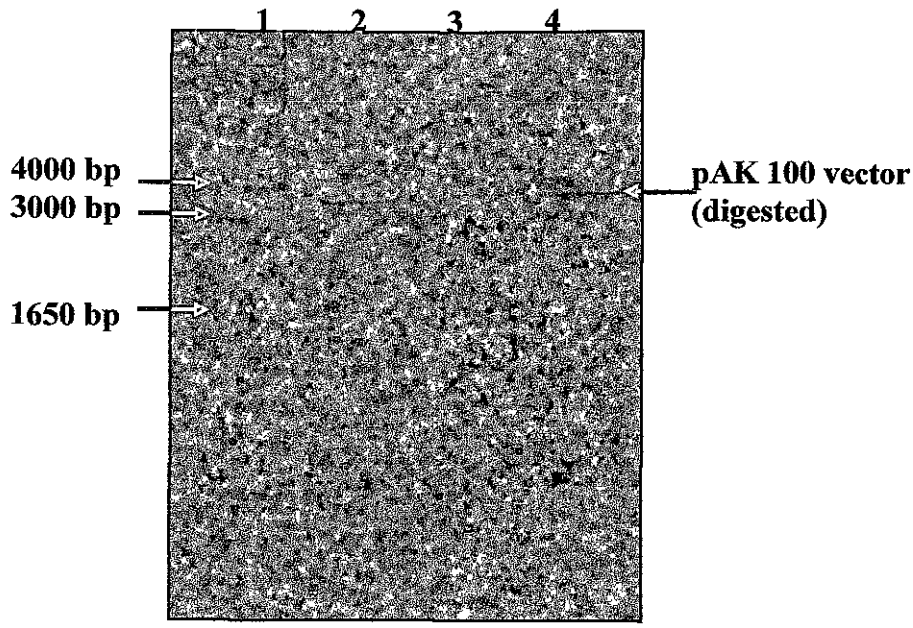

Figure 3.9 *Restriction digestion of pAK 100 vector. Lanes: (1) Bioline Hyperladder; lanes (2-4) The digested pAK 100 vector.*

Table 3.5 *Concentrations of DNA of the various components for library production*

Samples	Concentration of DNA (ng/μl)
Zilpaterol Heavy chain Mouse	40
Zilpaterol Light chain Mouse	50
Digested SOE-PCR product	40
pAK 100 vector	60

3.2.5 Production of single chain Fv antibodies to zilpaterol

3.2.5.1 First round selection and screening of functional scFv sequences to zilpaterol from zilpaterol recombinant library using phage ELISA

Production of phagemid particles from the zilpaterol recombinant antibody library was carried out as described in Section 2.4.1. After transformation of the ligation reaction into competent *E. coli* XL1-Blue cells, the resulting phage particles were then used in the first round of affinity selection of scFv antibodies by panning with zilpaterol-HSA as described in Section 2.2.4. After panning and elution from the immunotube a titration was carried out on the remaining phage resulting in a 1 x 10^5 phage particle count.

95 individual clones from the zilpaterol recombinant antibody library selected after the first round of panning, were grown separately and infected by helper phage, as described in Section 2.4.5. The recombinant scFvs, displayed on the surface of filamentous phage, were screened on a plate coated with zilpaterol-HSA (for antigen binding) in a typical phage ELISA as described in Section 2.4.6. Results from the phage ELISA showed that 8 of the clones analysed possessed affinity to the conjugate (Figure 3.10).

Eight positive clones, A1, A9, B1, E2, H5, E7, H7 and D11 were selected on the bases of the strength of their absorbance readings for characterisation with respect to their affinity for free zilpaterol. The scFv-displaying phage produced from these clones was used to conduct a simple competitive ELISA with free zilpaterol. Four of the eight clones (A9, B1, E2 and E7) demonstrated competition for free zilpaterol (Figure 3.11).

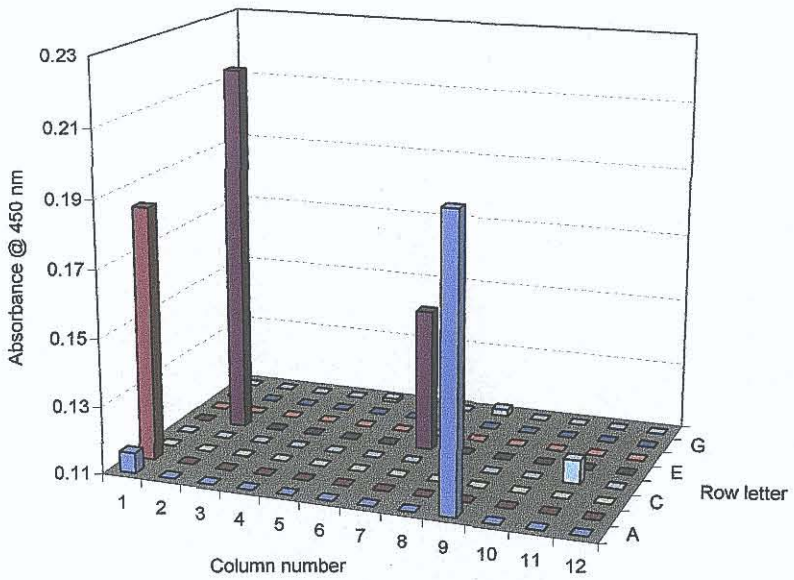

Figure 3.10 *Graphical representation of absorbance data from an ELISA carried out on 95 clones after the first round of panning. A1, A9, B1, E2, H5, E7, H7 and D11 scFv antibody clones were picked in order to study their ability to bind to free zilpaterol.*

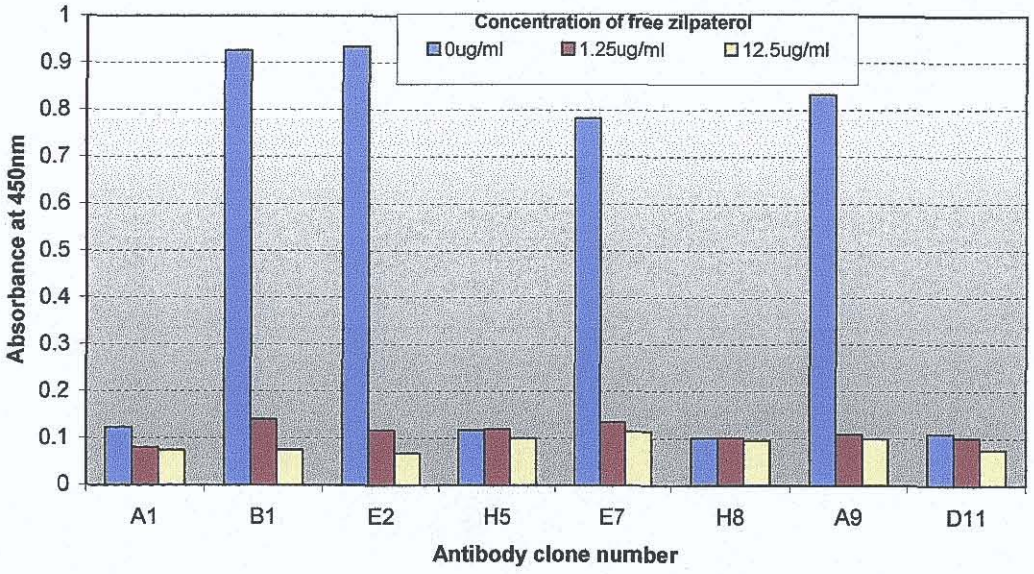

Figure 3.11 *Bar chart representing the results obtained from a competitive ELISA of eight phage-scFv antibody fragments (clones selected from panning, Figure 3.10) with free zilpaterol. ScFv clones B1, E2, E7 and A9 demonstrated better binding capabilities than the other clones, and were therefore chosen for further study.*

3.2.5.2 Digestion of A9, B1, E2 and E7 scFv antibody light and heavy chain genes and pAK 400 vector for production of soluble scFv antibody

Antibody light and heavy chain genes from clones A9, B1, E2 and E7 were digested with *SfiI* restriction enzyme as shown in Figure 3.12. The two main bands represent the digested pAK 100 vector, and the scFv fragment. The pAK 400 vector is used for the production of soluble scFv fragments. The digested pAK 400 vector is shown in Figure 3.13. This DNA was gel-purified as described in Section 2.3.3.4. Light and heavy chain genes from clones A9, B1, E2 and E7 were ligated into the digested pAK 400, as described in Section 2.4.9. These four ligation products were transformed into JM83 competent cells (Section 2.4.11), resulting in the production of four clones capable of producing soluble scFv antibodies.

3.2.5.3 Soluble expression of A9, B1, E2 and E7 scFv antibodies

Soluble scFv antibodies from A9, B1, E2 and E7 clones were produced in supernatant (see Section 2.4.12).

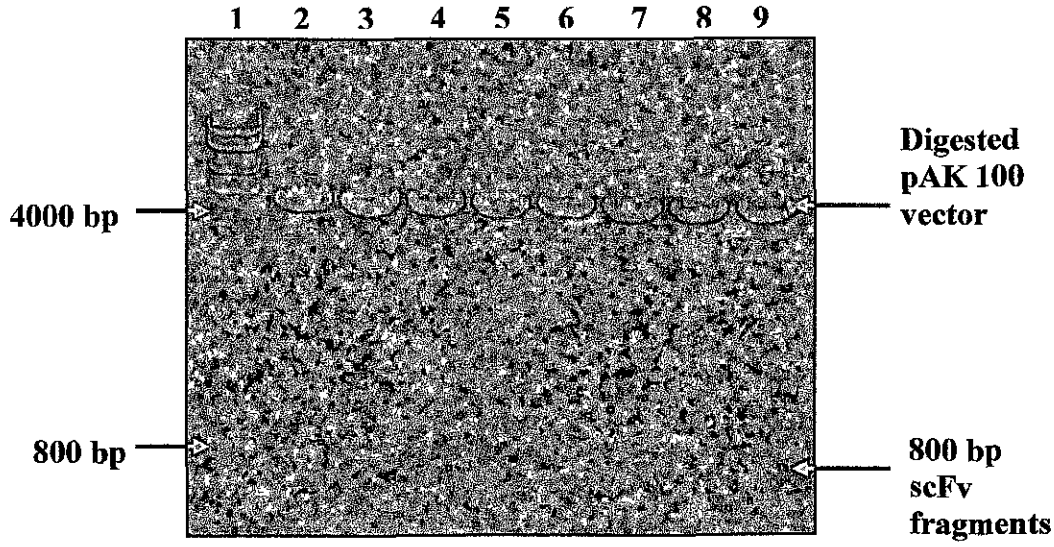

Figure 3.12 *Restriction digestion of pAK 100 vector purified from A9, B1, E2 and E7 clones Lanes: (1) Bioline Hyperladder; (2-3) A9 digested pAK 100 and scFv fragment; lanes (4-5) B1 digested pAK 100 and scFv fragment; lanes (6-7) E2 digested pAK 100 and scFv fragment and lanes (8-9) E7 digested pAK 100 and scFv fragment.*

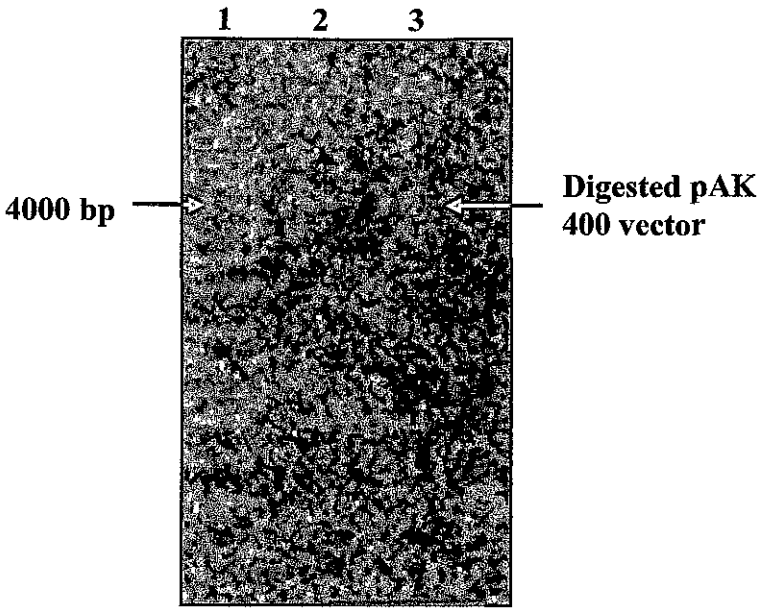

Figure 3.13 *Restriction digestion of pAK 400 vector. Lanes: (1) Bioline Hyperladder; lanes (2-3) Digested pAK 400 fragment. pAK 400 was restricted in order to provide ends to which the digested scFv DNA fragments could be annealed.*

3.2.6 Development of an scFv-based competitive enzyme-linked immunosorbent assay (ELISA) for zilpaterol

3.2.6.1 Checkerboard ELISA for determination of optimal zilpaterol coating concentration and optimal scFv antibody dilution

The working dilution of these antibodies was determined using the format already discussed in Section 2.4.13. Zilpaterol-HSA was coated on the wells of four microtitre plates at three different concentrations (5 μg/ml, 2.5 μg/ml, 1.25 μg/ml), with one row of wells per concentration. A fourth row of wells was left uncoated, and all four rows were blocked with 5% (w/v) milk marvel. Dilutions of cellular extract from the four clones A9, B1, E2 and E7 containing the scFv were prepared, with concentrations ranging from neat to 1/100. Each dilution of antibody was added to each concentration of conjugate on the microtitre plates. The results for each of the

four clones were plotted in Figure 3.14. The scFv produced from the B1 clone gave the strongest signal and was therefore chosen for further analysis and assay development. For the B1 scFv antibody, the optimal conjugate coating concentration was found to be 5 μg/ml, and the optimal scFv antibody dilution was determined to be 1/2.

3.2.6.2 Interday competitive ELISA for B1 scFv antibody characterisation

A competitive ELISA using the optimal coating concentration and scFv antibody dilution was performed as described in Section 2.4.15. In order to determine the inter-day assay variation, the ELISA was repeated three times over three separate days. The normalised mean values, which were calculated as the mean absorbance at each antigen concentration / absorbance in the presence of zero antigen concentration (A/A_0) over the three days were used to construct a calibration curve for the interday assay. Using Biaevaluation 3.1 software the calibration curve was plotted using the four-parameter equation. Comparisons were made between each zilpaterol concentration and its back-calculated counterpart concentration, as calculated from the calibration curve. The percentage accuracy for each concentration was calculated as the difference between the two (Table 3.6). Using these accuracy percentages to calculate the *limit of quantitation*, the limit of detection for free zilpaterol was defined as 78 ng/ml (Figure 3.15). The coefficients of variation (CVs) between the three replicates of each cell concentration were determined by expressing the standard deviation as a percentage function of the mean. The CVs are shown in Table 3.6. The interday CVs ranged from 1.66 to 9.48 %.

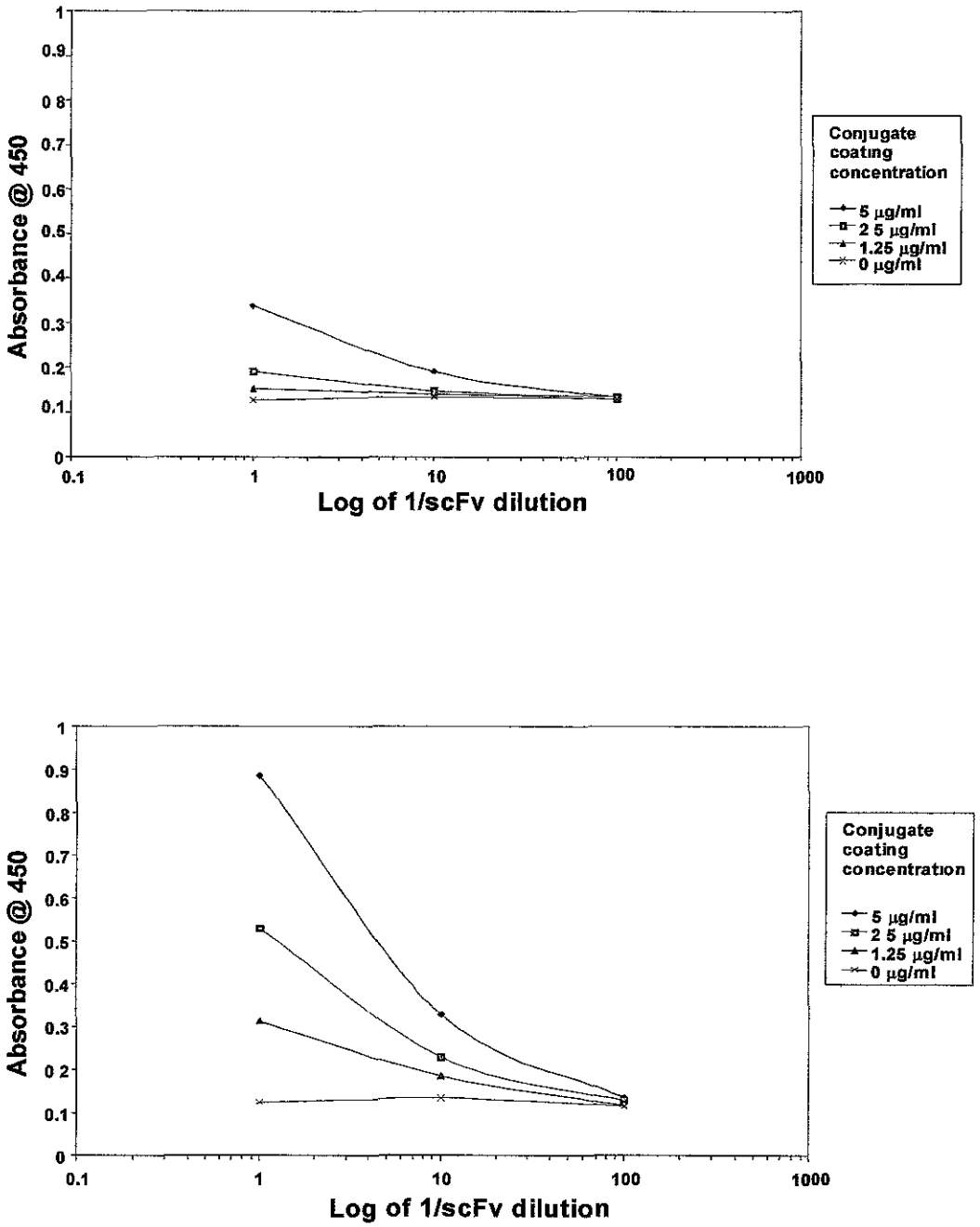

Figure 3.14 a *Checkerboard ELISAs performed using antibodies A9 (top of page) and B1(above) to determine the optimal zilpaterol coating concentration and optimal scFv antibody dilution for use. ScFv produced from the B1 clone gave the strongest signal. The optimal conjugate coating concentration for the B1 scFv was found to be 5 µg/ml, and the optimal scFv antibody dilution was determined to be 1 in 2*

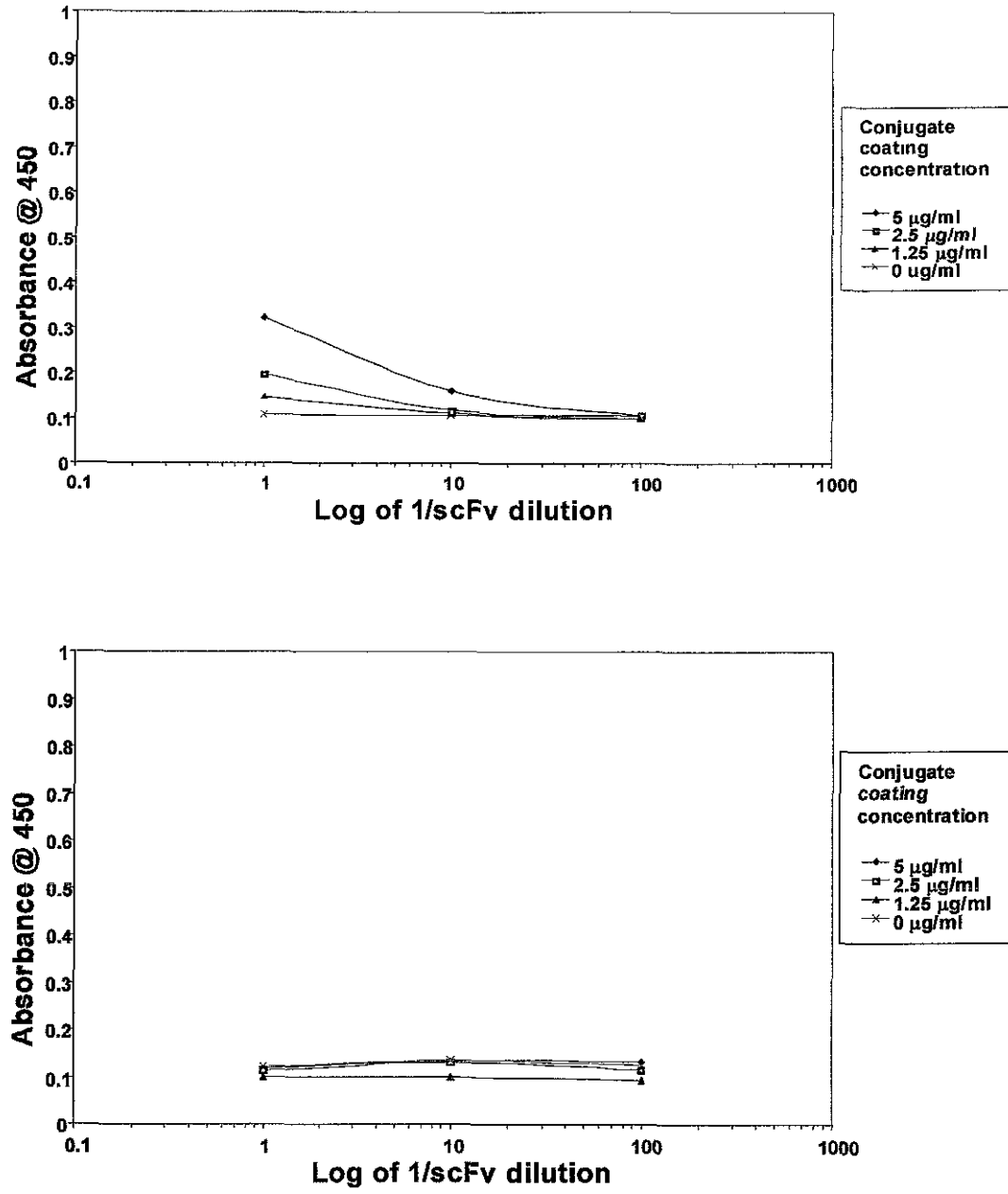

Figure 3.14 b *Checkerboard ELISAs performed using antibodies E2 (top of page) and E7 (above) to determine the optimal zilpaterol coating concentration and optimal scFv antibody dilution The response observed using these antibodies highlighted B1 (Figure 3.13 a) as the best antibody for assay development*

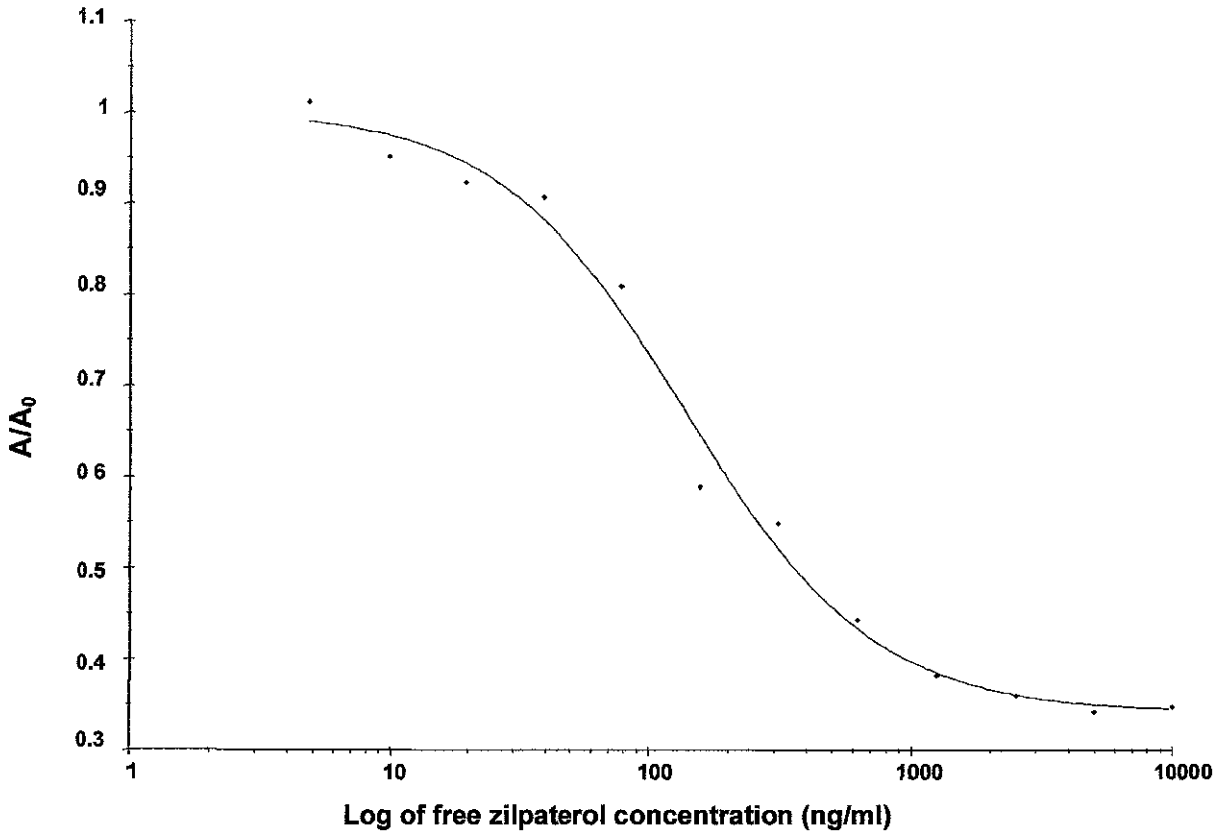

Figure 3.15 *Interday assay calibration curve for the B1 scFv antibody A 4-parameter equation was fitted to the data set using BIAevaluation 3.1 software. The inter-assay coefficients of variance are tabulated in Table (3 6). Each point on the curve is the mean of three replicate measurements analysed over three days.*

Table 3.6 *Interday assay coefficients of variation (CV's) and percentage accuracies for the B1 scFv antibody. Interday studies were conducted and coefficients of variance (a quantitative measure of precision) calculated using the equation % CV = (S.D./Mean) x 100 where for intermediate precision (interday), the S D. is computed from replicate (three replicates) analyses over three validation runs on three separate days.*

Conc. (ng/ml)	Back calculated conc. (ng/ml)	Calculated mean ± S.D.	Coefficient of variation (%)	Accuracy (%)
2500	2627.59	0.43±0.01	2.74	94.89
1250	1314.46	0.44±0.01	3.29	94.84
625	568.47	0.51±0.03	5.42	109.04
312.5	264.07	0.58±0.04	7.36	115.49
156.25	209.89	0.64±0.02	2.89	65.67
78.13	65.89	0.81±0.08	9.48	115.67
39.06	31.25	0.91±0.03	3.61	119.99
19.53	26.07	0.93±0.02	2.12	66.51

3.2.7 Development of a Biacore-based inhibition immunoassay for zilpaterol

3.2.7.1 CM5 Biacore chip directly immobilised with zilpaterol

A CM5 Biacore chip, which had been directly immobilised with zilpaterol, was obtained from Dr. Lisa Connolly of Queens University, Belfast.

3.2.7.2 Inhibition immunoassay for the detection of zilpaterol using the anti-zilpaterol scFv antibody

The protocol used to conduct an inhibition assay for free zilpaterol using the anti-zilpaterol scFv antibody is found in Section 2.6.5. To determine the interday assay variation, individual assays were performed each day for five days. The mean and percentage CV of the five results generated for each of the five assays was determined. Inhibition was observed for each individual assay. However, the percentage CV for the five results did not allow an accurate calibration curve to be constructed (results not shown). Figure 3.16 shows a single inhibition assay performed on the Biacore 3000. Figure 3.17 shows the linear range of the Biacore-based inhibition assay shown in Figure 3.10. Comparisons were made between each zilpaterol concentration and its back-calculated counterpart concentration, as calculated from the calibration curve. The percentage accuracy for each concentration was calculated as the difference between the two. Using these accuracy percentages to calculate the limit of quantitation, the range of detection for free zilpaterol was defined as between 15.6 and 125 ng/ml.

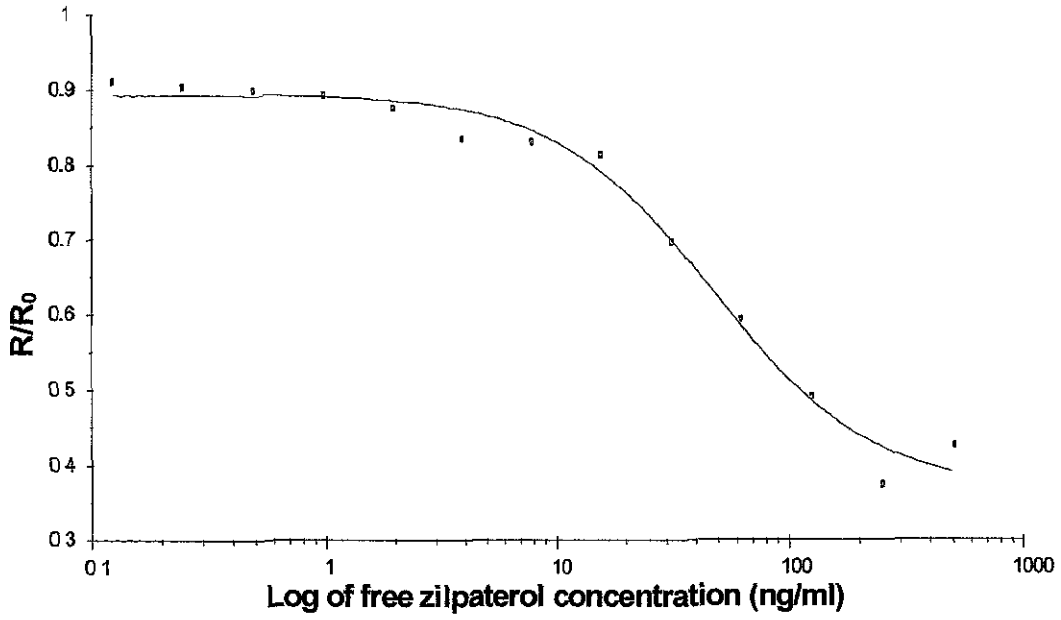

Figure 3.16 Biacore-based inhibition assay to determine the range of detection of free zilpaterol using the anti-zilpaterol scFv antibody. The range of detection was found to be from 15.6 to 125 ng/ml (R/R_0 represents the mean response unit at each antigen concentration / response unit in the presence of zero antigen concentration).

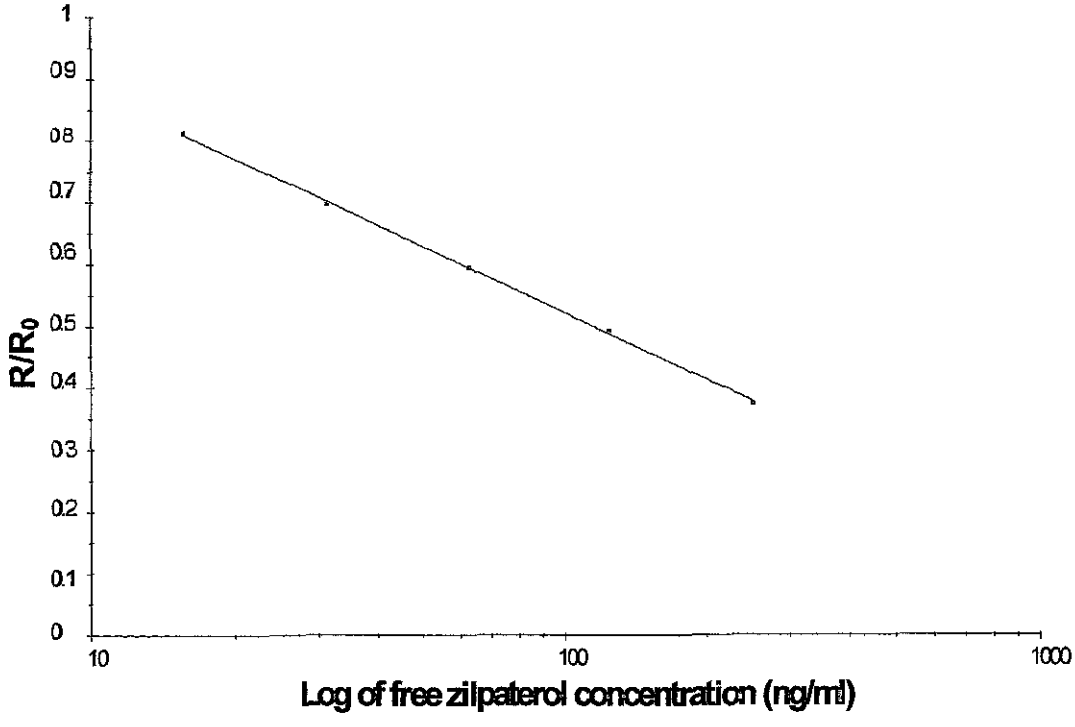

Figure 3.17 The linear range of the Biacore-based inhibition assay shown in Figure 3.16, with the range of detection from 15.6 to 125 ng/ml.

3.2.7.3 Biacore-based cross-reactivity study of scFv antibody

Cross-reactivity potential of the scFv was determined against four β-agonists, clenbuterol, mabuterol, salbutamol and ractopamine, as well as several other related steroid compounds in a Biacore inhibition assay format. The inhibition assay was performed as described in Section 3.2.7.2, with equal concentrations of the drug to be tested added in place of the zilpaterol. The displacement method described by Miller and Valdes (Miller *et al.*, 1992) was used to accurately estimate the levels of cross-reactivity, with the IC_{50} value defined as the analyte concentration that results in 50% inhibition of the antibody and the IC_{90} value defined as the analyte concentration that results in 90% inhibition. Levels of cross-reactivity were estimated at the IC_{50} (CR_{50}) as 100-fold the ratio between the IC_{50} values of zilpaterol and of the cross-reactant. IC_{90} (CR_{90}) levels of cross-reactivity were estimated as 100-fold the ratio between the IC_{90} values of zilpaterol and of the cross-reactant. Levels of cross-reactivity at the IC_{50} (CR_{50}) and IC_{90} (CR_{90}) are shown in Tables 3.7 and 3.8.

Table 3.7 *Cross-reactivity and specificity studies on scFv antibody against four β-agonists. The cross-reactivity potential was approximated at the IC_{90} value, which is the concentration of drug detectable at 90% A/A_0, and the IC_{50} value, which is estimated at 50% A/A_0. The CR_{90} and CR_{50} were then expressed as 100-fold the ratio of zilpaterol and of the cross-reactant.*

Drug	IC_{90}[a] (ng/ml)	IC_{50}[b] (ng/ml)	CR_{90}[c] (%)	CR_{50}[d] (%)
Zilpaterol	20	50	100	100
Mabuterol	50	-	40	-
Ractopamine	2500	-	1	-
Clenbuterol	20	-	100	-
Salbutamol	30	-	66	-

[a] Least detectable dose calculated at 90% A/A_0

[b] 50% inhibition concentration (50% A/A_0)

[c] Percentage cross-reactivity determined at IC_{50}

[d] Percentage cross-reactivity determined at IC_{90}

Table 3.8 *Cross-reactivity and specificity studies on scFv antibody against several other potential cross-reactants The cross-reactivity potential was approximated at the IC_{90} value, which is the concentration of drug detectable at 90% A/A_0, and the IC_{50} value, which is estimated at 50% A/A_0. The CR_{90} and CR_{50} were then expressed as 100-fold the ratio of zilpaterol and of the cross-reactant.*

Drug	IC_{90} (ng/ml)[a]	IC_{50} (ng/ml)[b]	CR_{90} (%)[c]	CR_{50} (%)[d]
Zilpaterol	20	50	100	100
α-estradiol	>1000	-	-	-
Zeranol	20	-	100	-
Nortestosterone	>1000	-	-	-
Norgesterone	>1000	-	-	-

[a] Least detectable dose calculated at 90% A/A_0

[b] 50% inhibition concentration (50% A/A_0)

[c] Percentage cross-reactivity determined at IC_{50}

[d] Percentage cross-reactivity determined at IC_{90}

3.3 Discussion

This chapter includes all assay development undertaken using both polyclonal and scFv antibodies to zilpaterol.

The anti-zilpaterol antibody R699 was used to develop a competitive ELISA for the detection of zilpaterol. This assay involved immobilisation of the polyclonal antibody onto the surface of a microtitre plate, followed by simultaneous addition of a fixed concentration of HRP-labelled zilpaterol and varying concentrations of free zilpaterol. The signal produced was inversely proportional to the concentration of free zilpaterol present in the sample. Initial investigation work was carried out to determine the optimal conditions for this assay. A competitive checkerboard ELISA was conducted to determine the optimal concentrations of coating antigen and of antibody for use in ELISA. The limits of detection in competitive ELISA's are a function of the antibody affinity, and the equilibrium between both the free and immobilised conjugate. As a result, too high a concentration of coating conjugate will shift the binding equilibrium in favour of binding to the ELISA plate and cause reduced sensitivity to free antigen in solution. Similarly, the concentration of antibody used must be the limiting factor. If this were not the case, antibody would be capable of binding to free drug and bound conjugate in excess. Therefore, it is essential to optimise both the loading concentration of solid-phase conjugate and the dilution of antibody. The optimal serum coating dilution was found to be 1/1600. The optimal range of free zilpaterol concentrations for use in the development of a competitive ELISA was determined to be between 0.24 and 500 ng/ml, while a dilution of 1/4000 of zilpaterol-HSA was optimal for use.

Further competitive ELISAs were then performed using various different buffering solutions (Sections 2.3.1.3.1 and 2.3.1.3.2) to determine the optimal coating and dilution buffer for use with the polyclonal antibody and zilpaterol, respectively. Differences in the binding capabilities of the polyclonal antibody to the immobilised zilpaterol conjugate were compared. Sodium acetate proved to be the optimal buffer for use in the assay.

Once these factors had been determined, a model competitive assay was developed where standards of free zilpaterol ranging from 0.48 to 500 ng/ml were prepared and mixed with a 1/2000 dilution of zilpaterol-HSA (final dilution 1/4000). Both the free and HSA-bound zilpaterol competed within this range. Free zilpaterol showed a range of detection between 0.24 to 31.25 ng/ml. The assay displayed excellent recognition of the unconjugated zilpaterol molecules and showed very low limits of detection. Interday assay variability statistics were then analysed in an attempt to assess the reproducibility of the assay. Interday assay coefficients of variation (CV's) were found to be between 2.75 and 13.24% (Table 3.1). This result indicates that this polyclonal antibody can be used to generate an accurate, specific and reproducible assay.

One of the initial steps in designing an immunoassay is the assessment of reactivity towards structurally similar molecules. (Wild, 1994). Cross reactivity is defined as a measure of the antibody response to structurally similar molecules. Serial dilutions of the cross-reactive analyte of interest are prepared with similar dilutions of specific antigen. A competitive ELISA carried out as described in Section 2.2.5. Tables 3.2 and 3.3 show the IC_{90} value, which is the concentration of drug detectable at 90% A/A_0, and the IC_{50} value, which is estimated at 50% A/A_0. These values were calculated using the displacement method described by Miller and Valdes (Miller *et al.*, 1992). Competitive assays were carried out with several other β-agonists, in addition to several other potential cross-reactants. Clenbuterol, mabuterol, salbutanol and ractopamine (the β-agonists), along with alpha-estradiol, 19-nortestosterone, zeranol, beta-estradiol and norethisterone were used in place of zilpaterol in the competitive assay to ascertain cross-reactivity. IC_{90} values, which is the concentration of drug detectable at 90% A/A_0, and the IC_{50} values, which is estimated at 50% A/A_0, for each of these beta agonists were calculated (Table 3.3). Low cross-reactivity levels indicate that the scFv is specific to the structure unique to the zilpaterol molecule.

The next step was the development of a Biacore-based Inhibition immunoassay for the detection of zilpaterol using R699 anti-zilpaterol polyclonal antibody and a CM5 Biacore chip, which had been directly immobilised with zilpaterol.

A model inhibition assay was developed (described in Section 2.6.4) for free zilpaterol. Standards of zilpaterol ranging from 0.02 to 500 ng/ml (final concentrations) were mixed with a 1/1600 dilution (final) of R699 antibody. These mixtures were allowed to incubate at 37°C for 30 minutes followed by injection over the zilpaterol-immobilised surface for 3 minutes. The surface of the chip was regenerated between cycles by passing pulses of 5 µl of 15 mM NaOH over the surface. The antibody response to the surface is inversely proportional to the concentration of free drug in solution. These binding responses were plotted in Figure 3.6 and showed that the detection range of R699 for free zilpaterol was between 490 pg/ml to 62.5 ng/ml (Table 3.4).

Production of an scFv antibody fragment specific for zilpaterol and use of this fragment in the development of an assay for the detection of zilpaterol was then carried out. The advantage in production and use of a recombinant antibody fragment in assay development is that the binding capability of the fragment can be improved using genetic engineering techniques (as outlined in the introductory chapter). The antibody fragments described in this chapter are the first recombinant antibodies produced to zilpaterol to my knowledge. The initial stages for the isolation of a specific scFv antibody fragment involved the immunisation of mice with zilpaterol-HSA and sacrifice of the animal once a suitable immune response had been generated. The spleen was subsequently removed and mRNA isolated from these spleen cells. This was carried out by Dr. Paul Dillon.

The isolated mRNA was used in the production of cDNA followed by the amplification of the light and heavy chain genes. The set of primers used were produced by Krebber *et al.* (1997). They incorporate all mouse V_H, V_λ and V_κ sequences collected by the Kabat data base (Kabat *et al.*, 1991) and combine extended primer sets described by Kettleborough *et al.* (1993). The PCR for the production of light and heavy chain genes was optimised and the conditions used are described in Section 2.3.4. Bands of light (375 - 402 bp) and heavy (386 - 400 bp) chain gene amplification can be seen in Figures 3.7. Results show that the bands for both light and heavy chain genes are very clear with minimal production of non-specific bands. This is a significant result as production of non-specific bands would

lower the concentration and diversity of the specific bands and decrease the overall diversity of the recombinant library produced using the PCR products.

The light and heavy chain genes were then assembled together by splice by overlap extension (SOE) PCR (Figure 3.8). The SOE-PCR product was purified and digested along with the pAK100 vector. This was followed by ligation of digested SOE-PCR product with digested pAK100, as described in Section 2.3.1. Attempts to clone digested heavy and light chain SOE-PCR products into the digested pAK100 vector produced low yields of transformants (5×10^3). However, affinity selection was conducted. Selection of scFv antibodies to zilpaterol was carried out by rounds of affinity selection panning to enrich for antibodies recognising zilpaterol. Screening of positive scFv-producing clones was performed by growing individual colonies separately and infecting them with helper phage as described in Section 2.4.1. The recombinant scFvs displayed on the surface of the filamentous phage, were tested for antigen binding in a typical phage ELISA. This would simultaneously show the recognition of scFv for antigen, while also demonstrating extracellular expression if culture supernatant were used.

Four clones found to be positive in phage ELISA were picked from each round of panning and further characterised by antigen competition studies to verify that the binding was antigen-specific. The clones were A9, B1, E2 and E7 (Figure 3.10). At this point B1 was picked for further characterisation and analysis as a soluble scFv in order to develop a model system for zilpaterol detection.

As the B1 clone possesses antigen-specific characteristics as shown by phage display, the next step was to produce a soluble scFv antibody. This was carried out by digestion of the pAK100 vector harbouring the light and heavy chain genes of the B1 clone and sub-cloning into the pAK400 vector for soluble expression in a JM83 cell line. Expression medium devoid of glucose was used to increase expression levels of recombinant protein (De Bellis and Schwartz, 1990). The pAK400 vector also contains a much stronger Shine-Dalgarno sequence (Krebber *et al.,* 1997), resulting in a further significant enhancement of protein expression.

The scFv was then used in the development of a competitive ELISA for zilpaterol. A checkerboard ELISA for determination of optimal loading ratios of conjugate and

optimal anti-zilpaterol scfv antibody dilutions was performed and results are shown in Figure 3.14. A zilpaterol concentration of 5 μg/ml was found to be optimal for use in this assay. A model competitive ELISA assay was developed for this antibody in PBS where standards of zilpaterol ranging from 4.88 to 10000 ng/ml were prepared and mixed with purified antibody at a dilution of 1/2. The B1 antibody showed a limit of detection of 78.13 ng/ml, as approximated from Figure 3.15 and Table 3.6. Interday assay variability statistics were then analysed in an attempt to look at the reproducibility of the assay. Interday assay coefficients of variation (CV's) for the B1 scFv were found to be between 1.66 to 9.48 %. This result indicated that this anti-zilpaterol scFv gives an accurate, specific and reproducible assay.

The next step was the development of a Biacore-based Inhibition immunoassay for the detection of zilpaterol using the B1 anti-zilpaterol scFv. A model inhibition assay was developed (described in Section 2.6.5) for free zilpaterol. Standards of zilpaterol ranging from 0.98 ng/ml and 1 μg/ml (final concentration) were mixed with 1/10 with cellular extract. These mixtures were allowed to incubate at 37^0C for 30 minutes followed by injection over the zilpaterol-immobilised surface for 3 minutes. The antibody response to the surface is inversely proportional to the concentration of free drug in solution. These binding responses were plotted in Figures 3.16 and 3.17 and show that the detection range of the B1 scFv for free zilpaterol was between 15.6 ng/ml and 125 ng/ml.

Cross-reactivity studies were then performed in order to measure the response of the B1 scFv to other beta-agonists and other potential cross reactants. Serial dilutions of the cross-reactive analyte of interest are prepared with similar dilutions of specific antigen and a competitive ELISA carried out as described in Section 2.2.5. Tables 3.7 and 3.8 show the IC_{90} and the IC_{50} values, as calculated using the displacement method described by Miller and Valdes (Miller *et al.*, 1992). Competitive assays were carried out with several other beta-agonists, in addition to several other potential cross-reactants. Clenbuterol, mabuterol, salbutanol and ractopamine (the beta-agonists), along with alpha-estradiol, 19-nortestosterone, zeranol, beta-estradiol and norethisterone were used in place of zilpaterol in the competitive assay already developed. IC_{90} values, which is the concentration of drug detectable at 90% A/A_0,

and the IC_{50} values, which is estimated at 50% A/A_0, for each of these beta agonists were calculated (Tables 3.7 and 3.8).

A comparison between the ranges of detection achieved for assays using the R699 polyclonal, and the recombinant scFv antibody (Table 3.9) shows that the polyclonal antibody demonstrated a higher degree of sensitivity than the scFv antibody. Likewise, a comparison between the cross-reactivity percentages calculated for the R699 polyclonal antibody, and the recombinant scFv antibody (Table 3.10) demonstrates that the polyclonal antibody displayed lower levels of cross reactivity compared with the scFv antibody.

Table 3.9 *Comparison between the range of detection achieved for the R699 Polyclonal Ab and the Recombinant scFv antibody*

	Limit of detection ELISA (ng/ml)	**Limit of detection Biacore (ng/ml)**
R699 polyclonal Ab	0.24	0.49
Recombinant scFv Ab	78	15.6

Table 3.10 *Comparison between the cross-reactivity values calculated for the R699 Polyclonal Ab and the Recombinant scFv antibody*

	R699 polyclonal Ab		**B1 scFv recombinant Ab**	
Drug	**CR_{90}[c] (%)**	**CR_{50}[d] (%)**	**CR_{90}[c] (%)**	**CR_{50}[d] (%)**
Zilpaterol	100	100	100	100
Mabuterol	0.1	-	40	-
Ractopamine	-	-	1	-
Clenbuterol	3	-	100	-
Salbutamol	0.1	-	66	-
α-estradiol	-	-	-	-
19-nortestosterone	2	-	100	-
Zeranol	-	-	-	-
β-estradiol	-	-	-	-
Norethisterone	-	-		

The antibody fragments described in this chapter are the first recombinant antibodies developed against zilpaterol to my knowledge. Further work involving genetic engineering of the scFv DNA was planned in order to improve the performance of the antibody fragment. However, this work could not be performed as no conjugate was available for testing. Comparison of these results with others described in the literature was not possible as references to other anti-zilpaterol antibodies could not be found.

Chapter 4

Cloning, expression and purification of a recombinant form of prostate-specific antigen protein

4.1 Introduction

4.1.1 Prostate-specific antigen

Prostate cancer (PCa) is the most frequently occurring cancer affecting men in developed countries (Bauvin *et al.*, 2003). Several factors, i.e. genetic, environmental and hormonal, are thought to influence the progression of prostate cancer. Despite advances in knowledge of various aspects of cancer, an effective treatment for prostate cancer has not yet been achieved. Research is, therefore, focusing on providing earlier and more accurate detection of the disease (Schmid *et al.*, 2004).

Prostate-specific antigen (PSA) is a 33kD tissue-specific glycoprotein that belongs to the glandular kallikrein gene family. It was first identified from seminal plasma in the early 1970s (Hara *et al.*, 1971) and was subsequently named prostate-specific antigen by Wang *et al.* in 1979. In 1980, it was first identified as present in the serum of men with prostate cancer. A group of proteases, the kallikreins, were subsequently discovered (Yousef *et al.*, 2001) and to date, 13 separate variants of the protein have been identified. All members of the kallikrein family have significant gene sequence homologies (Yousef *et al*, 2001). PSA was identified as a member of this family and, as such, was given the name human kallikrein 3 (hK3) (Rittenhouse *et al.*, 1998).

PSA is produced mainly by the prostate gland and its function is thought to be liquefaction of seminal fluid through enzymatic action. The active site of PSA is closely related to that of the chymotrypsin (Vihinen, 1994). PSA is not a cancer-specific protein. Its presence is not unique to cancerous cells, as it is present in normal prostate tissue (Jung *et al.*, 2000). Its use as a marker for prostate cancer is due to the elevated levels detectable in the serum of prostate cancer patients. Under normal conditions, i.e. in non-cancerous tissue, synthesis of PSA by the epithelial cells of the prostate gland is directed into the seminal fluid. PSA can only enter blood circulation by leaking into the extracellular fluid and diffusing into veins and capillaries. However, in cancerous prostatic tissue, tumour growth disrupts the basal cell layer and cell polarity is altered. The direction of PSA secretion is disturbed and PSA is released freely into the circulatory system. This explains the elevated PSA levels in the serum of prostate cancer patients (Stenman *et al.*, 1999). Even though

prostate cancer cells produce less PSA than non-cancerous cells, more leaks into the serum and therefore, elevated serum PSA is detected (Jung *et al.*, 2000). Serum from a non-cancer patient contains a PSA concentration of less than 2 μg/ml. Prostate cancer tissue generally contributes about ten times more to serum PSA levels than an equal amount of normal prostate tissue (Stamey *et al.*, 1987). Thus, elevated serum levels may be indicative of prostate cancer.

4.1.2 ELISA development for PSA detection

The practical application of PSA measurement in cancer detection has proved problematic. One major factor contributing to this stems from the fact that PSA is not a truly cancer-specific marker (Levesque *et al.*, 1995). Various other benign disorders have been shown to increase serum PSA levels, and the potential for false positive results has diminished the reliability of such PSA assays. One major disorder capable of increasing the level of PSA in serum is benign prostatic hyperplasia (BPH) (Anderson, 2002). BPH is a non-malignant growth of the prostate gland, characterised by similar distortion of the basal cell layer of the prostate gland and free release of PSA into blood circulation, as previously described for prostate cancer. Current commercially available assays can often fail to distinguish prostate cancer from such benign disorders, and, as a result, much research is underway to enhance specificity of PSA assays so as to eliminate the high percentage of false positives.

The discovery of various forms of PSA in serum, including the presence of complexes, has provided a new angle on the proteins role and significance as a disease marker in cancer. The predominant form of PSA is an α_1-chymotrypsin PSA complex (PSA-ACT), which appears to form 65-95% of the total PSA content of human serum (Zhang *et al.*, 1999). Uncomplexed PSA accounts for most of the remaining PSA in serum (5-35% of the total PSA content). Other complexes were also found to be present in trace amounts, such as PSA-API (α_1-protease inhibitor) and PSA-AMG (α_2-macroglobulin). The latter has proven difficult to detect due to its lack of immunoreactivity and its presence in serum has yet to be accurately quantified.

Detection of these different forms has proven highly significant since the discovery that the relative proportion of complexed to free PSA increases in patients with prostate cancer (Christensson *et al.*, 1993). This is thought to be due to the manner in which the PSA protein leaks into the blood circulation system. The presence of cancerous cells causes disruption of the basal border of the prostate gland epithelial cells. This results in loss of the normal secretory pathways and PSA is consequently released into the extracellular space and into the circulation. It is believed that this process of PSA release enables PSA to encounter and form complexes with ACT and AMG (Stenman *et al.*, 1999). Hence, levels of complexed PSA increase relative to free PSA in the serum of cancer patients. This information indicates a potential discriminatory factor that can be incorporated into PSA assays to increase specificity for prostate cancer detection. If specific antibodies against free and complexed forms of PSA could be developed and used in a prostate cancer assay format, then the potential of eliminating false positive results from benign disorders such as BPH is possible.

PSA is still recognised as the best available indicator for the presence of prostate cancer (Sarkar, 2002). Before the distinction between the various forms of PSA (i.e. free and bound forms) was established as an important factor in prostate cancer detection, antibodies generated did not distinguish between such forms, and the ELISAs developed detected total PSA levels only. The first immunoassay for PSA was described in 1980 (Kuriyama *et al.*, 1980). The format was a sandwich ELISA which was sufficiently sensitive to detect PSA in the serum of normal men and patients with BPH and prostate cancer. Following this, a sandwich ELISA was developed that detected total PSA only (Stowell *et al.*, 1991). Another sandwich ELISA used a polyclonal anti-PSA antibody coated to the surface of the ELISA plate for capture, and a monoclonal anti-PSA antibody, conjugated to HRP, for detection of total PSA (Khosravi *et al.*, 1995). Better sensitivity and accuracy was achieved. A limit of detection of 0.009 μg/l was reported. Assays evaluating the cross-reactivity of 10 anti-PSA monoclonal antibodies with the human Kallikrein 2 (hK2) were also conducted. These assays consisted of a sandwich ELISA and western blot transfer techniques. No cross-reactivity was observed in western blot analysis when hK2 was probed with anti-PSA antibodies. ELISA analysis concluded that 8 out of 10

antibodies analysed demonstrated cross-reactivity between PSA and hK2. However, no combination of monoclonal antibodies tested in a sandwich ELISA for hK2 could give a signal comparable to that for PSA in the same assay. The signal observed for hK2 was no greater than 0.1% of the PSA signal. It was concluded that despite the 80% sequence homology between PSA and hK2, a sizable subset of the immunogenic regions consist of surface epitopes that present either different amino acid residues or distinguishing tertiary structures in the two antigens. The determination of PSA levels using a sandwich ELISA would, therefore, not be significantly affected by cross-reactivity. (Corey *et al.*, 1997).

An ELISA capable of detecting the levels of uncomplexed, free PSA in serum was first reported in 1999 by Matsumoto *et al.* A range of monoclonal antibodies against PSA was first generated and two were selected. One was specific for free PSA and the other for both the free and complexed forms. Both were used to develop an ELISA for free PSA. The detection limit for free PSA established for this assay was 0.008 μg/l for complexed PSA. A year later, the same group published details of an ELISA detecting a complexed form of PSA, PSA-α_1-anti-chymotrypsin. (Matsumoto *et al.*, 2000). Three different monoclonal antibodies that recognised three distinct PSA epitopes were used in the ELISA. Two of the three monoclonal antibodies recognised two specific epitopes on the surface of total PSA; i.e. recognised the PSA protein regardless of whether it was free or complexed. The third antibody recognised an epitope only exposed on free PSA. Binding of this third antibody inhibited binding of the first two by blocking access to the epitopes to which they would bind. In this way, free PSA detection by the assay was blocked and a sandwich ELISA for the detection of complexed PSA was conducted using the other two monoclonal antibodies. This ELISA reported a detection limit of 0.19 μg/l. The result of this data is that commercial assays are now available that discriminate between free and bound forms of PSA. However, studies have shown significant differences in the sensitivities of these assays, indicating a lack of reliability (Oberpenning *et al.*, 2002). It is for this reason that current research is still intensely focused on the development of more reliable and accurate assays for the presence of prostate cancer.

One aspect of such research is the development of immunoassays that can demonstrate higher specificities to the various forms of PSA. Free PSA, is now known to consist of several slightly varying forms, including intact PSA (consisting of proPSA and inactive PSA), internally cleaved PSA, and several other newly identified forms (Mikolajczyk *et al.*, 1997; Peter *et al.,* 2001). Studies indicate that assays able to specifically detect these forms could provide further accuracy in discriminating prostate cancer from benign disorders. Researchers in the University of Turku in Finland are currently developing immunoassays against various forms of cancer-relevant free PSA. In 2000, Nurmikko *et al.* produced a PSA-specific monoclonal antibody that did not detect the internally cleaved form of PSA. Epitope mapping, peptide mapping, specificity, binding and affinity studies were conducted on these antibodies. A year later, the same group reported the development of an immunofluorometric sandwich assay for the measurement of intact, free PSA, but not internally cleaved forms (Nurmikko *et al.,* 2001). The detection limit of this assay was determined to be 0.035 μg/l. A similar immunofluorometric assay was also developed using europium (III) chelate nanoparticles. The detection limit achieved with assay was 0.04 ng/l. Because of the low sensitivity demonstrated, further optimisation of this assay is planned (Soukka *et al.,* 2001). To date, limited research seems to indicate that the ratio of internally cleaved to normal forms of PSA decreases in the serum of cancer patients. Hence, an immunoassay capable of quantifying these forms could potentially increase the accuracy of detection of prostate cancer.

Immunoassays utilising antibody fragments for the detection of PSA in serum have also been produced. Recombinant Fab fragments capable of detecting both free and complexed PSA have been reported (Eriksson *et al.*, 2000). The capture Fab, which bound both free and complexed PSA, was biotinylated and attached to streptavidin-coated mictrotitre wells. A Fab fragment that detected only free PSA, was labelled with a fluorescent europium chelate while a Fab detecting both free and complexed PSA was labelled with fluorescent terbium. Time-resolved fluorescence was used to measure both the europium and terbium signals in one well. Development of an immunoassay with europium- or terbium-labelled Fab fragments provided comparable results to assays conducted using monoclonal anti-PSA antibodies. Detection limits of 0.043 and 0.28 μg/l for both free and total forms of PSA were achieved.

Recombinant Fab fragments have also been used to capture and detect free and total prostate-specific antigen from serum in a single-particle immunoassay (Harma *et al.*, 2000). Thiol-Fab or thiolated monoclonal antibodies were covalently attached onto activated microparticles and free and total PSA were detected with europium- or terbium-labelled Fab fragments on a single microparticle using a microfluorometer. The limit of detection achieved was 0.35 μg/l.

Time-resolved fluorescence imaging was used for the immunohistochemical detection (i.e. tissue samples) of PSA and hK2 using lanthanide chelate-labelled monoclonal antibodies. Comparisons of the results obtained for both PSA and hK2 showed that signal intensity for PSA and hK2 correlated in benign tissue, while variation was observed in these signals between benign and malignant samples. This indicated that the assay is capable of comparing PSA and hK2 content in individual cells, and could potentially be used to indicate the presence of prostate cancer (Siivola *et al.*, 2000).

The cloning, expression and purification of a recombinant form of prostate-specific antigen protein is described in this chapter as very little work was reported in the literature for development of free and total PSA assays using antibody fragments. The PSA gene was inserted and expressed in *E. coli* cells. The PSA protein was purified by IMAC. A preliminary investigation of the feasibility of the use of a competitive ELISA for determining the concentration of PSA that was cloned and expressed as part of the research, was undertaken.

4.2 Results

4.2.1 Transfer of PSA gene insert into pQE-60 vector.

A vector was obtained from Dr. Evaggelia Emmanouilidou from the Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Greece (Emmanouilidou *et al.*, 2002) that contained the PSA gene insert. The vector, psa.pcDNA3, was used to transform competent JM83 cells, and several positive transformants were selected and cultures of these grown. The plasmid was then isolated from these cells, the gene encoding the PSA protein was restricted from the psa.pcDNA3 vector and PCR amplified using specifically designed primers (Figure 4.1). The PSA gene bands as well as the pQE-60 vector were restricted with BamH1 and Nco1 (Figure 4.2) and re-ligated into the high level expression vector, pQE-60. The ligated PSA gene and pQE-60 vector were then transformed into competent XL-10 Gold *E. coli* for controlled protein expression. Glycerol stocks of the resulting transformants were made and stored at -80^0C.

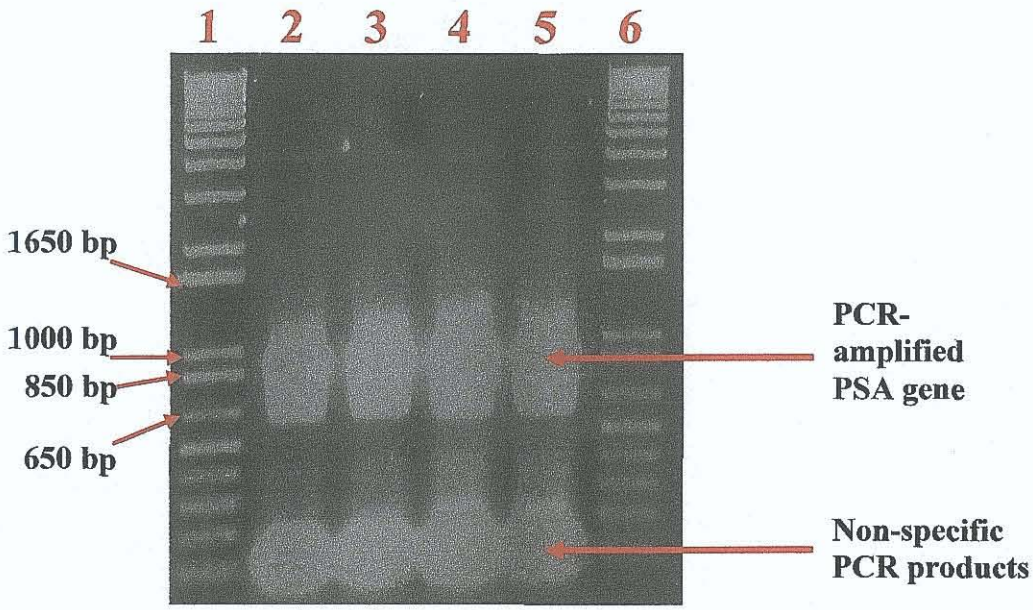

Figure 4.1 *Gel picture showing 850 bp bands containing the amplified PSA gene. Lanes 1 and 6 represent a Promega 1 Kb plus DNA ladder Lanes 2-5 represent DNA coding for PSA.*

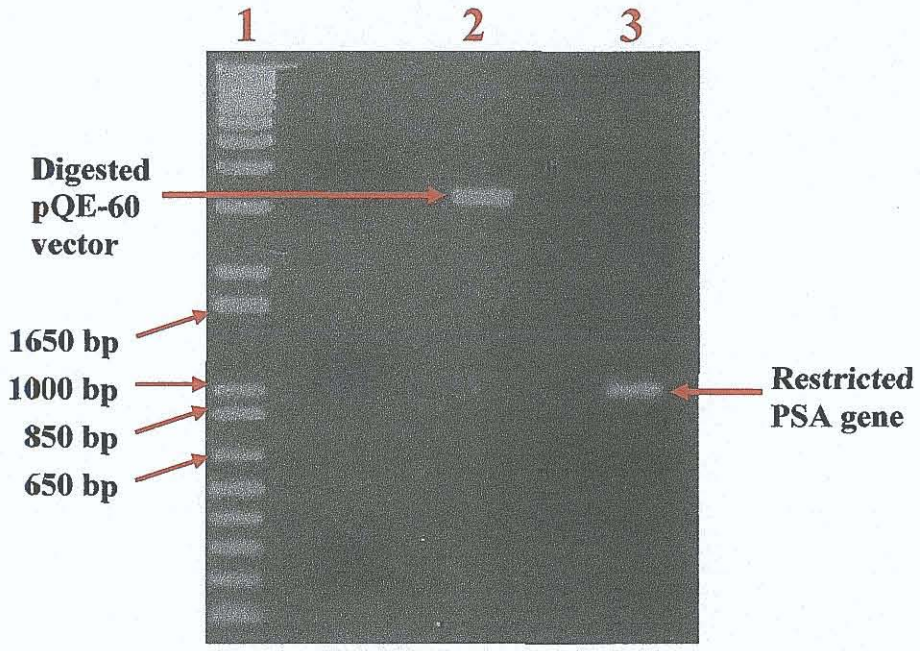

Figure 4.2 *BamH1 and Nco1 restriction digest of the PSA gene and the pQE-60 vector. Lane 1 represents a Promega 1Kb DNA ladder. Lane 2 contains restricted pQE-60 vector. Lane 3 contains the restricted PSA gene.*

4.2.2 Reamplification of PSA gene, ligation into pQE-60 and transformation into XL-10 Gold

Overnight cultures of the glycerol stocks already produced were made and the pQE-60 vector containing the PSA gene insert was isolated. This was then used as a template for another PCR amplification of the PSA gene using the newly designed primers (Figure 4.3). The newly amplified PSA gene was restricted with Sfi1 along with the pQE-60 vector (Figure 4.4) ligated and transformed into XL-10 Gold cells.

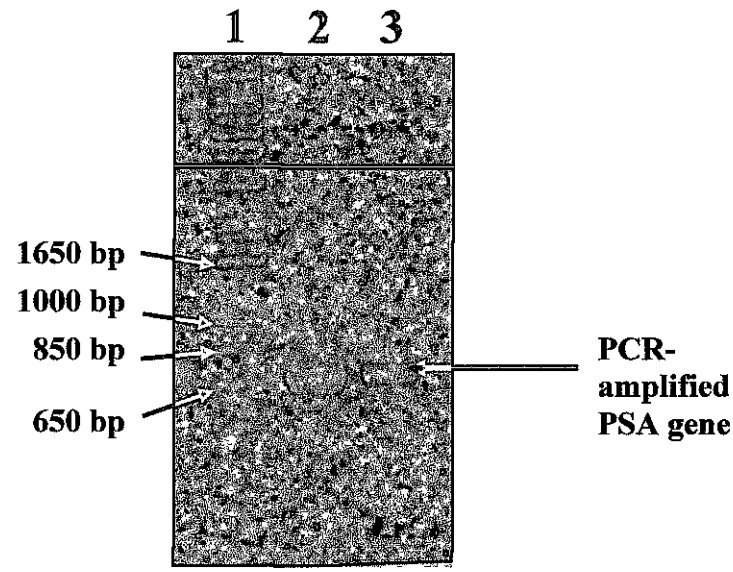

Figure 4.3 *Gel picture showing 850 bp bands containing the amplified PSA gene after PCR has been conducted using the newly designed primers. Lane 1 represents a Promega 1 Kb plus DNA ladder Lanes 2 and 3 represent DNA coding for PSA.*

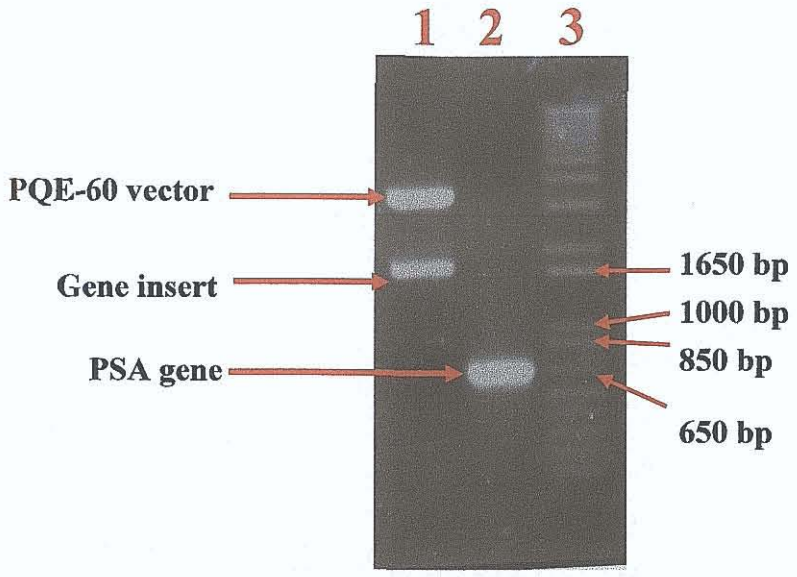

Figure 4.4 *Sfi1 restriction digest of the newly-amplified PSA gene and the pQE-60 vector. Lane 1 contains restricted pQE-60 vector. Lane 2 contains the restriction digested PSA gene. Lane 3 represents a Promega 1Kb DNA ladder.*

4.2.3 Time course analysis of PSA expression

Once PSA expression from the transformed XL-10 Gold cells (prepared as in Section 2.3.8) had been confirmed, a time course analysis on the level of protein expression was conducted to determine the optimal induction time for protein expression. This is important in order to get the balance between the amount of soluble protein in the cells, the formation of inclusion bodies, and protein degradation. By checking the recombinant protein present at various times after IPTG induction, the optimal induction period can be established. A 50 ml culture of the transformed XL-10 Gold cells in log phase was inoculated with IPTG and expression of PSA was induced. 1 ml samples were taken at one-hour intervals for up to 5 hours, and then following overnight induction. The cytoplasmic extract was isolated from each sample and SDS-page analysis was carried out (Figure 4.5). Western blot analysis was then conducted on alternate samples (i.e. sample T=0, T=2, T=4 and T=overnight) with the protein bands probed with a PSA-specific polyclonal antibody (Figure 4.6).

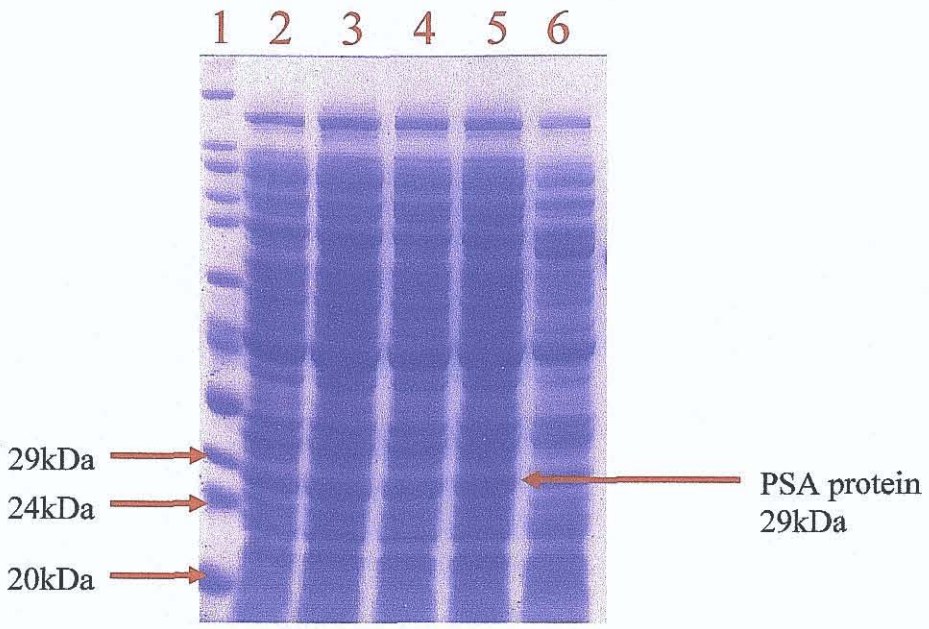

Figure 4.5 *Time-course expression of PSA in XL-10 Gold cells. Lane 1 contains molecular weight markers for protein qualification. Lane 2 represents protein expression 1 hour after induction, lane 3 after 2 hours, lane 4 after 3 hours, lane 5 after 4 hours, and lane 6 after overnight induction. A band at 29 kDa is visible and is most prominent after 4 hours of induction.*

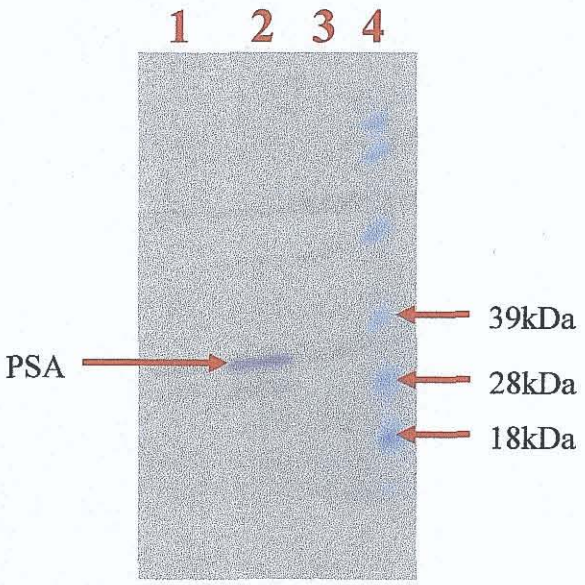

Figure 4.6 *Western blot analysis of time-course expression of PSA in XL-10 Gold cells. Lane 1 is the PSA present 2 hours after induction. Lane 2 is the PSA present 4 hours after induction. Lane 3 is the PSA presence after overnight induction. Lane 4 contains prestained molecular weight markers.*

4.2.4 Immobilised metal affinity chromatography (IMAC) purification of PSA

The IMAC purification technique was used to obtain a purified form of the PSA protein from the cytoplasmic extract, which contains a large number of contaminating proteins in addition to the PSA that have been produced by the host cell. Purification of PSA was conducted under denaturing conditions using IMAC resin supplied by QIAgen. The purified fractions were analysed by SDS-PAGE (Figure 4.7) and Western Blot (Figure 4.8) techniques.

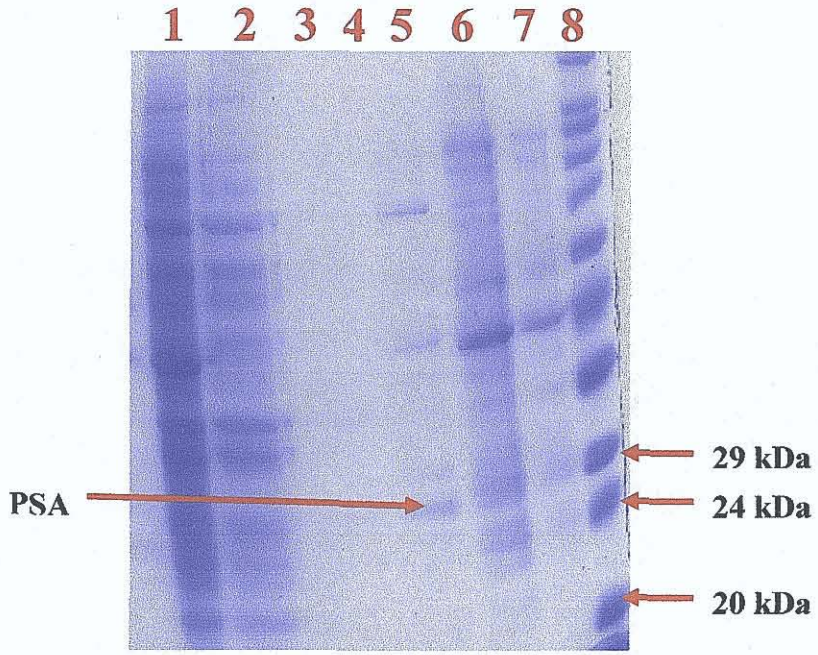

***Figure 4.7** SDS-PAGE analysis on the IMAC purification of PSA. Lane 1 is crude cytoplasmic lysate from XL-10 Gold following PSA expression. Lane 2 is flow-through from IMAC column following application of crude lysate. Lanes 3 and 4 are fractions collected from washings of the column. Lanes 5, 6 and 7 are column elutions 1, 2 and 3, respectively. A band PSA is visible in each of the 3 elutions.*

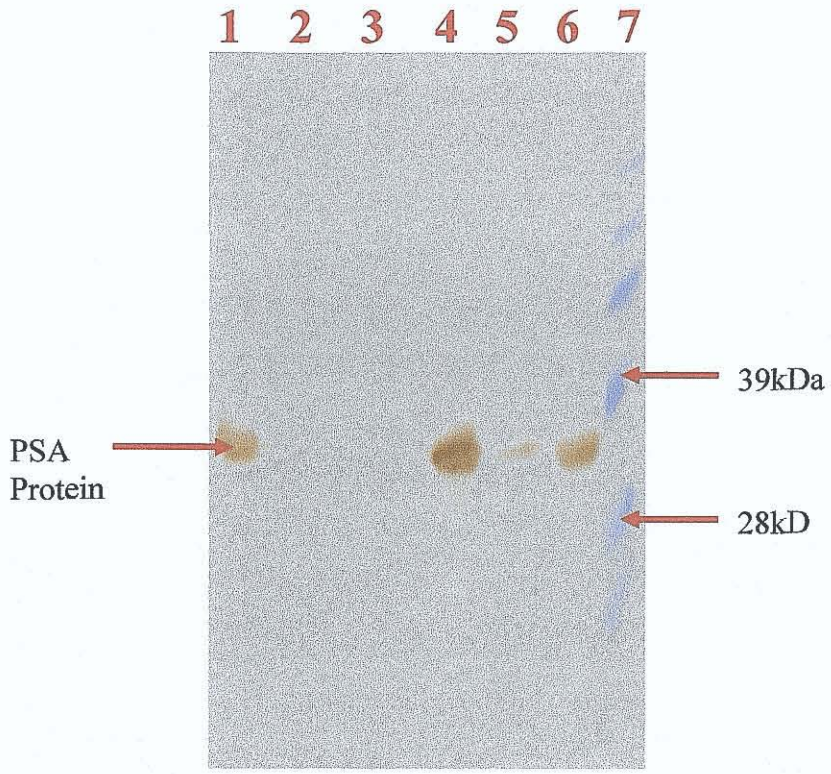

Figure 4.8 *Western blot analysis on the IMAC purification of PSA. Lane 1 is crude cytoplasmic lysate from XL-10 Gold following PSA expression. Lane 2 is flow-through from IMAC column following application of crude lysate. Lanes 3 and 4 are wash fractions 1 and 2, respectively. Lanes 5, 6 and 7 are elutions 1, 2 and 3, respectively. Lane 7 contains molecular weight markers.*

4.2.5 Development of a competitive enzyme-linked immunosorbent assay (ELISA) for PSA

A competitive ELISA was developed for the detection of the recombinant form of PSA using the commercial rabbit-anti-PSA polyclonal antibody. In order to determine the inter-day assay variation, the ELISA was repeated three times over three separate days. The normalised mean values, which were calculated as the mean absorbance at each antigen concentration / absorbance in the presence of zero antigen concentration (A/A0) over the three days were used to construct a calibration curve for the interday assay. Using Biaevaluation 3.1 software the calibration curve was plotted using the four-parameter equation.

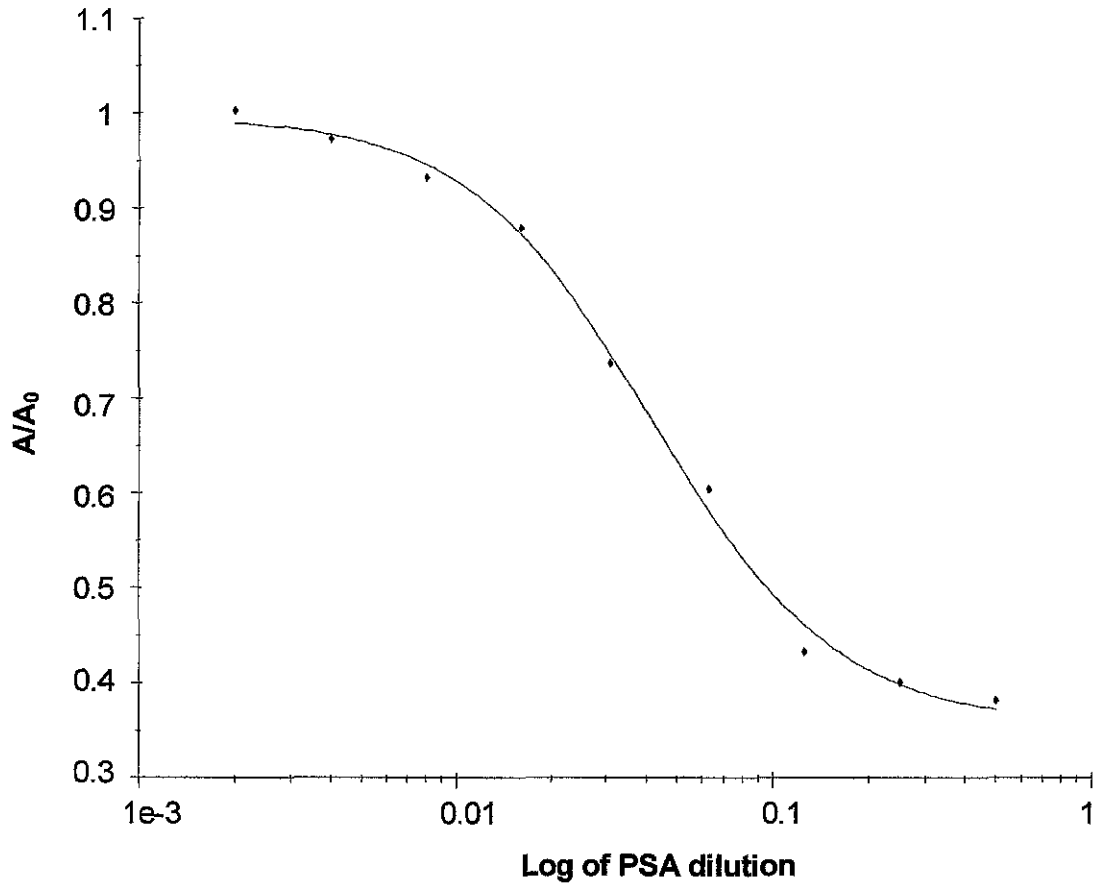

Figure 4.9 Inhibition ELISA for recombinant PSA

Table 4.10 *Percentage CV values calculated for the inhibition ELISA for recombinant PSA plotted in Figure 1*

PSA dilution	% CV
1/2	9.40%
1/4	10.76%
1/8	8.56%
1/16	6.46%
1/32	8.92%
1/64	8.47%
1/128	5.15%
1/256	6.76%
1/512	0.40%

4.3 Conclusion

This Section describes the successful cloning, expression and purification of PSA. The PSA gene insert was received in the psa.pcDNA3 vector, which was initially transformed into JM83 *E. coli* cells to allow for plasmid isolation and restriction of the vector for isolation of the PSA gene. The gene was then amplified by PCR, restricted and ligated into a fully purified and restricted pQE-60 expression vector. The vector was subsequently transformed into XL-10 Gold cells for soluble expression.

Expression of the PSA protein was initially analysed by conducting a time-course expression study. Clones were induced with IPTG for soluble PSA expression and samples taken every hour for 4 hours and then after overnight induction to analyse the optimal expression time. The results of this was analysed by SDS-PAGE and Western blot analysis. It was determined that 4 hours of induction was the optimal length of time after which PSA was to be isolated. The results of IMAC purification were also analysed by SDS-PAGE and Western blot, and purified PSA was visible when the elution fractions of the purification were observed.

Further work regarding purification was not possible due to the time constraints of this project. Future work in this area would involve further optimisation of the conditions for protein expression and purification, in order to obtain a pure, concentrated sample of PSA protein for analysis.

In addition, due to time constraints, only a basic investigation using recombinant PSA protein which was not possible to quantify, could be performed to determine if a competitive ELISA would demonstrate the required competition. Dilutions of the neat stock of recombinant PSA were used to perform the investigation (Section 4.2.4). A plot of the initial inhibition ELISA performed using commercial anti-PSA antibodies shows a definite inhibition trend (Figure 4.9). Since this work consists of only initial investigation into the development of a competitive immunoassay for PSA, further work in this area would involve quantification of purified recombinant protein, and development of sensitive ELISA and Biacore-based inhibition assays.

Future work could also involve use of the recombinant PSA protein for the immunisation and subsequent development of scFv antibody fragments, using the methods outlined in Section 2.2 to 2.4 for the development of such fragments to zilpaterol. Such antibody fragments would then be used to develop Biacore-based inhibition assays to PSA.

Chapter 5

Overall Conclusions

5.1 Overall conclusions

The research work described in this thesis is mainly concerned with the development of scFv antibody fragments and the use of these fragments along with polyclonal antibodies for the detection of the beta-agonist zilpaterol, and prostate-specific antigen (PSA).

The initial work carried out in this thesis was the characterisation of a polyclonal antibody for zilpaterol, provided by Dr. Lisa Connolly of Queens University, Belfast. The antibody was used to develop a competitive ELISA for the detection of zilpaterol. This assay involved immobilisation of the polyclonal antibody onto the surface of a microtitre plate, then simultaneous addition of a fixed concentration of HRP-labelled zilpaterol and varying concentrations of free zilpaterol. The signal produced was inversely proportional to the concentration of free zilpaterol present in the assay. A model competitive assay was developed which displayed excellent recognition of the unconjugated zilpaterol molecules and showed very low limits of detection. The interday assay variability statistics were determined. Cross-reactivity studies were also performed in order to assess the reactivity of the assay with structurally similar molecules. Competitive assays were carried out with several other beta-agonists, in addition to several other potential cross-reactants. Analysis of these results indicates that use of this polyclonal antibody gives an accurate specific and reproducible assay.

The next step was the development of a Biacore-based inhibition immunoassay for the detection of zilpaterol using R699 anti-zilpaterol polyclonal antibody. The binding response demonstrated showed very low limit of detection, indicating a highly sensitive assay.

The next section of the research was the successful production of an scFv antibody fragment specific for zilpaterol and use of this fragment in the development of an assay for the detection of zilpaterol. Libraries produced from pre-immunised spleen mRNA were subjected to rounds of affinity selection panning to select for and enrich for antibodies recognising zilpaterol. The scFv demonstrating the best binding characteristics was then expressed and used in the development of a competitive ELISA for zilpaterol.

A Biacore-based Inhibition immunoassay for the detection of zilpaterol using the B1 anti-zilpaterol polyclonal scFv was also generated. Cross reactivity studies were also conducted using Biacore to measure the response scFv to other β-agonsits and other potential cross-reactants.

Results showed that the assay developed was repeatable and reliable, and while improvement of the affinity of the scFv would result in a more sensitive assay, the ability of the scFv antibody to be used in such an assay format was confirmed. However, the antibody could be improved by established genetic engineering approaches.

Chapter 4 details the successful cloning, expression and purification of PSA. The results of a competitive ELISA for the detection of the recombinant form of PSA using the commercial rabbit anti-PSA polyclonal antibody are also described. While the concentration of the PSA protein used in the dilution could not be determined accurately, a plot of the results obtained indicated that the development of an inhibition ELISA using the recombinant form of the PSA is feasible.

Chapter 6

References

Anderson J. (2002). Does PSA testing influence the natural history of prostate cancer? *Eur. Urol. Suppl.*, **1**:3-10.

Bauvin E., Soulié M., Ménégoz F., Macé-Lesec'h J., Buémi A., Velten M., Villers M., Grosclaude P. (2003). Medical and non-medical determinants of prostate cancer management: a population-based study. *Eur. J. Cancer,* **13**(16):2364-2371.

Berek C. (1999). Affinity Maturation, in *Fundamental Immunology*, (4th ed.), Lippincott-Raven Publishers, Philadelphia, USA.

Blanca J., Munoz P., Morgado M., Mendez N., Aranda A., Reuvers T., Hooghuis H. (2005). Determination of clenbuterol, ractopamine and zilpaterol in liver and urine by liquid chromatography / tandem mass spectrometry. *Analytica Chimica Acta,* **529**:199-205.

Blazek D., Celer V., Navrátilová I., Skládal P. (2004). Generation and characterisation of single-chain antibody fragments specific against transmembrane envelope gp46 of maedi-visna virus. *J. Immunol. Methods,* **115**(1):83-92.

Bocca B., Fiori M., Cartoni C. and Brambilla G. (2003). Simultaneous determination of Zilpaterol and other beta agonists in calf eye by gas chromatography/tandem mass spectrometry. *JAOAC Int.,* **86**(1):8-14.

Brambilla G., Cenci T., Franconi F., Galarini R., Macri A., Rondoni F., Strozzi M., Loizzo A. (2000). Clinical and pharmacological profile in a clenbuterol epidemic poisoning of contaminated beef meat in Italy. *Toxicology Letters*, **114**:47-53.

Branden C. and Tooze J. (1998). *Introduction to Protein Structure* (2nd ed), Garland Publishing Inc., New York, USA.

Brennan J., Dillon P., 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**(1-2):327-42.

Christensson A., Bjork T., Nilsson O., Dahlen U., Matikainen M.T., Cockett A.T., Abrahamsson P.A., Lilja H. (1993). Serum prostate-specific antigen complexed to alpha 1-anti-chymotrypsin as an indicator of prostate cancer. *J. Urol.*, **150**:100-105.

Corey E., Buhler K.R., Vessella R.L. (1997). Cross-reactivity of ten anti-prostate-specific antigen monoclonal antibodies with human glandular kallikrein. *Urol.*, **50:**567-572.

Courtheyn D., Le Bizec B., Brambilla G., De Brabander H. F., Cobbaert E., Van de Wiele M., Vercammen J., De Wasch K. (2005). Recent developments in the use and abuse of growth promoters. *Anal. Chim. Acta.*, **143(2)**:151-160.

De Bellis D, and Schwartz I. (1990). Regulating expression of foreign genes fused to *lac*: control by glucose levels in growth medium. *Nucleic Acids Res.*, 18:1311.

De Franco, A. L. (1999). B-Lymphocyte Activation, in *Fundamental Immunology*, pp 225-261, (4th ed), Lippincott-Raven Publishers, Philadelphia, USA.

Delves, P.J. (1997). General Introduction, in *Antibody production-essential techniques*, p1-12, John Wiley and Sons, West Sussex, UK.

Dillon P.P., Manning B.M., Daly S.J., Killard A.J., O'Kennedy R. (2003). Production of a recombinant anti-morphine-3-glucuronide single-chain variable fragment (scFv) antibody for the development of a "real-time" biosensor-based immunoassay. *J. Immunol. Methods*, **276:**151-161.

Elliott, W. H. and Elliott, D. C. (2001). The Immune System, in *Biochemistry and Molecular Biology*, pp 451-463, Oxford University Press, UK.

Eriksson S., Vehniainen M., Jansen T., Meretoja V., Saviranta P., Pettersson K., Lovgren T. (2000). Dual-label time-resolved immunofluorometric assay of free and total prostate-specific antigen based on recombinant Fab fragments. *Clin. Chem.*, **46**(5):658-666.

Frazer, J.K. and Capra, J.D. (1999) Immunoglobulins: Structure and Function; in *Fundamental Immunology*, pp 37-74, (4th ed.), Lippincott-Raven Publishers, Philadelphia, USA.

Gosling J.P. (2000) Analysis by Specific Binding; in *Immunoassays*, pp 1-18, Oxford University Press, UK.

Hanes J. and Plückthun A. (1997). *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci. (USA)*, 94:4937-42.

Harma H., Tarkkinen P., Soukka T., Lovgren T. (2000). Miniature single-particle immunoassay for prostate-specific antigen in serum using recombinant Fab fragments. *Clin. Chem.*, **46**(11):1755-1761.

He M. and Taussig M.J. (1997). Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for *in vitro* display and evolution of antibody combining sites. *Nucleic Acids Res.*, 25(24):5132-4.

Hoogenboom H.R., Marks J.D., Griffiths A.D., Winter G. (1992). Building antibodies from their genes. *Immunol. Rev.*, **130**:41-68.

Hoogenboom H.R., De Bruine A.P., Hufton S.E., Hoet R.M., Arends J., Roovers R.C. (1998). Antibody phage display technology and its applications. *Immunotechnology*, **4**:1-20.

Hudson P.J. (1998). Recombinant Antibody Fragments. *Curr. Opin. Biotechnol.*, **9**(4):395-402.

Hurrell J.G.R. (1985). *Monoclonal hybridoma antibodies: Techniques and Applications*, Chapter 8, p. 154, CRC Press Inc., Boca Raton, Florida, USA.

Inoue, H., Nojima, H. and Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, **96**:23-28.

Jung J.Y., Kim H.K., Roh Y.T., Choi D.O.Y., Yoo T.K., Kim E.K. (2000). Long-standing ureteral metastasis secondary to adenocarcinoma of the prostate after bilateral orchiectomy. *J. Urol.* **164**(4):1298-1299.

Kane M.M. and Banks J.N. (2000). Raising Antibodies, in *Immunoassays,* pp 19-58, Oxford University Press, UK.

Kettleborough C.A., Saldanaha J., Ancell K.H. and Bendig, M.M. (1993). Optimisation of primers for cloning libraries of mouse immunoglobulin genes using the polymerase chain reaction. *Eur. J. Immunol.,* **23**:206-211.

Khosravi J.M., Papanastasiou-Diamandi A., Mistry J. (1995). An ultrasensitive immunoassay for prostate-specific antigen based on conventional colorimetric detection. *Clin. Biochem* , **28**:407-414.

Killard A.J., Deasy B, O'Kennedy R., Smyth M.R. (1995). Antibodies: Production, Function and Applications in Biosensors. *Trends. Anal. Chem.,* **14/6**:257-266.

King D.J. (1998). Preparation, structure and function of monoclonal antibodies, in *Applications and Engineering of Monoclonal Antibodies,* p14-18, T.J. International Ltd, Padstow, UK.

Knappik A., Ge L., Honegger A., Pack P., Fischer M., Wellnhofer G., Hoess A., Wolle J., Pluckthun A., Vimekas B. (2000). Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J. Mol. Biol.,* **296**(1):57-86.

Köhler G. and Milstein C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.),* **256**:495-497.

Krebber A., Bornhauser S., Burmester J., Honegger A., Willuda J., Bosshard H.R., Pluckthun A. (1997). Reliable cloning of functional antibody variable domains from hybridoma and spleen cell repertoires employing a re-engineered phage display system. *J. Immunol. Methods,* **201**:35-55.

Kuriyama M., Wang M.C., Papsidero L.D., Killian C.S., Shimano T., Valenzuela L., Nishiura T., Murphy G.P. and Chu T.M. (1980). Quantitation of prostate - specific antigen in serum by a sensitive enzyme immunoassay. *Cancer. Res.,* **40**:4658–4662.

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

Levesque M., Hu H., D'Costa M., Diamandis E.P. (1995). Prostate-specific antigen expression by various tumors. *J Clin. Lab. Anal.,* **9**:123-144.

Liddell E. (2001) Antibodies, in *The Immunoassay Handbook,* p118-139, (2nd ed), Nature publishing Group, New York, USA.

Male D., Cooke A., Owen M., Trowsdale J., Champion B. (1996). Advanced Immunology, 3rd ed., Times Mirror International Publishers Limited, London, UK.

Matsumoto K., Konishi N., Hiasa Y., Kimura E., Takahashi Y., Shinohara K., Samori T. (1999). A highly sensitive enzyme-linked immunoassay for serum free prostate-specific antigen (f-PSA). *Clin. Chim. Acta.*, **281**:57-69.

Matsumoto K., Konishi N., Samori T., Kimura E., Doi M., Kato S., Yuki Y. (2000). ELISA for a complexed antigen with a monoclonal antibody blocking reaction with the free antigen—assay-specific for complexed prostate-specific antigen. *J. Immunol. Methods,* **234**:99-106.

Max E.E. (1999). Immunoglobulins: Molecular Genetics, in *Fundamental Immunology*, pp 111-182, (4th ed.), Lippincott-Raven Publishers, Philadelphia, USA.

Mayforth R.D. (1993). Antibody structure, p 4-23, in *Designing Antibodies*, Academic Press Ltd., London, UK.

McCormack T., Keating G.J., Killard A.J., Manning B. and O'Kennedy R. (1998). Biomaterials for Biosensors, in *Principles of Chemical and Biological Sensors*, Diamond D. (ed), John Wiley and Sons, Chestester, England.

Mikolajczyk S.D., Grauer L.S., Millar L.S., Hill T.M., Kumar A., Rittenhours H.G., Wolfert R.L., Saedi M.S. (1997). A precursor form of PSA (pPSA) is a component of the free PSA in prostate cancer serum. *Urol.*, **50**:710-714.

Miller J., Valdes R., Jr. (1993). Methods for calculating cross-reactivity in immunoassays. *J. Clin. Immunoassay*, **15**:97-107.

Nelson P.N., Reynolds G.M., Waldron E.E., Ward E., Giannopoulos K., Murray P.G. (2000). Monoclonal Antibodies. *Mol. Path.*, **53**(3):111-117.

Nurmikko P., Vaisanen V., Piironen T., Lindgren S., Lilja H., Pettersson K. (2000). Production and characterisation of novel anti-prostate-specific antigen (PSA) monoclonal antibodies that do not detect internally cleaved Lys145-Lys146 inactive PSA. *Clin. Chem.*, **46**(10):1610-1618.

Nurmikko P., Pettersson K., Piironen T., Hugosson J., Lilja H. (2001). Discrimination of prostate cancer from benign disease by plasma measurement of intact, free prostate-specific antigen lacking an internal cleavage site at Lys145-Lys146. *Clin. Chem.*, **47**(8):1415-1423.

Oberpenning F., Weining C., Brandt B., De Angelis G., Heinecke A., Hamm M., Stieber P., Hertle L., Schmid H.P., Semjonow A. (2002) Combining Free and Total Prostate-Specific Antigen Assays from Different Manufacturers: The Pitfalls. *Eur. Urol.*, **42**:577-582.

Orum H., Andersen P.S., Oster A., Johansen L.K., Riise E., Bjornvad M., Svendsen I., Engberg J. (1993). Efficient Method for Constructing Comprehensive Murine Fab Antibody Libraries Displayed on Phage. *Nucleic. Acids. Res.*, **21**(19):4491-4498.

Osbourn J.K. (2000). Selection of Antibodies from Phage Display Libraries of Immunoglobulin Genes, in *Monoclonal Antibodies*, pp 67-90, Oxford University Press, UK.

Panayotou G., Waterfield, M.D. and End, P. (1993). Riding the evanescent wave. *Current Biology*, **3/12**:913-915.

Peter J., Unverzagt C., Krogh T.N., Vorm O., Hoesel W. (2001). Identification of precursor forms of free prostate-specific antigen in serum of prostate cancer patients by immunosorption and mass spectrometry. *Cancer Res.*, **61**:957-962.

Rittenhouse H. G., Finlay J.A., Mikolajczyk S.D., Partin A.W. (1998). Human Kallikrein 2 (hK2) and Prostate-Specific Antigen (PSA): Two Closely Related, but Distinct, Kallikreins in the Prostate. *Crit. Rev in Clin. Lab. Sci.*, **35**(4):275-368.

Roitt I., Brostoff J., Male D. (Eds). (1998). Immunology, 5th ed., Gower Medical Publishing, London, UK.

Sarkar P., Pal P.S., Ghosh D., Setford S.J., Tothill I.E. (2002). Amperometric biosensors for detection of the prostate cancer marker (PSA). *Int. J. Pharmaceutics,* **238**:1-9.

Shelver W.L. and Smith D.J. (2004). Enzyme-Linked Immunosorbent Assay Development for the β-Adrenergic Agonist Zilpaterol. *J Agric. Food Chem.*, **52**:2159-2166.

Shelver W.L., Kim H.J., Li Q.X. (2005) [1]. Development of a monoclonal antibody-based enzyme-linked immuosorbent assay for the beta-adrenergic agonist zilpaterol. *J. Agric. Food Chem.*, **53**(9):3273-80.

Siivola P., Petterson K., Piironen T., Lövgren T., Lilja H., Bjartell A. (2002). Time-resolved fluorescence imaging for specific and quantitative immunodetection of human kallikrein 2 and prostate-specific antigen in prostatic tissue sections. *Urol.*, **56**(4):682-688.

Smith G.P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, **228**:1315-1317.

Soukka T., Paukkunen J., Harma H., Lonnberg S., Lindroos H., Lovgren T. (2001). Supersensitive time-resolved immunofluorometric assay of free prostate-specific antigen with nanoparticle label technology. *Clin. Chem.*, **47**(7):1269-1278.

Stapleton S., Tully E. and O'Kennedy R. (2004). Immunoassays: Production of Antibodies, p 306-316, in *Encyclopedia of Analytical Science*, 2nd edition, Townsend A. (de.) Academic Press, New York, USA.

Stenman U.H., Leinonen J., Zhang W.M., Finne P. (1999). Prostate-specific antigen. *Semin. Cancer. Biol.*, **9**:83-93.

Stowell L.I., Sharman, L.E., Hamel K. (1991). An enzyme-linked immunosorbent assay (ELISA) for prostate-specific antigen. *Forensic. Sci. Int.*, **50**:125-138.

Tizard I.R. (1995). Immunology: An Introduction, (4th ed.), Saunders College Publishing, London, UK.

Tout N.L., Yau K.Y.F., Trevors J.T., Lee H. Hall J.C. (2001). Synthesis of ligand-specific phage-display scFv against the herbicide picloram by direct cloning from hyperimmunized mouse. *J Agric. Food. Chem.*, **49**:3628–3637.

Vaughan T.J., Williams A.J., Pritchard K., Osbourn J.K., Pope A.R., Earnshaw J.C., McCafferty J., Hodits R.A., Wilton J., Johnson K.S. (1996). Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat. Biotechnol.,* **14**(3):309–314.

Vihinen M. (1994). Modeling of Prostate-Specific Antigen and Human Glandular Kallikrein Structures. *Biochem. and Biophys. Research Comm.* **204**(3)1251-1256.

Wild D. (1994). The Immunoassay Handbook. 1st eds. Stockton Press, New York, U.S.A.

Yau K.Y.F., Lee H., Hall C. (2003). Emerging trends in the synthesis and improvement of hapten-specific recombinant antibodies. *Biotech. Advances,* **21**:599-637.

Yousef G.M., Bharaj B.S., Yu H., Poulopoulos J. and Diamandis E.P. (2001). Sequence Analysis of the Human Kallikrein Gene Locus Identifies a Unique Polymorphic Minisatellite Element. **285**(5):1321-1329.

Zhang W.M., Leinonen J., Vesalainen S., Nordling S., Stenman U.H. (1999). Measurement of the complex between prostate-specific antigen and α_1-protease inhibitor in serum. *Clin. Chem.,* **45**:814-821.