The Production of Recombinant single chain Antibody Fragments for the Detection of Illicit Drug Residues

A Thesis submitted for the degree of

Doctorate of Philosophy

By

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This thesis is dedicated to my Dad and Bernie.

Declaration

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

Signed: Joanne Bowen

Date: 9/5/05

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Presentations

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Brennan, J. and O'Kennedy, R. (2003). The production of novel genetically-derived scFv antibodies capable of illicit drug detection. Defence Science and Technology Laboratory collaboration meeting, DCU, 30th October.

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Abstract

Recombinant antibodies represent a more sensitive and specific detection tool for immunoanalysis. The research carried out for this thesis describes the production of genetically-derived single chain antibody fragments to detect illicit drugs.

A variety of novel recombinant antibody fragments against morphine-3-glucuronide, a metabolite of heroin has been produced. A monomeric, dimeric and enzyme-labelled scFv were characterised with respect to their binding abilities and cross reactivities. Monomeric scFv was successfully applied to a competitive ELISA format for the detection of morphine residues in saliva. The assay was used to positively identify morphine residues in the saliva of drug addicts attending a rehabilitation clinic. An inhibition assay to detect morphine in saliva was also developed using the BIAcore 3000 instrument, a surface plasmon resonance-based biosensor for detection of biomolecular interactions in 'real-time'. A novel sol particle lateral flow immunoassay was generated using monomeric and dimeric scFvs for the detection of M3G.

Recombinant antibody fragments against tetrahydrocannabinol were isolated from a naïve human library by a process known as biopanning. Phage-displayed antibodies have been fully characterised with respect to their binding capabilities and were successfully applied to an immunoassay for the detection of marijuana residues in saliva.

Abbreviations

A	Absorbance
AP	Alkaline phosphatase
APC	Antigen presenting cell
BCA	Bicinchoninic acid assay
BCIP/NBT	5–bromo–4–chloro–3–indolyphosphate/nitro blue tetrazolium chloride
BgG	Bovine-δ-globulin
BIA	Biomolecular interaction
BSA	Bovine serum albumin
BtG	Bovine thyroglobulin
C	Constant
Ca	Circa
CAP FUDT	College of American pathologists forensic urine drug testing
cDNA	Complementary deoxyribonucleic acid
CDR	Comlementarity determining region
CFU	Colony forming units
C_H	Constant heavy chain
C_L	Constant light chain
CM	Carboxy-methyl
Conc	Concentration
C.V.	Coefficient of variation
D	Diversity
DMSO	Dimethylsulphoxide
dsFv	Disulphide stabilised variable fragment
DTT	Dithiothreitol
E	Exponential
EDC	N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme multiplied immunoassay technique
EU	European Union

Fab	Antigen binding fragment
Fc	Antibody constant region
FCA	Freund's Complete Adjuvant
FICA	Freund's Incomplete Adjuvant
Fd	Antibody fragment consisting of one V_H and one C_{H1} domain
fd	A strain of filamentous bacteriophage
Ff	Family of filamentous bacteriophage
Fv	Antibody variable fragment
GC	Gas chromatography
Gly	Glycine
H	Heavy chain
HAT	Hypoxanthine aminopterin thymidine
H_B	Heavy chain back primers
HBS	Hepes buffered saline
H_F	Heavy chain forward primers
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
His	Histidine
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
HUCK	Human kappa constant domain
IDA	Iminodiacetic acid
IFN	Interferon
IgG	Immunoglobulin class G
IgA	Immunoglobulin class A
IgD	Immunoglobulin class D
IgE	Immunoglobulin class E
IgM	Immunoglobulin class M
IL	Interleukin
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl-β-D-galactopyranoside
J	Joining
K_A	Equilibrium association affinity constant
K_a	Association rate constant

K_D	Equilibrium dissociation affinity constant
K_d	Dissociation rate constant
KLH	Keyhole limpet haemocyanin
L	Light chain
LB	Luria-Bertani
L_B	Light chain back primers
LDD	Least detectable dose or lowest detectable dose
LE	Low expression
LED	Light emitting diode
L_F	Light chain forward primers
LFIA	Lateral flow immunoassay
Ln	Natural log
LOD	Limit of Detection
Log	Logarithm
LOQ	Limit of quantification
M	Slope
Mab	Monoclonal antibody
MAM	Monacetylmorphine
MBDB	3,4-methylenedioxyphenyl-2-butanamine or alternatively known as (N-methyl-1-(1,3 benzodioxol-5-yl)butanamine
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxy-N-ethylamphetamine
MDMA	3.4- methylenedioxymetamphetamine
MHC	Major histocompatability complex
MPBS	Marvel™ PBS
mRNA	Messenger ribonucleic acid
MS	Mass spectroscopy
M.W.	Molecular weight
M3G	Morphine-3-glucuronide
NE	Non-Expression
NEB	New England Biolabs
NHS	N-hydroxysuccinimide
NTA	Nitrotriacetic acid
OD	Optical density

o-PD	*ortho*-Phenylenediamine
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffered saline-tween 20
PCC	Pyridinium chlorochromate
PCR	Polymerase chain reaction
PDB	Protein data bank
PEG	Polyethylene glycol
pH	Log of the hydrogen ion concentration
pI	Isoelectric point
RAG	Recombinase-activating gene
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RSS	Recombination recognition sequences
RT-PCR	Reverse transcription polymerase chain reaction
S	Switch
SAMHSA	Substance abuse and mental health service administration
scAb	Single chain antibody
scback	Single chain back primer
S.D.	Standard deviation
S.E.	Standard error
Ser	Serine
scfor	Single chain forward primer
scFv	Single chain variable fragment
SDS	Sodium dodecyl sulphate
SOC	Super optimal catabolites
SOE	Splice by overlap extension
StAb	Stabilised single chain antibody
S/P	Saliva:plasma ratio
SPR	Surface plasmon resonance
TAE	Tris acetic acid ethylenediaminetetra-acetic acid
TB	Terrific Broth
TBS	Tris buffered saline

T_C	Cytotoxic T-cell
TdT	Terminal deoxynucleotidyltransferase
TED	Tris (carboxymethyl) ethylenediamine
TEMED	N,N,N′,N′-tetramethylethylenediamine
TES	Tris ethylenediaminetetra-acetic acid
Tet	Tetracycline
T_H	Helper T-cell
THC	Tetrahydrocannabinol
THC-COOH	Tetrahydrocannabinol carboxylic acid
TIR	Total internal reflection
TLC	Thin layer chromatography
TMB	3,3′,5,5′-Tetramethylbenzidine hydrocloride
TNF	Tumour necrosis factor
TSS	Transformation and storage solution
2 x TY	Two times tryptone yeast extract media
TYE	Tryptone yeast extract media
UV	Ultraviolet
up	Ultrapure
V	Variable
V_H	Variable region of heavy chain
V_L	Variable region of light chain
WHO	World Health Organisation

Units

µg	microgram
µl	microlitre
µM	micromolar
°C	degrees Celcius
AU	absorbance units
cm	centimetres
Da	dalton
g	grams
kDa	kilo dalton
Kg	kilogram
L	litre
m	metre
M	molar
mg	milligram
mins	minute
ml	millilitre
mm	millimetre
mM	millimolar
mol	mole
nl	nanolitre
nm	nanometre
nM	nanomolar
pg	picogram
pM	picomolar
p.s.i.	pounds per square inch
rpm	revolutions per minute
RU	response units
secs	second
U	unit
v/v	volume per unit volume
w/v	weight per unit volume

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Chapter 1: Introduction

1.1 Immunity and the Immune System

The immune system is the body's natural line of defence that works efficiently to protect against the invasion of foreign bodies. It is comprised of two defence systems, the innate and acquired immune systems. The two systems work simultaneously in order to maintain an individual's health and well-being.

1.1.1 Innate immunity

Innate immunity describes the body's natural ability to prevent and destroy infectious agents. Innate immunity fights pathogens using defences that are quickly mobilised and triggered by receptors that recognise a broad spectrum of pathogens. It is a non-specific but immediate response to foreign bodies, the efficiency of which is not enhanced through repeated exposure to the same foreign object. The system works on two levels, primary defence is brought about through physical and biochemical barriers to prevent entry of foreign particles. The secondary response involves a non-specific elimination of invading microbes.

Primary barriers to infection include the skin, which acts as an impermeable barrier to microbial invasion (Partidos *et al.*, 2003), and mucous membranes, which coat inner cavities of the body that are open to the exterior. These 'sticky' membranes prevent adherence of microbes to endothelial cells. Trapped foreign particles can then be expelled from the body through mechanical processes, such as coughing and sneezing. Bodily secretions also play an important role in preventing infection. The low pH of sebaceous secretions, for example, maintains the acidic environment of the skin, while, the presence of lysozyme, a hydrolytic enzyme, provides anti-microbial protection (Roitt *et al.*, 1994).

The secondary line of defence involves non-specific elimination of organisms that have penetrated the body's outer barriers. The innate immune system has specialised killer cells that destroy invading micro-organisms by a process known as phagocytosis. This process of engulfing and ingesting foreign bodies is carried out by two categories of specialised cells, microphages and macrophages. Microphages or

polymorphonuclear neutrophils are a short-lived cell that circulates in the blood, except when recruited to a site of acute inflammation. They are the first on the scene to fight infection and provide the major defence against pyogenic bacteria. Macrophages are long-lived phagocytes, derived from bone marrow promonocytes that are present in connective tissue, lungs, spleen and lymph nodes. They primarily defend against organisms capable of living within the host cell, for example intracellular pathogens, which display surface markers on the cell's exterior. The Complement system is a triggered enzyme cascade of serum proteins that may be activated by the innate immune response. It facilitates phagocytosis by making microbes more recognisable to the immune system, attracting phagocytic cells to a microbe and stimulating the ingestion process. Extracellular killer cells, such as eosinophils, are also involved in innate immunity. They contain destructive enzymes, such as proteinases and peroxidases, and are responsible for attacking larger invaders that cannot be physically phagocytosed. Natural killer cells have the ability to recognise cell-surface changes, like viral infection. They bind to the infected cells and destroy them by apoptosis. Inflammation causes increased vascular permeability and facilitates chemotaxis of phagocytes, making it integral to the innate immune response.

1.1.2 Acquired immunity

Acquired immunity is a specific immune defence that adapts itself to previously unseen molecules, producing specific memory cells to prevent against further infection. It can be further divided into the humoral and cellular immune systems. The humoral immune system utilises antibody producing-B lymphocytes to combat infection of bodily fluids. The cellular immune system fights intracellular pathogens using cytotoxic T-cells (T_C) and helper T-cells (T_H), derived from activated T-lymphocytes in the thymus (Reinhardt and Jenkins, 2003)

1.1.2.1 Cell mediated immune response

Cell mediated immunity (Figure 1.1) has evolved to deal with infection that cannot be reached through humoral antibodies, like intracellular pathogens, such as viruses. T-

cells originating in the thymus circulate permanently between the blood and lymphatic systems. There are three types of T cell involved in cell mediated immunity: helper T-cells (T_H cells), derived from CD^+4 T-cells, killer T-cells, also known as cytotoxic T-cells (T_C cells), derived from CD+8 T-cells and memory T-cells. T-cells surface receptors require the antigen to be 'presented' at the cell surface by an antigen presenting cell (APC), such as macrophages or dendritic cells. Proteins of the Major Histocompatability Complex (MHC) perform this antigen presentation process (Reinhardt and Jenkins, 2003).

Cytotoxic T-cells (T_C cells) recognise an antigen in association with a class I Major Histocompatability Complex molecule (MHC I), which is made by all nucleated cells in the body. If an infected cell is recognised by a T_C cell, they signal the cell to become apoptotic and commit suicide, thus killing that cell along with any intracellular pathogens. Each T_C cell contains granules containing perforin and a set of serine proteases, called granzymes. The T_C cell, releases the contents of these granules after attaching to the target cell. Perforin enlarges the pores in the membrane of the target cell, facilitating the entry granzymes and the induction of apoptosis (Benjamini *et al.*, 2000). T_C cells may also release cytokines like γ-interferon, to reduce the spread of viruses to adjacent cells (Roitt *et al.*, 1994).

Helper T-cells recognise antigens presented on a cell surface in association with a MHC class Π molecule, produced only by APCs. Helper T-cells are divided into two primary types, T_{H1} or T_{H2} cells, depending on the type of cytokines they produce. T_{H1} cells recognise antigens presented on the surface of macrophages and serve to enhance cell-mediated immunity through the release of cytokines such as interleukin-2 (IL 2), γ interferon and tumour necrosis factor beta (TNF β). Collectively these cytokines stimulate the production of T_C cells, natural killer cells and activate macrophages and neutrophils. T_{H2} cells recognise antigens presented on B-lymphocytes and synthesise cytokines such as interleukins 4, 5, 10 and 13, that stimulate antibody production, enable antibody class switching and activate eosinophils. Memory cells are cells derived from CD^+4 T-cells that persist following antigenic exposure. They are long-lived antigen-specific memory cells that will mature and replicate, upon re-exposure to the same antigen-MHC complex.

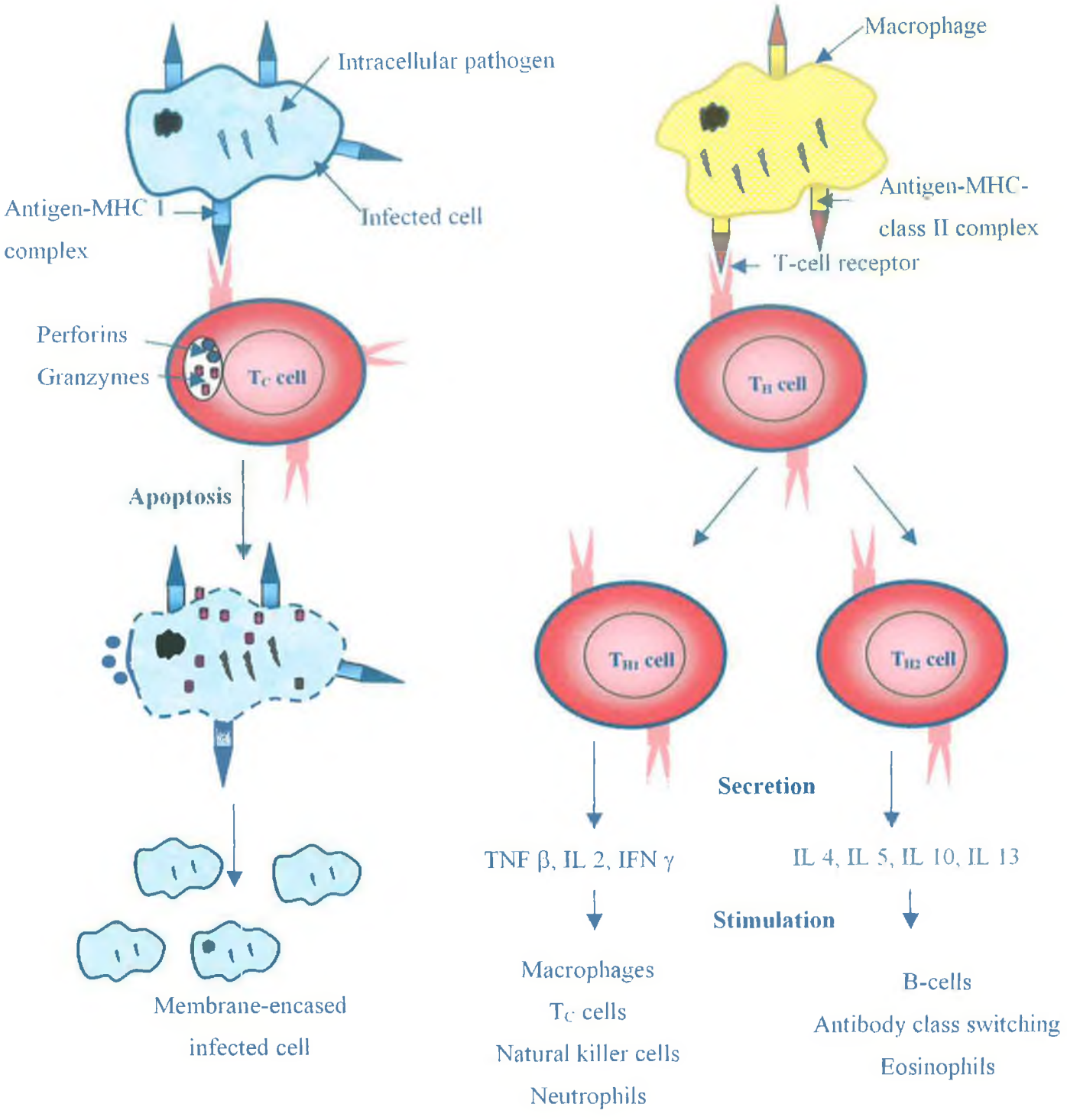


Figure 1.1: *Diagrammatic overview of cell mediated immunity. T_C cells recognise a foreign antigen in association with an antigen-MHC class I complex. These cytotoxic cells signal the infected cell to become apoptotic and commit suicide. T_H cells recognise an antigen in association with a MHC class II complex, displayed on the outside of an antigen presenting cell. T_H cells are subdivided into types 1 and 2, depending on the type of cytokines they secrete. These cytokines function in the stimulation of other cells involved in the immune response.*

1.1.2.2 The humoral immune response

The humoral immune response is mediated by B-lymphocytes, which originate in the bone marrow. The cells of this system respond differently depending on the type of antigen that is encountered. Thymus-independent antigens comprise large bacterial proteins and antigens that contain repetitive epitopes. In response to this type of antigen, each B-lymphocyte displays a membrane bound immunoglobulin, with a single specificity, on its surface, which acts as an antigen receptor. The 'best fit' antibody receptor will develop into an antibody-forming plasma cell that secretes an antibody, identical to the receptor. In this manner it is the antigen that effectively selects the correct antibody for the immune response. This form of humoral response is independent of T-cell activation and produces predominantly poor affinity-IgM type antibodies, that do not give rise to memory cells (Roitt *et al.*, 1994). The majority of antigens contain single discrete epitopes that cannot be crosslinked directly by the B-cell receptor. These antigens are described as thymus-dependant antigens and require T-cells and B-cells to cooperate together to generate an immune response, a process known as T-cell mediated humoral response (Figure 1.2). Both B-cells and T-cells must be activated and respond to epitopes of the antigen that are physically linked (Benjamini *et al.*, 2000). The T_H cell secretes cytokines that stimulate the B-cell to proliferate and differentiate into an antibody-producing cell. Firstly, the antigen that binds the B-cell receptor is internalised by the B-cell. The B-cell then acts as an antigen-presenting cell, displaying the antigen on its surface, as part of an antigen-MHC class II complex. It is now available for recognition by a T_H cell. A number of adhesion pairs strengthen the interaction, upregulate the proliferation of B-cells and promote antibody class-switching. The type of cytokine secreted by the T_H cell determines the class of immunoglobulin produced. If interleukin 4 is secreted, B-cells switch to IgE production, whereas if γ interferon is secreted by the T_H cell B-cells switch to IgG production (Benjamini *et al.*, 2000).

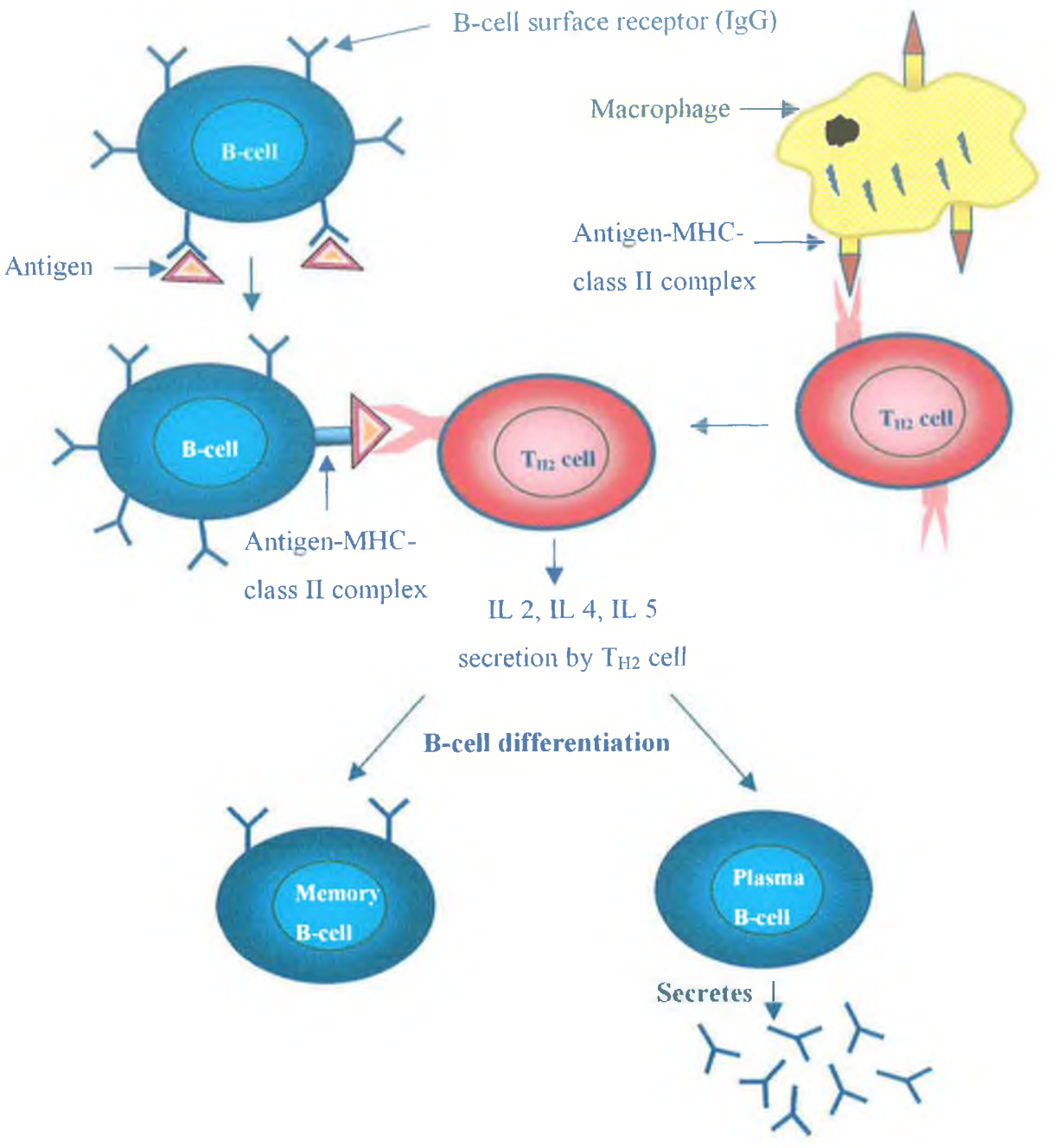


Figure 1.2: *Schematic representation of the main features of the T-cell mediated humoral immune response. The foreign antigen is internalised and displayed on the surface of an antigen presenting cell, in association with a MHC class II complex. B-cells recognise and bind antigen in solution with antibodies displayed on their surface. T_{H2} cells secrete cytokines, such as IL 2, 4 and 5, that simulate B-cell proliferation. This results in B-cell differentiation into plasma and memory B-cells. Plasma cells have the ability to produce large quantities of soluble antibody and memory cells. These are long-lived antigen-specific B-cells that will mature and replicate. They will persist within the body and elicit a stronger immune response, upon re-exposure to the same antigen.*

1.1.3 The anatomy of the immune response

Maturation, differentiation and proliferation of lymphocytes take place in the organs of the lymphoid system. This system can be sub-divided into primary and secondary lymphoid organs. Primary lymphoid organs are those in which B and T-cells mature into antigen-recognising effector cells. In humans, this takes place in the bone marrow and the thymus. Both B-lymphocytes and T-lymphocytes are produced from stem cells in the bone marrow. B-lymphocytes mature in the bone marrow, while T-lymphocytes migrate to the thymus for maturation. These cells migrate from the primary lymphoid organs, through the bloodstream, to the secondary or peripheral lymphoid tissues, where antigen differentiation and proliferation takes place (Benjamini *et al.*, 2000). Peripheral lymphoid organs comprise of highly organised areas of lymphatic tissue. Such areas include the spleen and lymph nodes in addition to scattered accumulations of tissues, such as mucosal-associated lymphoid tissue (MALT). It is in these areas that mature lymphocytes interact with antigen and differentiate to synthesise specific antibodies. Foreign antigens are carried to the secondary lymphoid organs via draining lymphatics, where they are exposed on the surface of these specialised cells. Both T- and B-lymphocytes circulate rapidly around the secondary lymphatic organs. This increases the probability that an antigen-specific lymphocyte will encounter the target antigen. Upon interaction, the lymphocytes become activated and the acquired immune response, with antigen specificity is triggered.

1.2 Antibody Structure

An antibody or immunoglobulin is a serum soluble glycoprotein produced by B-lymphocytes in response to an invading foreign antigen. An antibody molecule is composed of four polypeptide chains, consisting of two identical heavy (H) and two identical light (L) chains, held together by interchain disulphide bonds, and stabilised by non-covalent interactions. The N terminal domains of both heavy and light chains are highly variable between antibodies, while the remaining domains are relatively constant, although they differ between isotypes (Male *et al.*, 1996). There are five distinct classes (isotypes) of antibody, IgA, IgM, IgD, IgE and IgG, differentiated by structural variations in their heavy chain (Zaleski *et al.*, 1983). Immunoglobulin light chains are designated as either kappa (κ) or lambda (λ) class and are folded into two globular domains. There are five classes of heavy chain, gamma (γ), mu (μ), alpha (α), delta (δ) and epsilon (ξ). Heavy chains are comprised of either four or five domains, with IgM and IgE containing five domains, while IgG, IgA and IgD contain four heavy chain domains.

IgM is the first class of antibody to be produced in the primary response to antigen. This class of antibody is a pentamer of the basic four-chain polypeptide chain. It has low affinity but due to its valency of 5, it possesses high avidity for multivalent antigens. IgM antibodies are found on the surface of mature B-lymphocytes and act as an antigen-specific receptor. They are also efficient agglutinating antibodies and are involved in Complement activation. IgA is secreted selectively across seromucousal surfaces, appearing in saliva, tears, nasal fluid, sweat, gastrointestinal and genitourinary tracts. IgA defends exposed external surfaces of the body against attack (Roitt *et al.*, 1994). IgE triggers inflammatory responses, recruiting plasma factors and effector cells, protecting sites susceptible to trauma and pathogen entry. IgD is primarily a cell surface receptor on the surface of blood lymphocytes. Its expression varies during B cell differentiation. IgG is the most abundant type of antibody produced by the immune system, representing 70-80% of total IgG produced (Roitt *et al.*, 1994 and Male *et al.*, 1996) and 15% of total protein (Benjamini *et al.*, 2000). IgG is the most persistent of all antibody classes, with a half-life of approximately 23 days (Benjamini *et al.*, 2000). It is the most versatile type of

antibody, capable of toxin neutralisation, Complement activation and opsonisation. The structure of an antibody is generally represented by the immunoglobulin G (IgG) molecule as shown in Figure 1.3, although the exact structure varies depending on the isotype,

An IgG molecule consists of two gamma (γ) heavy chains and two light chains, either kappa (κ) or lambda (λ). Heavy chains have a molecular weight of approximately 50 kDa and consist of one variable region and three constant domains. It is these constant regions that determine the antibody isotype. Light chains consist of one variable and one constant domain and have a molecular weight of approximately 25 kDa. Therefore, a whole IgG molecule has an apparent molecular weight of approximately 150,000 Da.

Constant regions are essential in biological effector functions, whereas, the specificity of an antibody is encoded in the variable heavy (V_H) and variable light (V_L) chain regions. Within these variable domains are three regions of approximately 110 amino acid residues that display a high variability in amino acid sequence between antibody molecules. These 'hypervariable' regions are also referred to as complementarity determining regions (CDRs), subdivided into CDR1, CDR2 and CDR3 in heavy and light chains. These hypervariable regions are brought together by the folding of the variable domain into loops at the tip of the molecule. This forms the three-dimensional antigen-binding site, which is located at the end of each antibody arm. Antigen binding is also facilitated by the flexibility of the hinge region, an area composed primarily of prolines and cysteines found between heavy chain constant domains. This allows the angle between each 'arm' to alter from 60-180°, enabling the two binding sites to work independently from one another. Framework regions also confer antigenic specificity to the molecule, acting as a scaffold to hold the hypervariable loops in the correct position for antigen binding (Faber *et al.*, 1998)

Other areas of functional importance include the C_{H1}, C_{H2} and C_{H3} regions. The C_{H1} region is involved in binding the complement C4b fragment, while each C_{H2} domain contains carbohydrate binding sites, the nature and number of which varies between isotypes (two in the case of human IgG_1). The C_{H3} domain possesses the effector

functions of the immunoglobulin molecule that allow it to interact with the rest of the immune system (Stockinger and Lemmel, 1978)

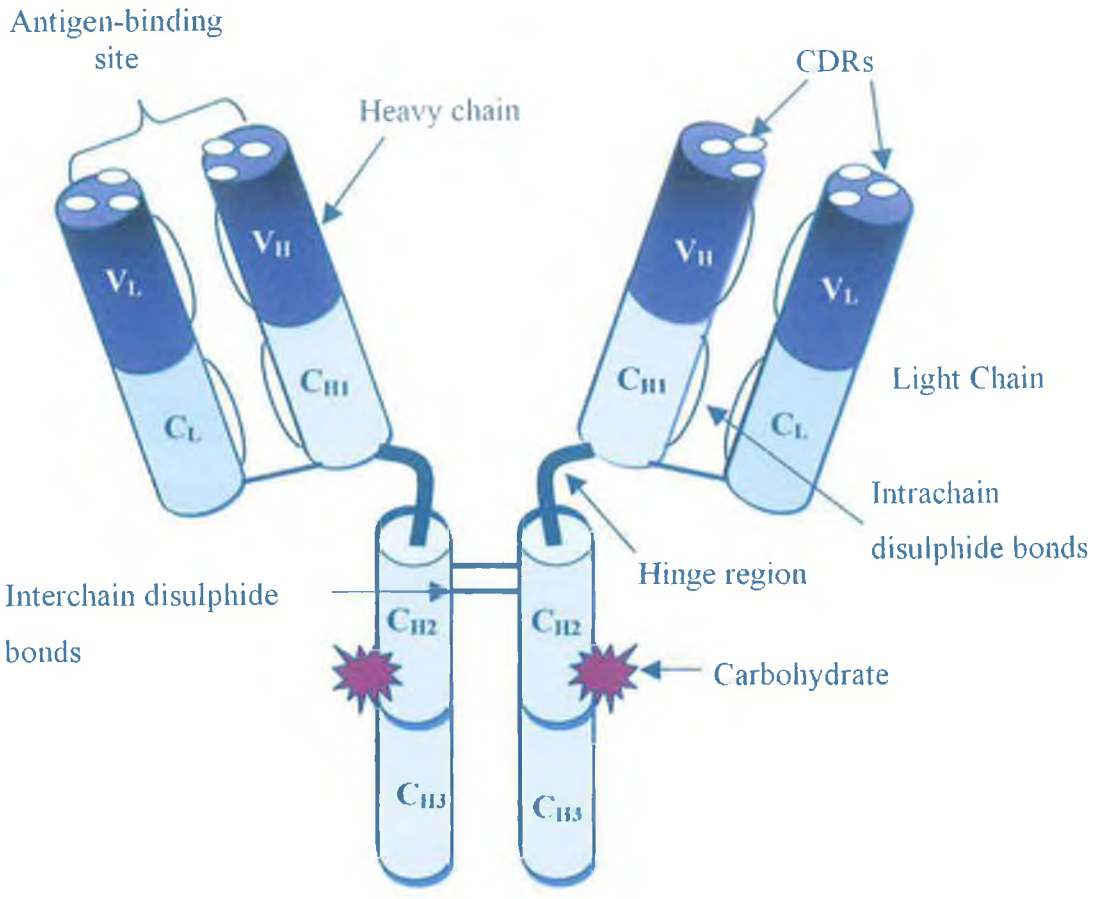


Figure 1.3: *Diagrammatic representation of an immunoglobulin G (IgG) molecule. The IgG molecule is composed of two identical light chains and two identical heavy chains. The light chains are composed of a variable (V_L) and constant (C_L) domain. The heavy chain consists of one variable (V_H) and three constant (C_{H1}, C_{H2} and C_{H3}) domains with a hinge region connecting the C_{H1} and C_{H2} regions. The heavy and light chains are connected via disulphide bonds; disulphide bonds are also present in the constant and variable regions. The CDRs at the amino terminal of the variable domains confer antigenic specificity and contain considerable amino acid sequence variation.*

1.3 Genetic Basis for Antibody Diversity

It is estimated that there is a diversity of B and T-cells with an antigenic specificity ranging from 10^{15} to 10^{18} in any given individual (Benjamini *et al.*, 2000). If every single one of the millions of antibodies produced by an individual were encoded by an individual and unique gene for each of its heavy and light chains, then the immune response would require millions of genes to encode for it alone.

Dreyer and Bennett (1965) first proposed the hypothesis of genetic recombination in antibody formation. This explains how this diversity is achieved, without utilising an enormous number of germ line genes. Clusters of genes on three chromosomes encode the three types of antibody chains, κ, λ and heavy chains. Kappa chain genes are located on chromosome 2, lambda chain genes are clustered on chromosome 22 and heavy chain genes are found clustered as a gene family on chromosome 14.

Light chain genes are assembled from three distinct gene segments, the V (variable) segment, a J (joining) segment and a C (constant) segment. In a B-cell, hundreds of V gene segments lie clustered in a region on the same chromosome but up to a million base pairs away from the C segment (Watson *et al.*, 1992). In other cells, however, this V gene is not found in the vicinity of the gene segment. It is the variable regions, encoded by V and J gene segments, that encodes for the diversity of the antibody light chain. Any V gene can be joined to any J segment located upstream of the C gene segment. The region of the V-J join forms the third hypervariable loop (Male *et al.*, 1996). This leads to the loss of the intervening stretches of DNA, including introns and exons. Since any V gene can recombine with any J region, the number of possible combinations is greatly increased.

Three distinct gene segments encode heavy chain genes. In addition to V and J gene segments, variable heavy chain genes are also encoded by a D or 'diversity' segment. The D and J regions code for the third hypervariable loop of the heavy chain. The production of the heavy chain therefore requires two recombination steps, firstly the D_H segment is joined to the J_H segment and then the V_H segment is joined to the DJ_H segment (Figure 1.4). This gives rise to the V_H domain. The human heavy chain is

encoded by 50 V_H genes, 20 D_H genes and 6 J_H genes, making the total number of possible gene permutations enormous.

During the recombination process of heavy and light chain genes, the various V, D and J regions are brought into contact with each other through a looping out process, mediated by a lymphoid-specific recombinase enzyme complex (Roth and Craig, 1998). The complex is a product of two recombinase-activating genes RAG 1 and RAG 2. The RAG proteins recognise short sequences in the coding regions called recombination recognition sequences (RSS), consisting of a conserved heptamer and nonomer, separated by non-conserved spacer sequence of either 12 or 23 nucleotides (Schlissel, 2003). These proteins break the double stranded DNA between each RSS and it's corresponding coding sequence, yielding two coding ends and two signal ends. The coding ends are covalently closed DNA hairpins and signal ends are blunt and 5′ phosphorylated. These are joined by proteins involved in the nonhomologous DNA end joining pathway (Lieber *et al.*, 2004). The joints formed between coding ends are imprecise and contain short deletions, palindromic duplications or non-templated nucleotide additions (Schlissel, 2003). The addition of such nucleotides in the N region is referred to as insertional diversity and is mediated by the enzyme terminal deoxynucleotidyltransferase (TdT) (Benjamini *et al.*, 2000). The imprecision in joining V and J or V, D and J segments gives rise to further diversity in antibody variable regions. This is termed junctional diversity.

Somatic hypermutation introduces further diversity into the antibody sequence. This occurs at a rate of 10^{-5} to 10^{-3} per base, per generation, over the lifespan of the B-cell. This frequency is a million times higher than in other cells (Li *et al.*, 2004). The mutations are predominantly point mutations in the V(D)J unit of antibody V genes occurring in mutation hotspots, defined by specific sequence motifs. Somatic hypermutation results in the production of antibodies with increased affinities for the target antigen (Benjamini *et al.*, 2000).

Following stimulation of a B-cell by recognition of a specific antigen and stimulation by T_H-secreted cytokines, a further genetic rearrangement of antibody genes gives rise to antibody class or isotype switching (Figure 1.5). This process allows the expression of an antigen-specific binding site with a different C region gene,

facilitating antibody mediation of different effector functions. Each C_H region contains μ, δ, ε, γ and α genes, separated by repeating GC pentameric sequences called switch (S) regions, (except δ, which contains no S region). Class switch recombination involves Cμ being replaced by Cγ, Cε or Cα segments to generate IgG, IgE and IgA antibodies respectively (Li *et al.*, 2004). Following antigen and cytokine stimulation, the V(D)J segment linked to Cμ rearranges its DNA to the S region in front of the desired C gene. In the process, the intervening C region DNA is excised. Therefore, a cell, which has undergone class switching, loses its ability to revert back to producing the original class of antibody. This mechanism of class switching is unique to antibody heavy chains and allows an antibody with a single antigenic specificity to associate with a variety of different effector functions (Benjamini *et al.*, 2000).

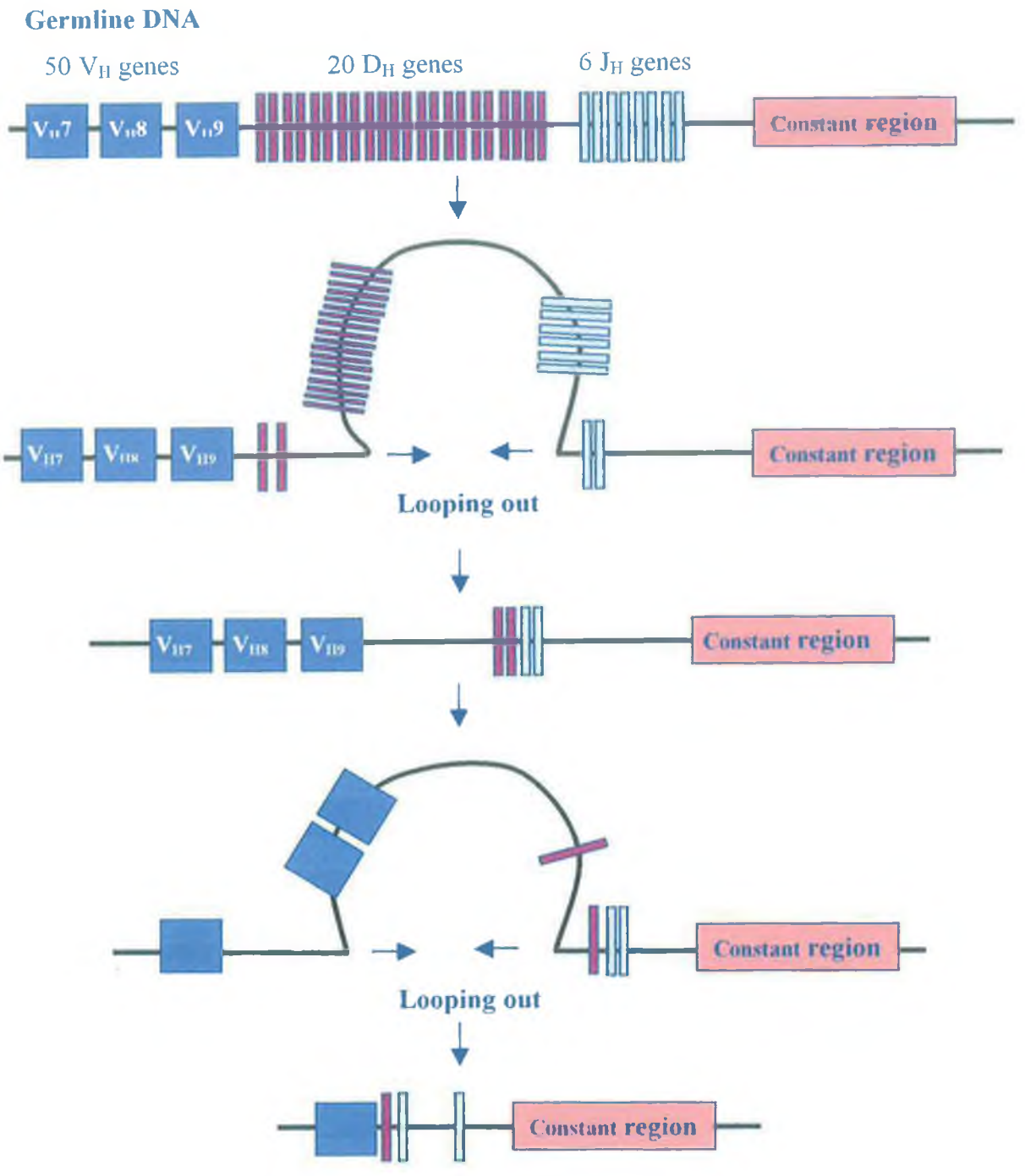


Figure 1.4: *Schematic representation illustrating the process of V(D)J recombination in the synthesis of an antibody's heavy chain. Heavy chain genes consist of variable (V), diversity (D), joining (J) and constant (C) gene segments. The first step in recombination involves bringing D and J regions in contact and the intervening DNA is excised through a looping out process, facilitated by the enzyme recombinase. The second recombination step involves the V and DJ regions being brought into contact. This coding mechanism determines the antibody's specificity. The constant regions determine the isotype or class of antibody produced.*

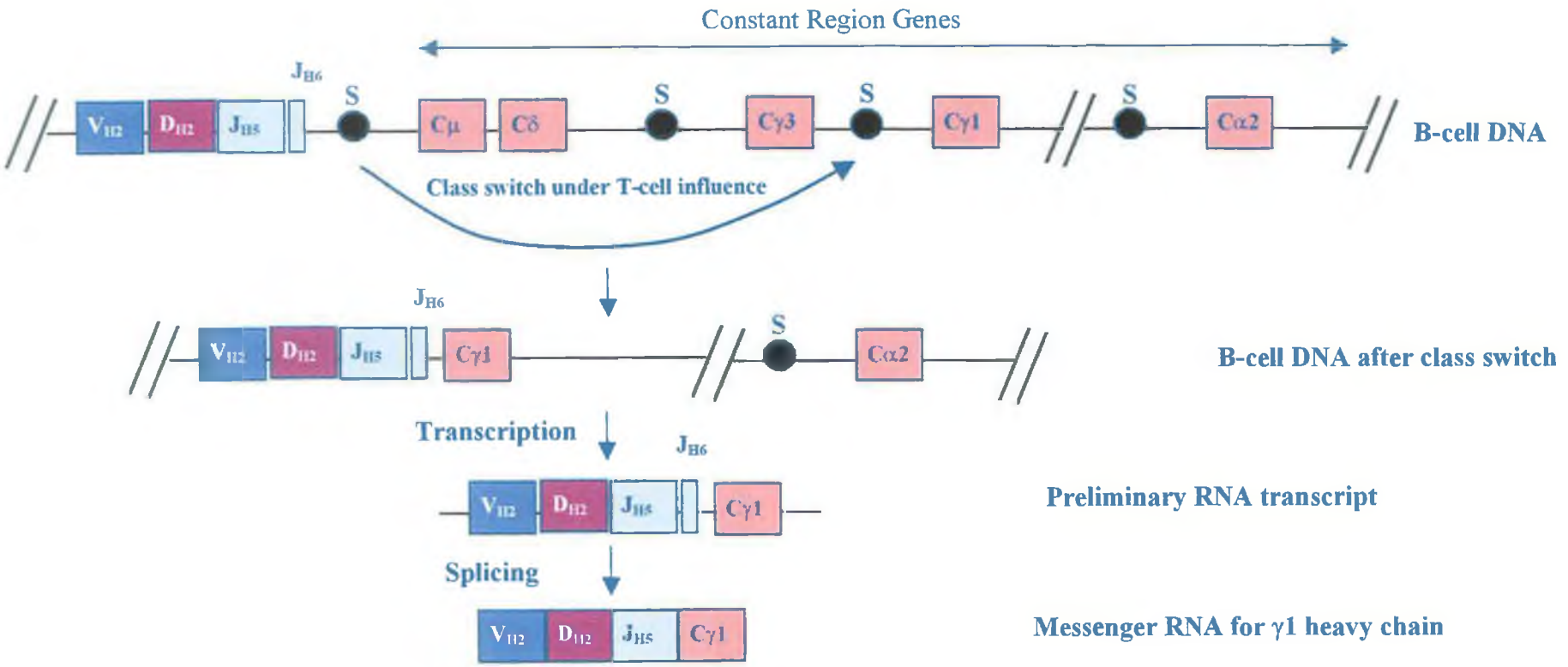


Figure 1.5 *Mechanism of class switching in immunoglobulin synthesis. The letter S corresponds to the switch region, upstream of each heavy chain constant region. Adapted from Benjamini et al. (2000).*

1.4 Antibody Production

1.4.1 Antigens and immunogens

An antigen is defined as a molecule that contains distinct sites or epitopes that are recognised by the immune system, leading to the generation of an immune response. In order for a molecule to elicit an immune response it must possess certain characteristics that make it immunogenic. The immune system must be able to discriminate the molecule as 'non-self', i.e. it must be of foreign origin. The molecule must be large enough to be seen by the immune system in order to stimulate antibody production. Large molecules, such as proteins, are capable of eliciting a good immune response. However, smaller molecules of less than 5 to 10,000 M.W. are poorly immunogenic (Clementi *et al.*, 1991). This type of antigen is termed a hapten, and can be defined as a low-molecular weight molecule that contains an antigenic determinant, but which is not itself antigenic unless complexed with an antigenic carrier.

The process of coupling a hapten to a large protein is referred to as conjugation. Various carrier proteins such as bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet haemocyanin (KLH) and thyroglubulin (THY), have been successfully used for this purpose. Synthetic and natural polymers have also been employed (Stapleton *et al.*, 2004). The point of conjugation will be decided by the functional groups available on the hapten and carrier. The orientation of the hapten for presentation to the immune system will also have to be considered. It is generally accepted that exposed sites away from the carrier act as antigenic epitopes to the immune system. Various procedures for conjugating haptens to carrier proteins have been documented including standard amine coupling via EDC/NHS chemistry, ethylenediamine linkage, diazonium coupling and active ester couplings (Hermanson, 1996).

Structurally analogous compounds have also been successfully employed in generating antibodies. Salamone *et al.* (1998) generated antibodies with a broad

specificity to cannabinoid metabolites using a derivatised benzpyran structure linked to bovine thyroglobulin (BTG) to elicit an immune response. The structure was designed to direct the immune response to the conserved epitopes of cannabinoid metabolites present in urine. In this thesis, EDC/NHS carbodiimide coupling was employed to conjugate M3G to a range of carrier proteins. EDC activates the carboxyl groups on M3G, forming a reactive carboxyl intermediate that is susceptible to attack from amine groups present on the protein carrier. NHS stabilises the reactive intermediate, preventing re-hydrolysis. One of the advantages of EDC/NHS-mediated coupling is that the molecules are coupled by cross-linking. This method eliminates the production of any antibodies with a specifity for a linker, as it does not introduce a linking region between hapten and carrier.

1.4.2 Adjuvants

Adjuvants are stimulators of the immune system and are generally employed during immunisation to enhance the immune response. Adjuvants function in protecting the antigen by emulsifying it in oil, slowing its release and prolonging its exposure to the immune system. Adjuvants also contain non-specific immune system stimulators, which generate an response, attracting macrophages and other cells to the site of immunisation. The most commonly used adjuvants are Freund's complete and incomplete adjuvants, FCA and FICA, respectively. FCA, is used for the initial immunisation and contains heat-killed *Mycobacterium tuberculosis* as an immune response stimulator. FICA is a less toxic and is used for subsequent immunisations ('boosts'), as it does not contain *mycobacteria,* rendering it a less severe adjuvant for immunisation purposes. Other adjuvants include hunter's TiterMax, liposomes, the RIBI adjuvant system and *Bordetella pertusis* (Stapleton *et al.*, 2004).

1.4.3 Polyclonal antibodies

Immunisation of an animal with a multivalent antigen will elicit the immune response to produce antibodies against this foreign molecule. If injections are repeated over a period of weeks, the animal will produce antibodies specific to this target. Following

immunisation, the blood of the host animal will contain a heterogeneous mixture of antibodies, each recognising different epitopes of the immunogen with different specificities and affinities. These antibodies are referred to as polyclonal due to their heterogeneous nature. Relatively large animals such as donkeys, rabbits, goats and sheep have been used for the generation of polyclonal antibodies. Using this approach antibodies have been successfully generated against a wide variety of targets (Fitzpatrick, 2001; Daly, 2001; Dillon, 2001; Fanning, 2002; Fitzpatrick, 2002; Leonard, 2003 and Dunne, 2004). Polyclonal antibodies however, may be considered to have a limited supply due to batch-to-batch inconsistencies, even between antibodies produced in hosts of the same species. The heterogenicity of the antibody population may also lead to high levels of cross-reactivity with structurally related molecules. Due to ethical considerations, the use of animals for antibody generation is also a concern.

1.4.4 Monoclonal antibodies

The generation of monoclonal antibodies employing hybridoma technology was first reported by Kohler and Milstein (1975). This allowed the production of an immortal cell line, secreting a homogeneous antibody population, with a single defined specificity and affinity for the target antigen. The process involves the immortalisation of a single B-cell through fusion with an immortal myeloma cell.

For the production of a monoclonal antibody, the immune response of an animal host is first primed with the immunogen of interest. The immune response is monitored until the desired titre of specific antibody is achieved. The spleen is removed and antibody-producing spleenocytes are harvested. Spleenocytes are the end product of B-cell differentiation and secrete antibodies with a defined specificity, however these cells will die under standard cell culture conditions. Myeloma cells are tumorigenic B-lymphocytes that can survive extended periods in culture. Commonly used myeloma cell lines include X63-Ag8.653 and Sp2/0-Ag14, which are derived from MOPC-21 cells. These cell lines have lost the ability to produce antibody (Stapleton *et al.*, 2004). The myeloma cells are fused to antibody-producing spleenocytes with

the aid of polyethylene glycol (PEG). PEG promotes membrane bridging and facilitates cell fusion and the transfer of nuclei. The resultant fusion product is termed a hybridoma or *hybrid*-myel*oma*. By-products of the fusion process include unfused spleenocytes and myeloma cells; spleenocytes fused with spleenocytes and myeloma-myeloma cell fusions.

Antibody-secreting hybridomas are selected using HAT (hypoxanthine, aminopterin and thymidine)-supplemented media (Figure 1.6). The presence of aminopterin blocks a cell's ability to synthesise the DNA building blocks of purines and pyrimidines, through the main *de novo* synthesis pathway. Mammalian cells, like spleenocytes, are capable of DNA synthesis via the salvage pathway. Using the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), and in the presence of hypoxanthine and thymidine, these cells can synthesise the necessary DNA building blocks via the salvage pathway of nucleic acid synthesis. Myeloma cells deficient in this enzyme are used in the fusion, rendering them incapable of DNA synthesis via this salvage pathway. The aminopterin present in the HAT media blocks DNA synthesis via the usual, *de novo* pathway. This leads to the death of any unfused myeloma cells. Successfully fused hybridomas will inherit the HGPRT enzyme from the spleenocyte and the trait of immortality from the myeloma cell. This results in an immortal antibody secreting cell line.

Hybridomas secreting the antibody of interest are isolated to monoclonality by a process known as cloning out by limiting dilution. Cloning out of hybridomas is a critical step in the process of monoclonal antibody production. Non-secreting hybridomas will have a growth advantage over antibody secretors and need to be eliminated as early as possible. The heterogeneous hybridoma cells are seeded at low densities (typically 1 or <1 cell/ml) in 96 well plates. The supernatants from each well are tested for antibody activity. Positive wells are divided and sub-divided a number of times to ensure monoclonality of the cell-line. A flow diagram illustrating the process of monoclonal antibody production, from immunisation to cloning out, is shown in Figure 1.7.

Alternatively, monoclonal antibodies may be produced by *in vitro* immunisation (Borrebaeck, 1983). Naïve spleen cells are exposed to the antigen for 5-9 days before

fusion, as described above. Antibodies produced using *in vitro* immunisation are as a result of a primary response and are generally lower affinity IgM-type antibodies. However, as this system does not require a lengthy immunisation period, the time scale of antibody production is reduced, along with the quantity of immunogen required. Bonwick *et al.* (1996) successfully generated antibodies to flucofluron and sulcofuron, using this method. However, the lack of papers in the literature suggests that there has been very little success in the development of assays using antibodies produced by *in vitro* technology.

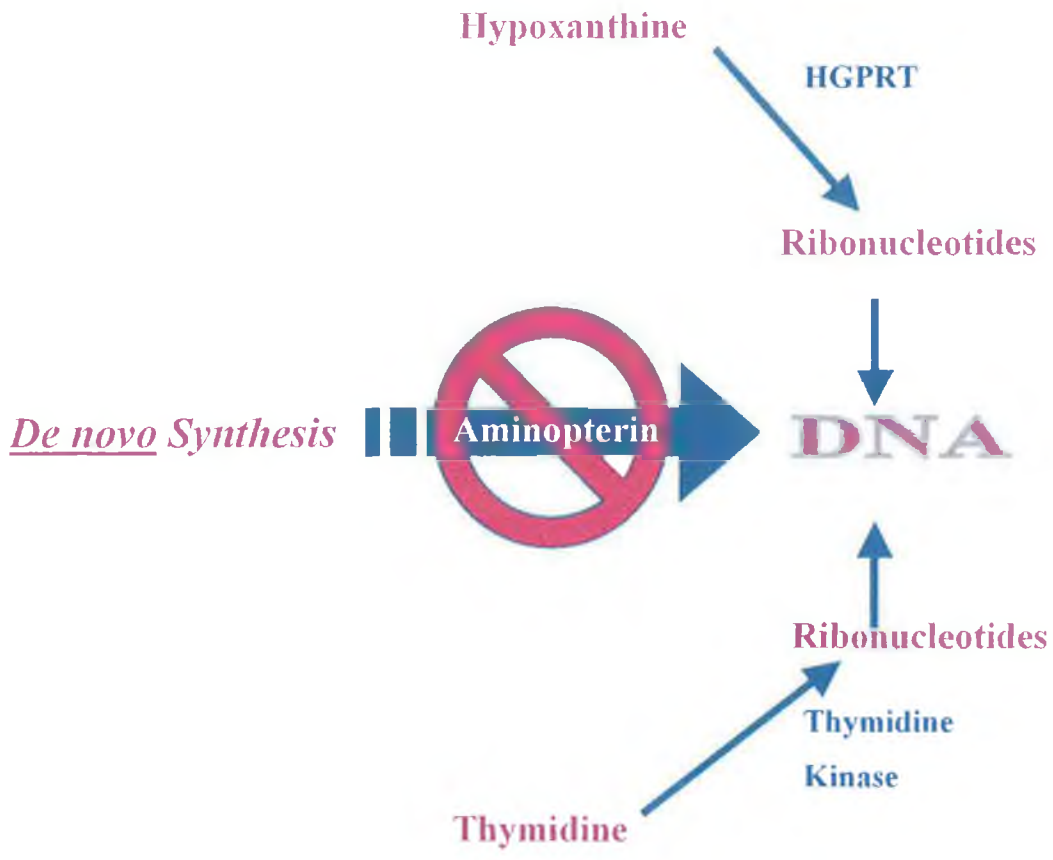


Figure 1.6: *De novo synthesis of DNA is blocked by aminopterin present in HAT-supplemented media. Cells that possess the enzymes hypoxanthine guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK), convert to the salvage pathway for synthesis of nucleic acids. These cells utilise the hypoxanthine and thymidine present in the HAT media to synthesis DNA and will therefore survive. However, cells lacking this enzyme will die off in the presence HAT because they are unable to produce the building blocks for nucleic acid synthesis.*

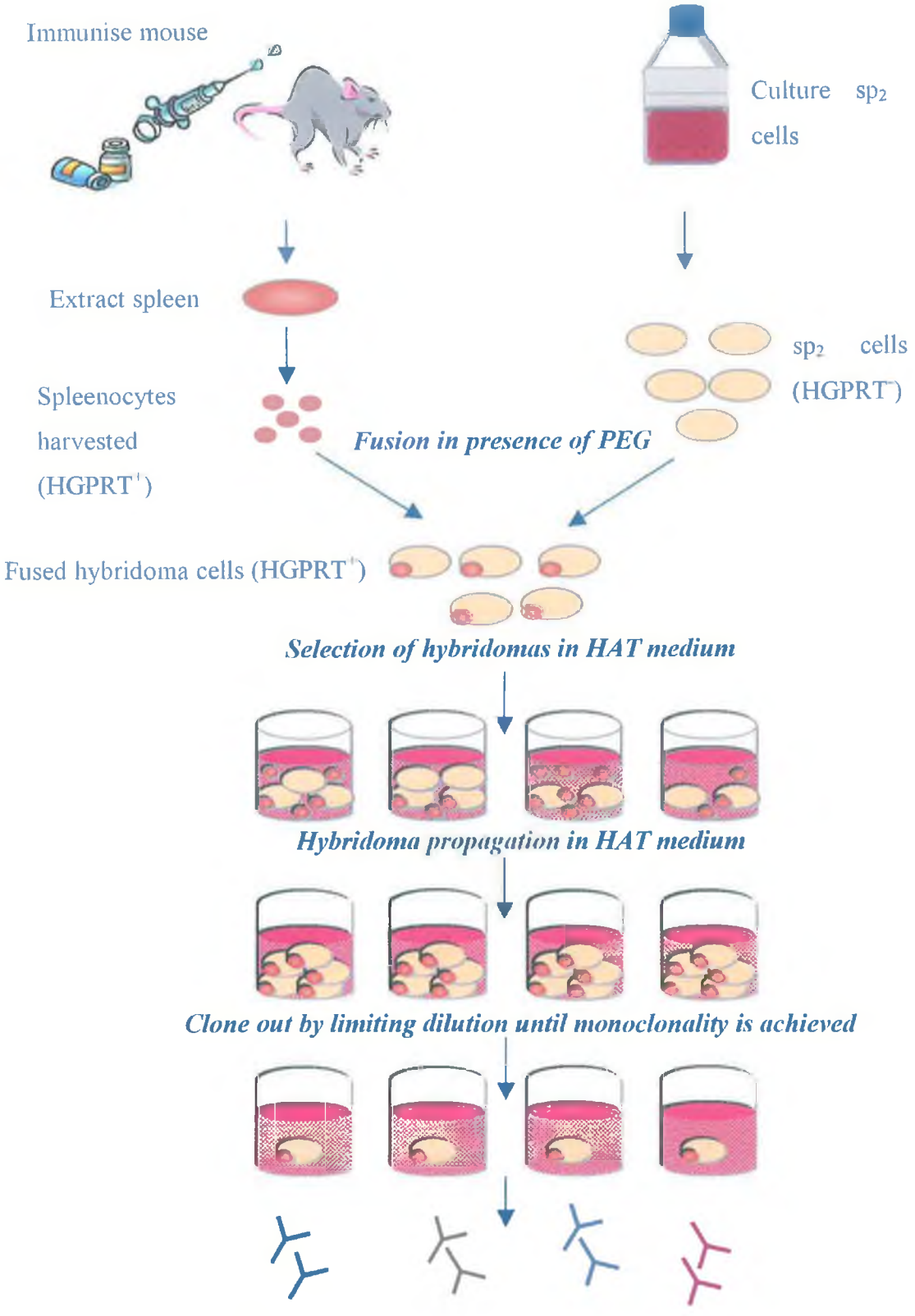


Figure 1.7: *Flow diagram illustrating the principle steps involved in the production of a murine monoclonal antibody.*

1.4.5 Antibody Fragments

A whole antibody molecule can be broken into several fragments using chemical, enzymatic or genetic means. Research conducted by Rodney Porter (1959) demonstrated that proteolytic treatment of the IgG molecule with the enzyme papain resulted in the formation of three fragments. Papain cleaves the amino terminal side of the disulphide bridge connecting the two heavy chains of the antibody, resulting in the production of two univalent antigen binding Fragments (Fab) and one crystalline Fragment (Fc), all of roughly equal size. Nisonoff *et al.* (1960) later demonstrated that an enzyme called pepsin, cleaved the carboxyl side of the antibody's disulphide bond. This results in the formation of one bivalent antibody fragment or $F(ab')_2$ and one Fc portion. Together these discoveries led to a new branch of antibody production. Following peptidic digestion of two antibodies, each with specificity for a different antigen, half the digestion product of one antibody can be mixed with an equal amount of the other antibody to form a bispecific antibody, possessing dual specificity for each of the two antigens (Nolan and O'Kennedy, 1990).

Over the last 15 years, advances in genetics and molecular biology have facilitated the growth of a different field of antibody production. Recombinant antibody technology has revolutionised the field of antibody engineering, allowing the production of monoclonal antibodies derived from a variety of species including humans (Griffiths *et al.*, 1994), mice (Dillon[B] *et al.*, 2003), rabbits (Li *et al.*, 2000), chickens (Andris-Widhopf *et al.*, 2000), sharks (Dooley *et al.*, 2003), camels (Ghahroudi *et al.*, 1997) and sheep (Charlton *et al.*, 2000). Various antibody fragments, including the Fab, Fv and scFv, can also be produced using recombinant DNA engineering (Figure 1.8). The Fv fragment is the smallest fragment that contains the complete antigen-binding site, consisting of one V_L and one V_H domain. The Fv fragment may be unstable for use in therapeutics or immunoanalysis because it lacks the inter-chain disulphide bond, which is present in the Fab fragment (Glockshuber *et al.*, 1990). Protein engineering may be used to stabilise the Fv fragment. The introduction of a disulphide bond, synthetic peptide linker or both, have been employed to stabilise the fragment, generating a disulphide stabilised Fv (dsFv), single chain Fv fragment (scFv) or stabilised antibody (StAb), respectively (Brinkmann *et al.*, 1993; Freund *et*

al., 1993 and Dooley *et al.*, 1998). Other fragments include the Fd fragment, consisting of one V_H and one C_{H1} domain, and a fragment CDR, the smallest antibody fragment capable of binding to an antigen.

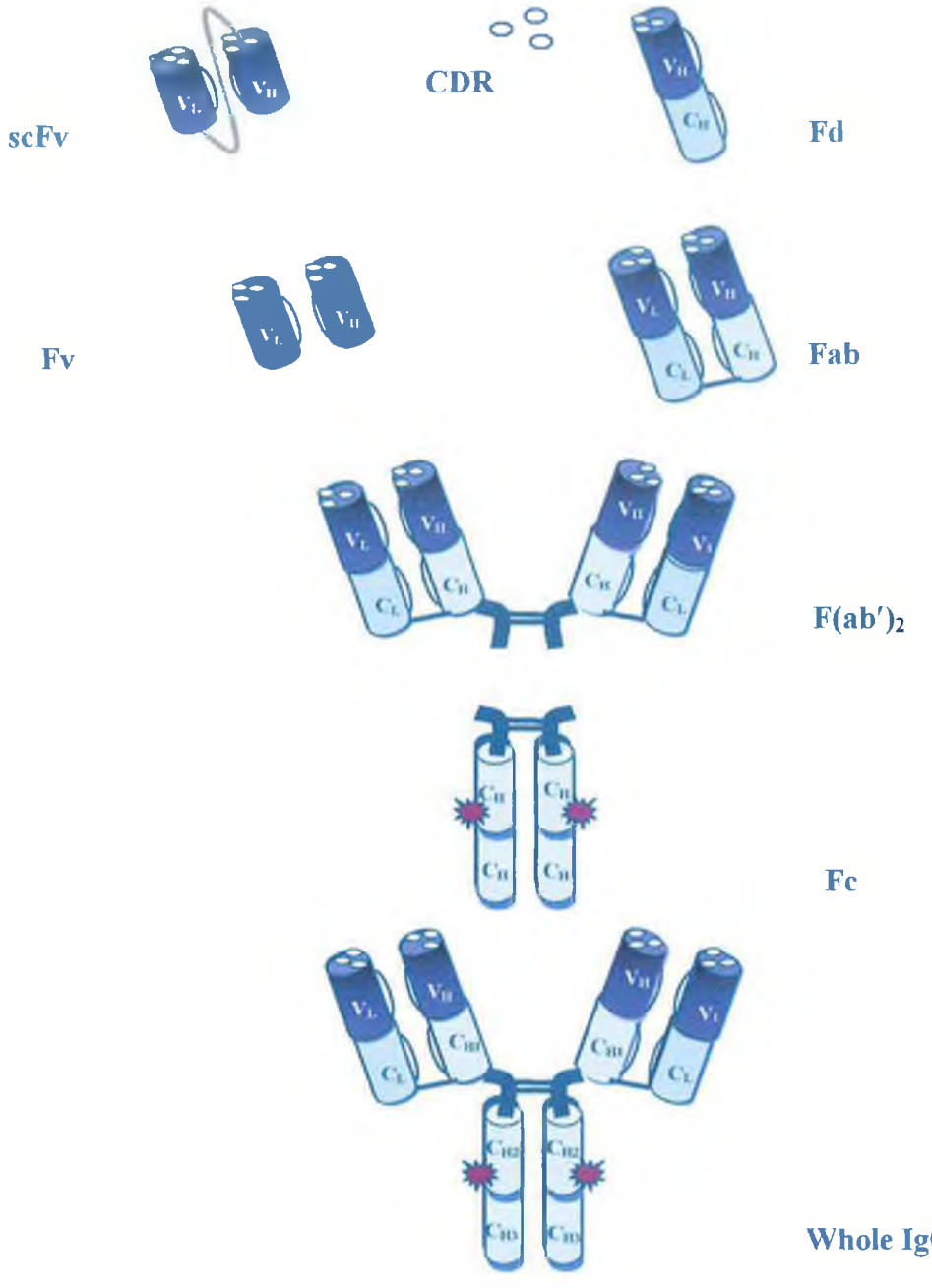


Figure 1.8: *Diagramatic illustration of antibody and antibody fragments which can be produced by genetic, chemical or enzymatic means. The antibody may be broken up into either Fab (single antigen-binding Fragment) or F(ab')$_2$ (two antigen-binding Fragments) and Fc (crystalline Fragments) regions. Fab Fragments may be further broken up into Fv (variable Fragment), scFv (variable Fragment, stabilised with a synthetic linker) and CDR regions, which are the smallest fragment capable of antigen binding.*

1.5 Recombinant Antibody Production

1.5.1 Principle of phage display

The principle of phage display was first used to express cloned peptides displayed on the surface of bacteriophage (Smith, 1985). Antibody variable domains, from a murine anti-lysozyme antibody, were then expressed as fragments, displayed on the surface of phage by McCafferty *et al.* (1990). The success of phage display technology in the area of antibody engineering is largely due to the physical coupling of genotype to phenotype. The protein displayed on the virus surface is physically linked to the genetic material encoding it, which remains packaged inside the virion coat. This facilitates selection and amplification of desired protein functions, while simultaneously selecting the genetic sequence encoding it.

Bacteriophage or phage, are viruses that infect gram-negative bacteria. The Ff family of filamentous phage (strains M13, fd and f1) are routinely used in phage display. These non-lytic filamentous phage consist of a single stranded genome enclosed in a protein coat, covered with thousands of copies of pVIII. At one end of the virion there are surface proteins pIII (3 to 5 copies) and pVI (5 copies), involved in host cell binding and the termination of the assembly. All of these proteins have been employed for phage display, although pVI has only rarely been used. Other phage proteins that are not employed in phage display include pVII and pXI (Rodi and Makowski, 1999 and Azzazy and Highsmith, 2002). Foreign DNA can be inserted into the phage genome attached either to gene III, VI, or VIII.

Ff bacteriophage infect male or donor *E. coli* that harbour the F′ episome (e.g. XL-1 Blue or TG1), using the F′ pilus as a surface receptor. The F′ plasmid is a large (94.5 kb) plasmid that is maintained at low copy number within the host cell. It has genes that are needed for its replication and for its ability to transfer DNA to a recipient. Cells carrying the F′ plasmid produce a filamentous structure known as F′ pili. These structures are involved in conjugative transfer of DNA between cells. This involves DNA transfer from a donor (F+) to a recipient (F-) cell by direct physical contact

between the cells. Cells lacking the F′ plasmid are known as female or acceptor cells, while cells harbouring the plasmid are known as male or donor cells.

Bacteriophage infection of a bacterial cell is mediated mainly the pIII protein. This phage coat protein consists of 2 aminoterminal domains (N1 and N2) and one carboxyterminal domain. The N1 and N2 domains interact together to form a bi-lobal structure that protrudes from the tip of the phage particle. Infection of the bacterial cell is initiated by the binding of the N2 domain to the tip of the bacterial F′ pilus. This triggers the F′ pilus to retract into the bacterial cell membrane, allowing the N1 domain to bind the carboxyterminal of the bacterial membrane protein Tol A, which is the coreceptor required for infection (Reichmann and Hollinger, 1997). The Tol A protein forms part of the Tol QRA complex, which is partially responsible for the stabilisation of the bacterial outer membrane. Tol A consists of three domains, an N terminal domain (D1) that anchors it to the cytoplasm, a central domain (D2), which spans the periplasmic space and a carboxyterminal domain (D3), which is associated with the outer membrane (Lubkowski *et al.*, 1999). It is the carboxyterminal domain (D3) of the Tol A protein that interacts with the N1 domain of the phage pIII protein. This is thought to cause the Tol A protein to adapt a more compact conformation, bringing the outer and inner membranes into closer proximity. At this stage the pIII N2 domain is also thought to interact with the D3 domain of Tol A, leading to the insertion of the pIII protein into the inner membrane and the subsequent release of the phage DNA into the bacterial cytoplasm (Karlsson *et al.*, 2003). It is by this mechanism that the phage genome is translocated into the host cell, where it undergoes replication utilising both phage and host proteins. The phage single stranded DNA is converted to replicative form DNA. The transcription of phage genes begins and amplification proceeds by rolling circular replication. The phage proteins are packaged into a fully functioning virion particle, expressing the scFv as a fusion protein on the surface. Phage particles are released from the cell when the entire molecule has been encapsulated in pVIII and the minor coat proteins have been attached to the ends of the virion particle.

F′ conjugative plasmids are a natural host for replication of this phage family. Initially, complete phage vectors or bacteriophage, which carried all the genetic

information required for the phage life cycle, were used as the display vector. Now small plasmid vectors or phagemids, which contain the appropriate packaging signal and cloning sites, have become a more popular type of vector for display (Daly[A] *et al.*, 2001). Phagemids have high transformation efficiencies, making them ideal for generating large antibody repertoires. When using phage to display antibody fragments, phagemids usually consist of the DNA encoding an scFv fused to the gene encoding a phage coat protein (gIII or gVIII protein). Other vector features include an antibiotic resistance marker, an origin of replication, a promoter and a tag to aid in purification. Helper phage such as M13K07 of VCS-M13 are also required to supply all necessary structural proteins for correct packaging of the phage particle. The *lacZ* promoter is the most common promoter used to control expression. Expression may be suppressed by the addition of the catabolic repressor, glucose, or induced by addition of isopropyl-β-D-galactopyranoside (IPTG). Upon induction, the scFv–coat fusion protein is incorporated into new phage particles that are assembled in the bacterium. Ths scFv is then displayed on the phage surface, while the genetic information encoding the scFv remains within the phage particle (Figure 1.9).

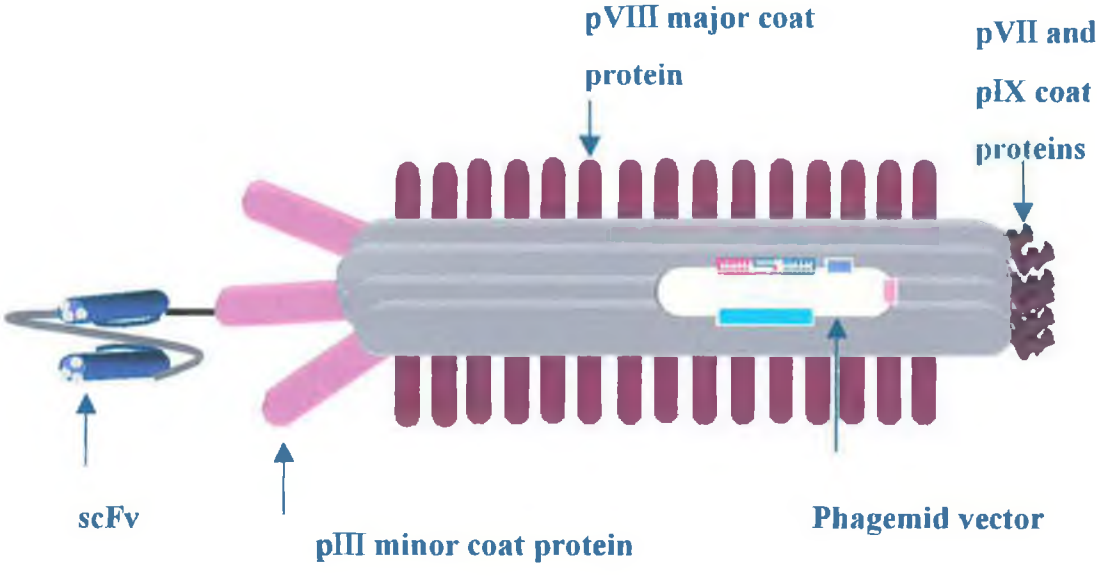


Figure 1. 9: *Schematic illustration of a filamentous phage displaying an scFv fused to the pIII minor coat protein. The genes encoding the scFv are present in a phagemid vector that remains inside the phage coat. The phage coat is made up approx. 3000 copies of pVIII encapsulating the extended single stranded DNA of the phage.*

1.5.2 Construction of a phage display library

To construct an scFv library, a source of variable antibody genes is required. This can be derived from B-lymphocytes, extracted from the spleen, peripheral blood or bone marrow or from a hybridoma cell line-secreting antibodies of interest. The mRNA encoding antibody genes is extracted and reverse transcribed to yield cDNA. There are sufficiently conserved sequences within the J segments and at the 5′ end of V genes to allow the design of degenerate forward and back primers, respectively (Hoogenboom *et al.*, 1992). Variable heavy and light chain genes are amplified separately using a multiplex PCR involving this 'universal' primer set, designed to be specific for the species from which the antibody is derived. The products are then assembled into one fragment using a linking PCR reaction. A commonly used reaction is a splice by overlap extension (SOE) PCR, using outer flanking primers that have sequence homology to the 3′ end of V_H and the 5′ end of V_L. These primers introduce a linking region, like the $(Gly_4Ser)_4$ linker commonly used in scFvs. The primers also code for specific restriction sites at their 5′ end, to facilitate cloning of the fragment into a phage expression vector (Krebber *et al.*, 1997). Following restriction of the scFv fragment and Phagemid vector, the two are ligated together and transformed into donor (male) *E. coli* cells harbouring the F′ pillus (e.g. XL-1 Blue or TG1). Library size is limited by transformation efficiency (up to size of about $1x10^8$). To ensure that the diversity of the repertoire is maintained, this transformation step is crucial and is normally carried out by electroporation. The transformed bacterial cells are then grown to exponential phase and infected with a helper phage (VCSM13 or M13K07), which will replicate inside the cells and emerge as antibody displaying phage particles. An overview of the construction of a phage display library is detailed in Figure 1.10.

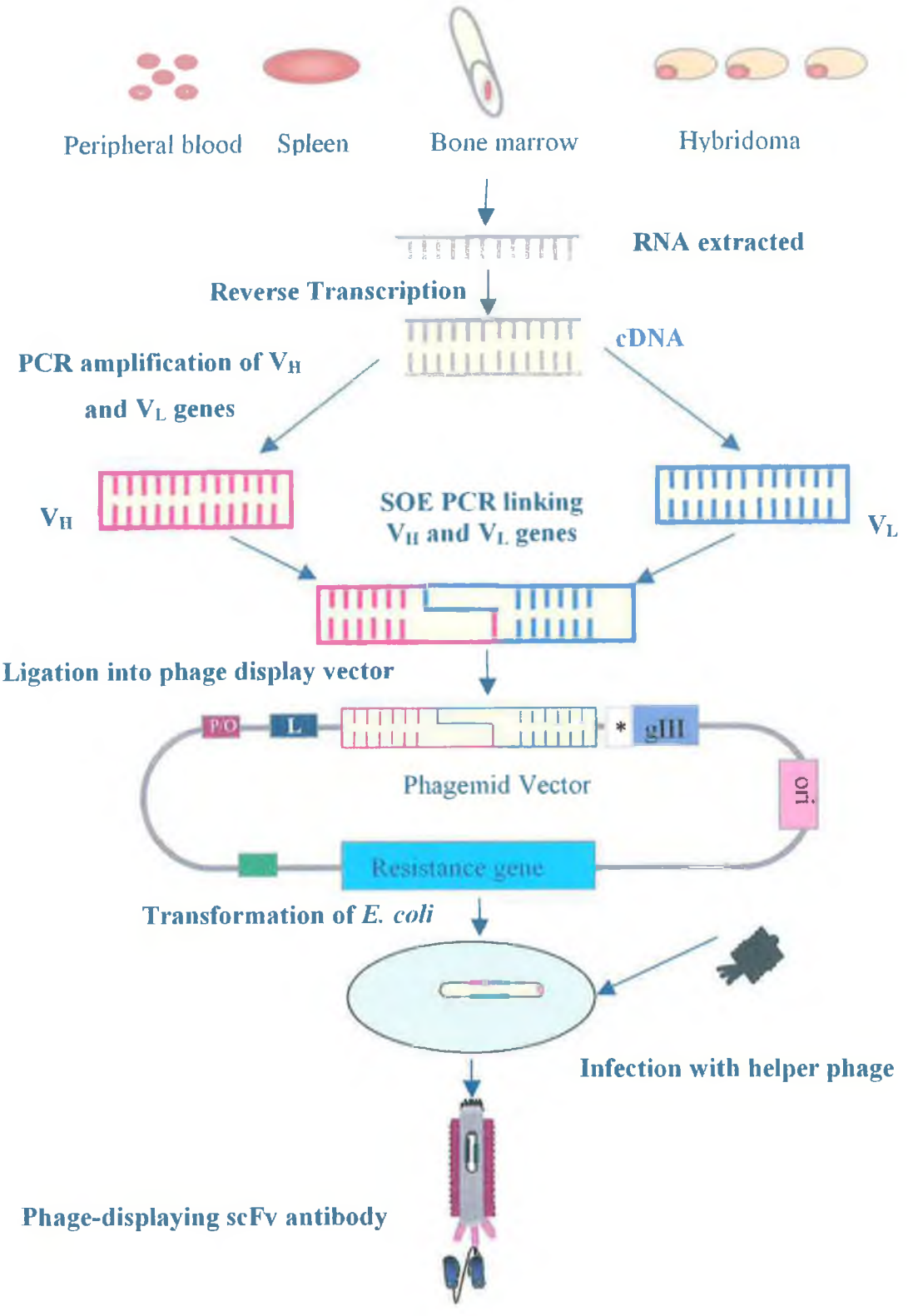


Figure 1.10: *Schematic overview detailing the steps involved in the generation of an antibody phage display library. Following isolation of mRNA, heavy and light chain antibody genes are amplified by PCR. Both fragments are spliced together and the DNA insert is ligated into a phagemid vector. This is transformed into E. coli, which following infection by helper phage, will express antibody fragments on the surface of phage particles.*

1.6 Natural Antibody Repertoires

1.6.1 Naïve libraries

The primary immune response comprises a vast array of IgM antibodies against a large variety of antigens. The diversity encoded by this primary immune response can be harnessed to generate a 'single pot' antigen-unbiased antibody repertoire. Naïve libraries are created using V-genes amplified from either total RNA or IgM RNA of non-immunised donors. Peripheral blood lymphocytes, bone marrow, spleen cells, and tonsils B-cells have all been used as sources of V-genes for library generation. Antibodies against a range of different antigens can be selected including, self, non-immunogenic and toxic antigens.

The major advantage of a 'single pot' repertoire is that once generated, it can be used as a 'universal' source of antibodies. It may also serve as a source of human antibodies for therapeutic purposes. Antibodies from a naïve library have not undergone affinity maturation by the immune system and can, therefore, suffer from poor binding kinetics. This may be overcome by creating larger repertoires. The larger the library size, the larger the number of clones and diversity encoded will be. This increases the probability of isolating a high affinity antibody to any given target. A library of 10^7 clones created by Marks *et al.* (1991), yielded antibodies with affinities in the range of 10^{-6}-10^{-7} M, while a larger repertoire comprising of 1.4×10^{10} yielded antibodies with K_D<10 nM (Vaughan *et al.*, 1996).

1.6.2 Immunised libraries

Antibody libraries may be generated using antibody genes isolated from the B-cells of an immunised host or immune donors. Immunisation primes the immune system so that IgG genes will be enriched for antigen-specific antibodies that will be affinity matured within the host's immune system. Immunised libraries have been created from a variety of species including mice (Dillon[B] *et al.*, 2003), rabbits (Li *et al.*, 2000), chickens (Andris-Widhopf *et al.*, 2000), sharks (Dooley *et al.*, 2003), camels (Ghahroudi *et al.*, 1997) and sheep (Charlton *et al.*, 2000). The process of immunising a host animal requires a lengthy time period and ethical concerns exist over the use of animals. The immune system bias of antibody V-genes towards target antigen mean that only a relatively small library is generated and each repertoire can only be used to select for antibodies against that specific antigen. One such example of a pre-immunised murine library created against M3G is described by Dillon[B] *et al.* (2003), which employed an optimised phage display system (Krebber *et al.*, 1997), for cloning of antibody V-genes.

1.6.2.1 The Krebber system of phage display

Krebber *et al.* (1997), have engineered an optimised phage display system for the generation of single chain antibody Fragments (scFv's) from hybridomas or spleen cells. The system was optimised for robustness, vector stability, tight control of scFv-Δgene III expression, primer usage for PCR amplification of variable genes, scFv assembly strategy and subsequent directional cloning using a rare single cutting restriction enzyme. A compatible vector series to aid purification, detection, multimerisation and modification is also described. The Krebber system has been employed for the generation of specific scFv's with high affinity for the major metabolites of heroin (Dillon[B] *et al.*, 2003).

The primers detailed by Krebber *et al.* (1997), encompass all possible variations of murine heavy and light chain genes collected in the Kabat database (Kabat *et al.*, 1991), combined with an extended primer set described by Kettleborough *et al.*

(1993). These primers encode a shortened version of the FLAG sequence (DYKD), which may be used as a handle for detection/purification (Prickett *et al.*, 1989). Heavy and light chain genes are amplified separately and annealed in the orientation V_L-$(Gly_4Ser)_4$-V_H, using a splice by overlap extension PCR. *Sfi I* recognises the sequence GGCCNNNNNGGCC, where N can be any nucleotide. This recognition site is introduced by the heavy chain forward and scback primers and is very rare in antibody sequences.

The pAK vector series can be used for phage display or for the expression of the soluble antibody in a variety of formats (Figure 1.11). Each vector consists of a chloramphenicol resistance gene and a tetracycline resistance 'stuffer' gene, which will be replaced by the genes encoding the variable heavy and light genes of the antibody. The tetracycline resistance cassette is flanked on either end with an *Sfi I* restriction site for cloning. All vectors contain a *Pel B* leader sequence; a *lac* promoter/operator to allow repression of translation by the addition of glucose; a *lac* repressor gene to ensure independent *lac* promoter repression; and a strong upstream terminator (t_{HP}), which in combination with the *lac* promoter eliminates background expression prior to induction with IPTG.

The phagemid vectors pAK 100 and 200 have been engineered to contain a truncated version of gIII phage coat protein (250-406) in order to avoid immunity to superinfection. This ensures complete product repression prior to helper phage infection. The pAK 100 vector also encodes a c-myc tag for detection and an amber codon that facilitates switching between expression of phage-bound and soluble scFv, simply by changing the expression host. When phage are grown in a *SupE*, suppressor strain of *E. coli*, the amber codon is read as glutamine and the antibody fusion protein is displayed on the phage. In non-suppressor strains, the amber stop is read as a stop codon and soluble protein is secreted into the culture media (Hoogenboom *et al.*, 1992). The pAK 200 vector does not contain this amber codon. As suppression is never complete, this will result in a higher proportion of antibody-phage fusions but the scFv gene will need to be re-cloned into another vector prior to soluble expression.

The pAK 300 vector is the most basic plasmid for soluble antibody expression. It encodes a C-terminal hexahistidine tag for purification using Immobilised Metal

Affinity Chromatography (IMAC) (Janknecht *et al.*, 1991; Lindner *et al.*, 1997 and Müller *et al.*, 1998) or for detection using an anti-his tag antibody. The pAK 400 vector contains a much stronger Shine Dalgarno sequence (SD_{T7g10}) for increased expression and a hexahistidine tag. The pAK 500 vector contains a single chain double helix (scdHLX) for dimerisation. This consists of a C-terminal flexible hinge isolated from murine IgG3 and a helix 1-turn-helix 2, followed by a pentahistidine tag. The resultant homodimerised minibody shown in Figure 1.12 is reported to have increased antigen affinity (Pack *et al.*, 1993). The pAK 600 vector encodes the bacterial alkaline phosphatase gene to enable direct detection and facilitate dimerisation by scFv-AP fusions (Pack *et al.*, 1993 and Müller *et al.*, 1998). This results in the production of a dimeric, bifunctional scFv, as shown in Figure 1.12.

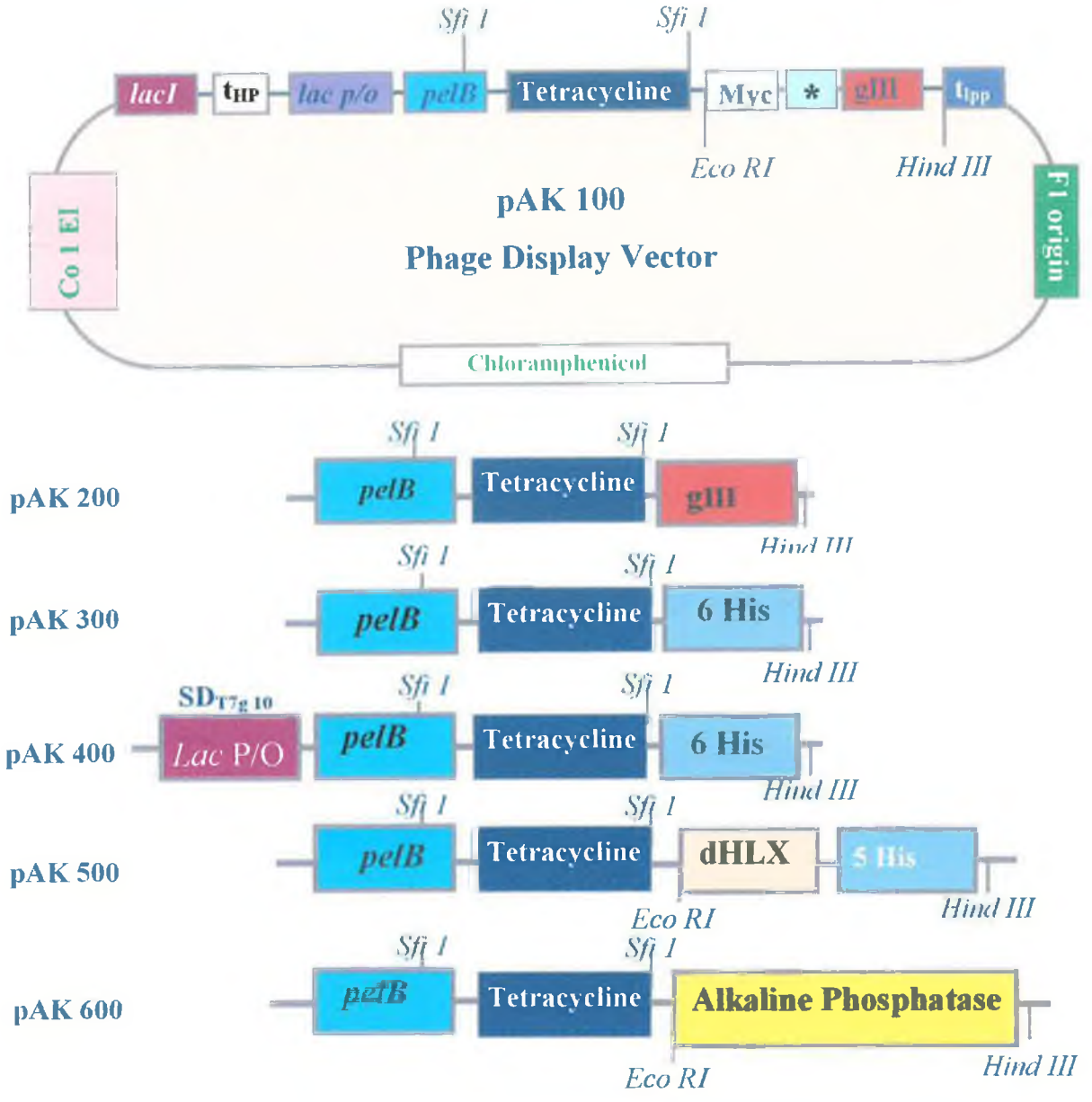


Figure 1.11: The *pAK compatible vector series for phage display and soluble expression. All vectors contain a tetracycline 'stuffer' gene and a chloramphenicol antibiotic resistance gene, in addition to a lacI repressor gene (LacI), a strong upstream terminator (t_{HP}), the lac promoter/operator (Lac p/o) and a pelB leader sequence (pelB), modified to contain an Sfi I site and a downstream terminator (t_{1pp}). The pAK 100 and 200 phagemid vectors encode a truncated version of the gIII phage coat protein (gIII). pAK 100 also contains a c-myc tag (myc) and an amber codon (*). Soluble expression vectors, pAK 300 and 400 encode a 6 His tag for purification and detection. In addition, pAK 400 also encodes a strong Shine Dalgarno sequence (SD_{T7g10}) for increased expression levels. pAK 500 encodes a 5 His tag and a helix for dimerisation and pAK 600 encodes the bacterial alkaline phosphatase gene for direct detection and dimerisation. (Adapted from Krebber et al., 1997.)*

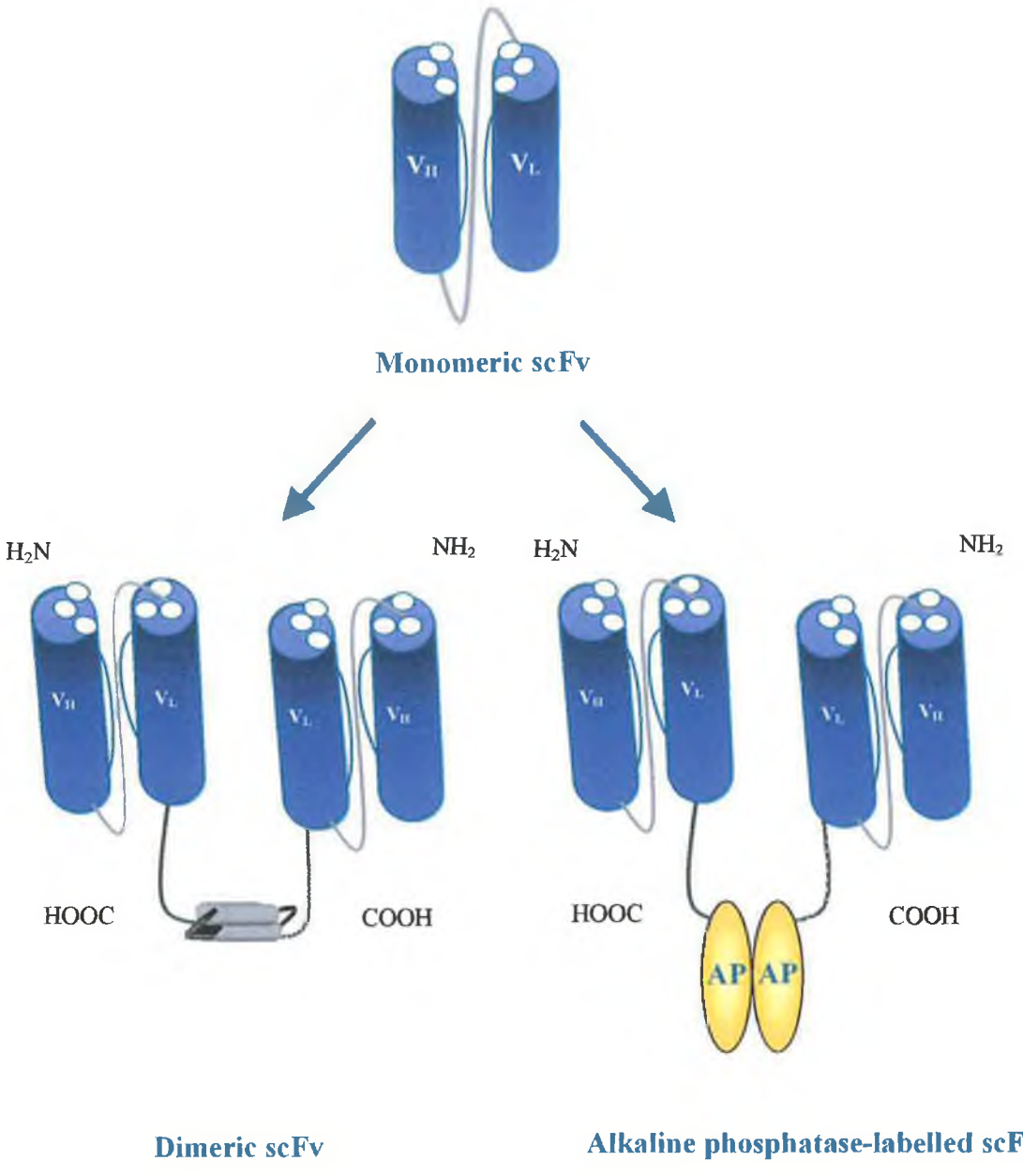


Figure 1.12: *Schematic diagram of the monomeric, dimeric and alkaline phosphatase-labelled scFvs produced using pAK 400, 500 and 600 expression vectors, respectively. The monomeric scFv consists of a variable heavy and light chain domain connected via a glycine-serine linker; the dimeric scFv comprises two scFv fragments connected via a single chain double helix (scdHLX) and the bifunctional scFv consists of two alkaline phosphatase-labelled scFvs dimerised through enzyme fusions of the alkaline phosphatase (AP).*

1.7 Synthetic Antibody Libraries

Synthetic antibody libraries are constructed *in vitro* through the assembly of V-gene segments and D/J regions. Antibody repertoires can be subclassed as fully or semi-synthetic, where the library randomisation is created in a single framework. Synthetic libraries are constructed from V-genes that have not been rearranged by the immune system. Using the natural germline V-genes as a scaffold, the CDR regions (usually CDR3 loops, as these correspond to the regions of highest natural diversity) are partially randomised to mimic 'natural diversity'. The CDR regions may undergo partial or complete randomisation using oligonucleotide-directed mutagenesis or PCR-based methods. Synthetic libraries can be specifically designed towards end use, for example the geometry of the binding site can be designed to match the shape of the antigen group, protein or hapten. Longer CDRH3 lengths yield higher affinity hapten binders, as a general rule, whereas a flat planar binding-pocket is better for binding large antigens (Strachan *et al.*, 2002). The *in vitro* nature of assembly allows the contents, degree of variability and overall diversity of the antibody repertoire to be controlled. One disadvantage of synthetic libraries is the possibility that the repertoire may be dominated by antibody domains that fold incorrectly or prove toxic to the expression host. Molecular cloning techniques may also introduce a high number of stop codons, or lead to truncated antibody sequences, if not tightly regulated.

In this study, the library used for the generation of scFv antibodies to THC is derived from the largest synthetic library constructed to date consisting of 6.5×10^{10} Fab-displayed on the surface of phage (Griffiths *et al.*, 1994). The library was constructed using a repertoire of $>10^8$ heavy chains and 8×10^5 light chains, both containing randomised CDR regions. To maximise library size, bacteria containing a 'donor' heavy chain repertoire, encoded on a plasmid, were infected with an 'acceptor' light chain repertoire encoded by phage. This combinatorial infection facilitates *in vivo* recombination within the bacterium through Cre catalysed recombination at *lox*P sites. This library has been used successfully for the isolation of antibody fragments against a variety of antigens such as foreign antigens and human antigens with affinities in the region of 4-217 nM (Griffiths *et al.*, 1994). ScFvs have also been isolated against small haptens like environmental contaminants including microcystin-LR, a

hepatotoxin produced by cyanobacteria, (McElhiney *et al.*, 2000, and Strachan *et al.*, 2002) and the herbicide, 2,4-dichlorophenoxyacetic acid (Brichta *et al.*, 2003);

1.8 Selection Procedures for Isolation of Phage-displaying Specific Antibodies

The design of an appropriate selection strategy is a pre-requiste for the isolation of scFvs with the desired binding kinetics and specificity for the target antigen. The process involves enriching the library for clones with specific binding capabilities for the antigen of interest, while depleting the repertoire of non-specific aberrant phage-displayed clones.

Traditionally, 'biopanning' on surface-immobilised antigen is a very popular method of selection (Figure 1.13). Specific phage retained on an antigen coated solid surface, e.g. an immunotube (Krebber *et al.*, 1997) or immunoaffinity column (Hoogenboom and Winter, 1992). Non-specific phage are removed through stringent washing. Specific phage are eluted using acidic elution, with compounds like glycine-HCl (Dillon[B] *et al.*, 2003); by alkaline elution, with compounds like triethylamine (de Bruin *et al.*, 1999); by passive bacterial elution (Wind *et al.*, 1997) or by competition with excess antigen (Charlton *et al.*, 2001), or a structurally similar antigen (Strachan *et al.*, 2002). The eluted phage can then be amplified in bacteria and subjected to further rounds of enrichment.

While this method may prove simple, fast and effective, it suffers from some drawbacks. Firstly, phage may be selected against an antigen in a conformationally-altered immobilised state and may be incapable of binding free antigen in solution. Secondly purified antigen is required for the selection process to be specific. It must also be taken into account that large quantities of antigen may be required for immobilisation over a number of selection rounds. Thirdly, unless elution conditions are carefully chosen, a bias may be created towards the selection of low affinity binders. It may be difficult to discriminate between clones with similar affinities. This is particularly important for non-specific stripping elutions like acid/base. Including an initial subtractive panning step may be used to increase specificity for

the target antigen. This involves a preliminary round of panning against blocking solution and/or protein carrier to eliminate any phage that bind to these proteins, prior to exposure to antigen-coated surface. A subtractive procedure, in association with flow cytometry cell sorting, was used by de Kruif *et al.* (1995), to isolate scFvs specific for subsets of blood leukocytes. Phage were incubated with a heterogeneous population of blood cells. Phage bound to each subset of cells were sorted by flow cytometry and eluted. In this manner heterogeneous phage were absorbed by non-selected cells, enriching the population for those specific to target subset of cells.

Commercially available streptavidin-coated magnetic beads can be used to select for binders in solution. A biotinylated derivatised antigen is mixed with a heterogeneous phage population and incubated for the desired period of time. Immunomagnetic separation can then be used to isolate specific binders. Vaughan *et al.* (1996) used solution capture on soluble biotinylated antigen to isolate high affinity antibodies to doxorubicin (K_D= 5.8 nM) and fluorescein (K_D=0.3 nM) from a naïve human library. Alternatively if biotin disulphide is used to link antigen to biotin, the phage-antigen complex can be eluted with dithiothreitol (DTT), which disrupts the disulphide bond between antigen and biotin (Harrison *et al.*, 1996). Osbourn *et al.* (1998) describe a novel pathfinder selection strategy that involves enzyme-catalysed reporter deposition for the biotinylation of phage in close proximity to the target antigen. Phage can then be retrieved on streptavidin-coated magnetic beads for amplification and further enrichment.

Antibody libraries can also be engineered to include a specific protease cleavage site between the antibody heavy chain and the gIII phage protein. Ward *et al.* (1996) describe enzymatic cleavage to elute human antibodies against tetanus toxoid and keyhole limpet haemocyanin. This strategy does not rely on disrupting the antibody:antigen interaction. Therefore, cleavage is independent of the antibody's affinity allowing recovery of high affinity binders. Malmborg *et al.* (1996) utilised BIAcore to isolate Fab antibodies against hen egg lysozyme and phenyloxazolone on the basis of their dissociation rate constants. Phage were injected over an antigen-coated sensor chip surface and elutions of dissociating phage, collected at different time points, were used to select for antibodies with the slowest dissociation rate.

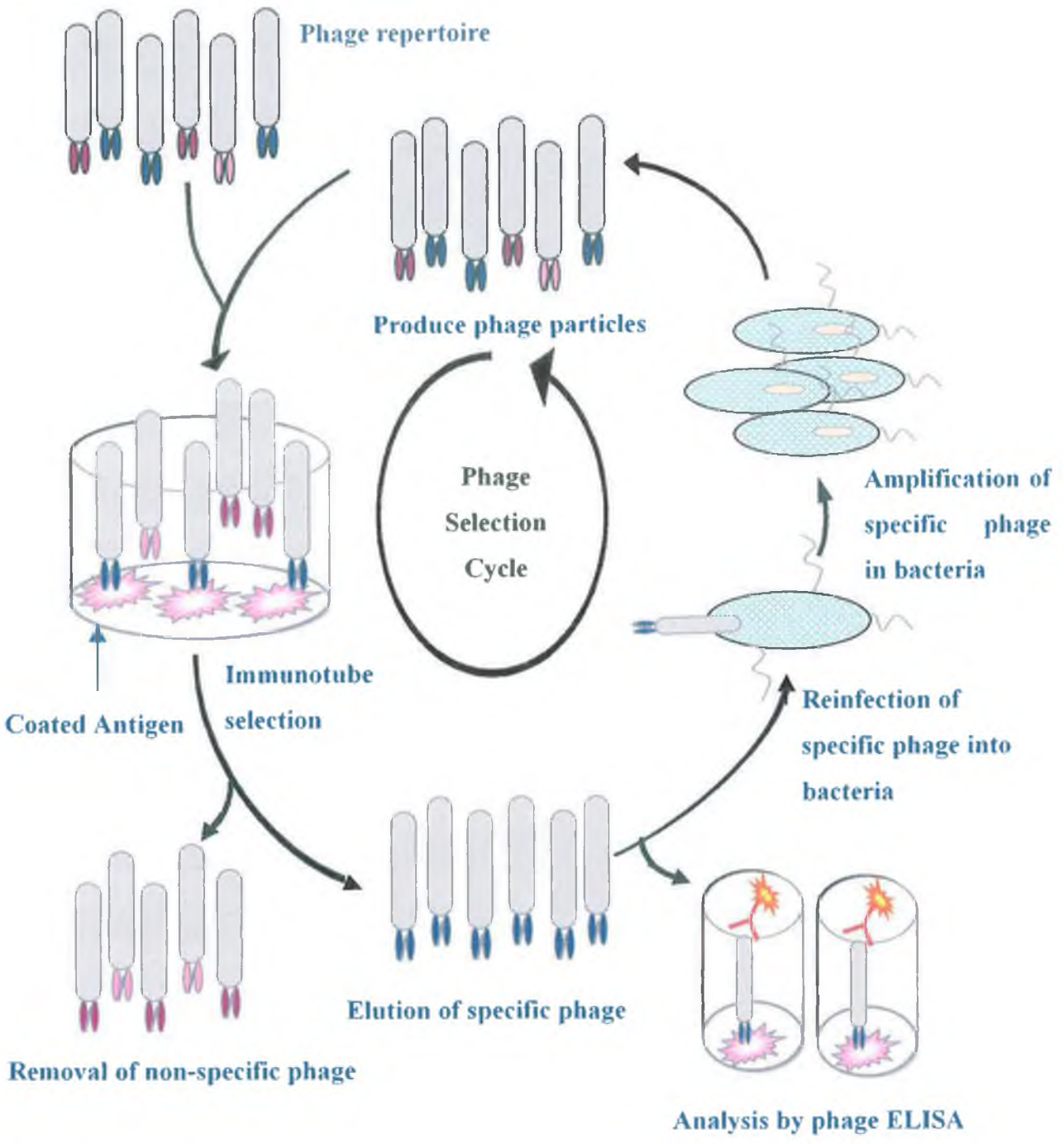


Figure 1.13: *Schematic illustration of a typical selection process for the enrichment of antigen-specific phage clones. The phage repertoire is incubated with the antigen of interest coated on a solid support. Phage that display antigen-specific antibodies will bind, while non-specific phage are removed by washing. Positive binders are eluted and reinfected into bacteria for further rounds of selection and enrichment. Individual clones are analysed by monoclonal phage ELISA.*

1.9 Soluble Expression

Bacterial expression systems are a popular method for the production of all functional antibody fragments (Fv, Fab, $F(ab')_2$, and scFv) because unlike whole antibodies, which require post-translational modifications, antibody fragments can be successfully produced as fully functional antigen binding molecules. *E. coli* is the strain of choice, due to its ease of manipulation, rapidity of growth and economical efficiency.

Following selection of antigen-specific phage antibodies, the same antibody can be produced in a soluble form directly from the same phagemid if it 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, encoding the pIII phage coat protein. An amber stop is read by *SupE* strains as a glutamine and therefore, transcription continues leading to the expression of an antibody-phage fusion protein. In non-supressor strains (HB2151 and JM83), this amber codon is read as a stop codon, bringing transcription to a halt and resulting in the secretion of soluble antibody. This process mimics the switch of B-cells between expression of surface bound and secreted antibody (Hoogenboom *et al.*, 1992). Non-suppressor strains of *E. coli* are routinely used to express large quantities of soluble antibody by eliminating glucose from the growth media and induction of expression with IPTG. Suppressor strains of *E. coli* (XL-1 Blue or TG1) may be forced to express small quantities of soluble antibody if in the absence of glucose and by induction with IPTG (Harrison *et al.*, 1996).

A leader sequence directs the expressed scFv to the periplasmic space of *E. coli*, thereby mimicking the eukaryotic secretory pathway. This signal sequence is cleaved during transportation through the cytoplasmic membrane and the secreted antibody is correctly folded with a homogenous N terminus. A *pelB* sequence is commonly used for this purpose (Krebber *et al.*, 1997 and Lemeulle *et al.*, 1998). A proportion of periplasmic scFv may leak out into the culture medium. Although the molecular cause is unknown, it seems to be dependant on the primary sequence of the antibody (Kipriyanov *et al.*, 1997). One disadvantage associated with periplasmic expression is the formation of insoluble antibody aggregates after transport to the periplasm

resulting in incorrect antibody folding. Kipriyanov *et al.* (1997) have demonstrated that the ratio of soluble versus insoluble scFv was dependant on the strength of the promoter and that the aggregation of the scFv in the periplasm could be reduced through the addition of sucrose just prior to induction. Inducer concentration, as well as incubation conditions can also be used to alter the level of secreted scFv. This is achieved by up-regulation of the *LacZ* promoter with the addition of increased concentrations of IPTG, resulting in a greater expression of soluble scFv into the medium. Reduction of the incubation temperature decreased antibody secretion into the medium, but had no effect on the yield of soluble scFv in the periplasm. ScFvs can also be produced without a leader sequence, as cytoplasmic inclusion bodies (Cho *et al.*, 2000). However, problems may arise during the delicate re-folding step, due to the incorrect formation of intermolecular disulphide bridges.

For production of scFvs on a large scale, the gene is often recloned into a specifically designed expression vector such as pIMS 147 (Strachan *et al.*, 1999 and Hayhurst *et al.*, 2003) or pAK 400 (Krebber *et al.*, 1997). The pIMS 147 vector encodes enhanced expression cassettes such as the skp periplasmic chaperone (Bothmann and Plückthun, 1998), to aid in protein solubility and decrease toxicity, the human Cκ domain for detection and the hexahistidine tag for purification. The pAK-compatible vector series encode enhanced expression, dimerisation or direct detection, via an enzyme label, as discussed in Section 1.6.2.1.

Antibody fragments have also been successfully expressed in eukaryotic systems. Such systems have the capability for post-translational modifications, like antibody glycosylation, as performed by a mammalian cell *in vivo*. Yeast expression systems offer the advantage that protein folding pathways and codon usage are closely related to that of mammalian cells, therefore, eliminating host environment biases and toxicity that may be associated with prokaryotic systems. The expression system is useful for the generation of therapeutic antibody products as expressed proteins are free from toxic cell wall pyrogens, associated with *E. coli* expression systems, and potentially oncogenic or viral nucleic acids as with mammalian expression systems. Yeasts such as *Schizosaccharomyces pombe* (Davis *et al.*, 1991), *Trichoderma reesei* (Eldin *et al.*, 1997), *Pichia pastoris* (Freyre *et al.*, 2000) and *Saccharomyces cerevisiae* (Shusta *et al.*, 1998) have been used to successfully express antibody

fragments. Antibody fragments have been successfully expressed in mammalian cells, such as Chinese Hamster ovary (CHO) cells (Dorai *et al.*, 1994), COS cells (Jost *et al.*, 1994 and Ridder *et al.*, 1995) and myeloma cells (Williams *et al.*, 2003). Myeloma cells do not require a serum media for growth, making them amenable to continuous and batch production systems, as well as simplifying the purification procedure. Antibody fragments can be recloned into expression vectors encoding antibody constant domains, facilitating antibody 'humanisation' and introducing effector functions encoded by the Fc portion (Mahler *et al.*, 1997; Persic *et al.*, 1997) and facilitating class switching (Boel *et al.*, 2000). Bacillovirus/insect cell expression systems yield soluble antibodies that are devoid of any human pathogens or toxic compounds (Brocks *et al.*, 1997 and Lemeulle *et al.*, 1998). Plant expression systems allow for bulk production of antibody fragments, without the requirement for fermentors or sterile culture conditions. ScFv's have been functionally expressed in different plant subcellular compartments including cytoplasm, apoplasts, endoplasmic reticulum, and in the seeds of transgenic plants. Various plants have been used to express recombinant proteins including tobacco (*Nicotiana tabaccum and N. benthaniana*) cereals (barley, rice, maize and wheat), legumes (peas, soyabean and alfalfa), and fruit and root crops (potato, tomato) (Stöger *et al.*, 2005). A humanized antibody against herpes simplex virus has been expressed in the soyabean plant (Zeitlin *et al.*, 1998). ScFvs against carcinoembryonic antigen has been expressed in peas (Perrin *et al.*, 2000), wheat (Stöger *et al.*, 2000) and rice (Torres *et al.*, 1998). Barley, potato and tobacco have been used to express a diagnostic antibody for the detection of anti-HIV-1 antibodies in human blood (Schunmann *et al.*, 2002). The scFv was directed against glycophorin and fused to an epitope of the HIV virus. The fusion protein could be used as a replacement for the SimpliRed™ HIV diagnostic agent. The tobacco plant using *Agrobacterium*-mediated transformation has also been widely reported for soluble expression of antibody fragments (Strachan *et al.*, 1998 and Hendy *et al.*, 1999).

1.10 Purification of scFv antibodies

Several different approaches have been taken to purify scFvs. The first type of purification method is derived from strategies used to purify whole antibodies. The principles are based on the intrinsic properties of the antibody like antigen-binding, isoelectric point, hydrophobicity, antibody class and fragment size. These methods include antigen-affinity chromatography (Lawrence *et al.*, 1998 and Brennan *et al.*, 2003), ion exchange chromatography (Kretzschmar *et al.*, 1996 and Williams *et al.*, 2003), hydrophobic charge introduction chromatography (Williams *et al.*, 2003), protein L, A and LA chromatography (Isaksen and Fitzgerald, 2001 and Williams *et al.*, 2003), and size exclusion chromatography (Kretzschmar *et al.*, 1996 and Laroche-Traineau *et al.*, 2000), which is normally used in tandem with other purification techniques.

The second group of purification methods are based on tags, which are genetically introduced into the scFv gene or plasmid to provide a handle for purification. These methods have proven more popular due to their universal applicability to different antibodies and recombinant proteins. Tag strategies that have been employed for antibody purification include human constant light chain tagging (Longstaff *et al.*, 1998 and Strachan *et al.*, 1998), chitin binding domain antibody fusions (Blank *et al.*, 2002), tagging with a four-amino acid flag tag (Wels *et al.*, 1992; Knappik and Plückthun, 1994 and Einhauer[B] and Jungbauer, 2001), c-myc tagging (Laroche-Traineau *et al.*, 2000), tagging with a nine amino acid streptavidin tag (Dübel *et al.*, 1995 and Skerra and Schmidt, 1999), antibody tagging by *in vivo* biotinylation (Tully, E., personal communication) and histidine tagging, as used in immobilised metal affinity chromatography (IMAC) (Harper *et al.*, 1997; Kerschbaumer *et al.*, 1997; Lindner *et al.*, 1997; Müller *et al.*, 1998).

IMAC was first described for the separation of serum proteins, by Porath *et al.* (1975). Since then, IMAC has established itself as a universal purification strategy for many recombinant proteins and antibody fragments. It boasts many advantages over the more traditional chromatographic techniques including higher ligand stability, higher protein loading, milder elution conditions, lower cost and complete recovery of ligand

following regeneration (Arnold, 1991). IMAC involves the insertion of the genes encoding the short histidine (His) peptide tag (containing 4-6 histidine residues) into the scFv sequence (Müller *et al.*, 1998). Insertion at the N or C terminus ensures that the antigen-binding site is not affected (Casey *et al.*, 1995), although C terminal His tags are often preferred for recombinant antibodies (Müller *et al.*, 1998).

The IMAC column consists of transition metals such as Ni^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+}, chelated by, iminodiacetic acid (IDA) nitrotriacetic acid (NTA), or tris (carboxymethyl) ethylenediamine (TED) (Arnold, 1991). Histidine containing proteins bind to the metal-chelate support via their non-protonated imidazole nitrogens. A high ionic strength buffer is employed to reduce non-specific electrostatic interactions. Specifically bound protein may be eluted using EDTA, to chelate metal ions; low pH, which confers a positive charge on the His residues so they are incapable of binding metal ions; or by addition of imidazole or metal ions, which compete with His for metal binding. The advantage of the latter system being that it does not denature the protein (Janknecht *et al.*, 1991). A gradient of increasing imidazole concentration is often employed to improve the purity of eluted protein.

1.11 Affinity Maturation of scFvs

The sequential rounds of mutation and affinity selection that occur within B-cells of the immune system have been mimicked in the area recombinant antibody technology using a process referred to as affinity maturation. Antibody affinity can be improved *in vitro* using a variety of techniques including site-directed mutagensis, CDR shuffling, chain shuffling or error prone PCR. The effects of the introduction of the individual mutations are analysed by a process of selection and screening. Mutations can also be introduced *in vivo* using *E. coli* mutator cells.

1.11.1 Site-directed mutagenesis

Site directed mutagenesis involves substitutions of the amino acids in one or more of the CDR regions and subsequent selection, to isolate higher affinity binders. Schier[B] *et al.* (1996) sequentially introduced random substitutions into the CDR3 area of heavy and light chains using oligonucleotide-directed mutagenesis. Light chain randomisation resulted in a 16-fold increase in antibody affinity. The highest affinity antibody was then used as a starting point to assess the effect of heavy chain randomisation. The overall effect of heavy and light chain mutagenesis led to a 1200-fold increase in antibody affinity, with an ultra low K_D in the picomolar range. Dong *et al.*, (2003) employed oligonucleotide mutagenesis of CDR H1 and 2, and CDR L3 to increase the affinity of an scFv recognising mouse neural cell adhesion molecule L1 by a factor of 60. They went on to dimerise the mutated scFv, which increased the affinity by a further factor of 5.5.

Lewis *et al.* (1995) reported a 9-fold increase in affinity for a highly evolved scFv against HIVgp-120, which had previously undergone both *in vivo* and *in vitro* affinity maturation. A three-step process of optimised residue substitution was employed for mutagenesis. This involved identifying residues that were non-critical for binding by sequential alanine substitution, followed by off-rate determination. Alanine is the chosen amino acid as it minimises disruption to the tertiary structure of the antibody. When substitutions result in a significant loss of activity, the residue is considered as operationally critical and is believed to be solvent exposed, possibly in contact with ligand. These residues are considered 'near-optimised' already and are by-passed for mutagenesis. Instead, positions that have a less direct effect on binding, perhaps those that are involved in access to the antigen-binding site, are targeted for mutagenesis.

A similar method, termed 'parsimonious mutagenesis', was used by Schier[A] *et al.* (1996) to increase the affinity of an scFv against the glycoprotein tumour antigen c-erbB-2, into the nanomolar range. In this method, amino acids involved in antigen binding are identified from the CDR regions using mutagenic oligonucleotides. These oligos are specifically designed to minimise coding sequence redundancy and limit

the number of amino acid residues, which do not retain parental structural features, thereby minimising the probability of non-viable structures.

1.11.2 Antibody CDR and chain shuffling

Chain shuffling mutagenesis involves the recloning of the antibody gene for one chain (e.g. V_H), with a repertoire of genes for the other chain (e.g. V_L) (Marks *et* al., 1992). This results in a mutant library with a V_H chain specific for the target and random V_L chains. At this stage the library is screened for specific binders and the one with the highest affinity is subjected to another round of chain shuffling. This time the new V_L chain is recombined with a repertoire of V_H chains. Depending on the source of V_H (specific to antigen or naïve), the CDR H3 may be kept constant to retain specificity. Park *et al.* (2000) improved the affinity of an scFv isolated from a naïve library against PreS1 of Hepatitis B by heavy chain shuffling with heavy chain genes derived from two healthy individuals. The mutated scFv exhibited a 6.5-fold increase in affinity. Another form of chain shuffling involves the shuffling of individual CDR regions. In order to conserve the specificity of the parental antibody, the CDRH3 is kept constant. Ellmark *et al.* (2002) shuffled an scFv against CD-40 using genes derived from a human antibody library. They maintained the CDR H1 and 3 regions constant, while chain shuffling the other CDR regions. The mutated library contained scFvs with an 8-37-fold improvement in affinity.

1.11.3 Error prone PCR

Error prone PCR is a mutagenesis approach that randomly introduces mutations throughout the antibody-encoding gene, effectively resulting in gene scrambling. DNA polymerases that lack proof-reading capabilities are often used when cloning antibody genes from hybridomas or an existing antibody fragment. This results in random insertions, deletions and substitutions in the nucleotide sequence. Many of these changes will be detrimental to the antibody; however, increased diversity and affinity can result. The level of affinity introduced compares favourably with that observed during the *in vivo* secondary immune response, which is typically in the

region of 1-3 orders of magnitude higher in affinity (Lewis *et al.*, 1995). Zhang *et al.* (2004) used a commercially available random mutagenesis kit (Stratagene, employing Mutazyme® polymerase) to affinity mature a HIV-1 neutralising scFv. Using sequential antigen panning on a number of HIV-related glycoproteins, it was possible to broaden the specificity and increase the neutralisation properties over those of the parental Fab.

1.11.4 In Vivo mutation using mutator Cells

In vivo affinity maturation can be carried out through the propagation of an antibody-encoding display vector in *E. coli* mutator cells. These conditional mutants produce single base substitutions, preferentially and predominantly, at a rate of 10^5 the rate of normal cells. The rate of mutation and ratio of transversions to transitions can be controlled using culture conditions (Coia *et al.*, 1997). One disadvantage with this system is that mutations in vector sequences, unassociated with antibody genes sequences, are possible. Mutations in areas such as antibiotic resistance genes, origins of replication and promoters, controlling expression, will be detrimental to the vector and will result in the loss of potentially good binders. Also, mutations to the gene to be affinity matured may also result in loss of expression of that gene e.g. stop codons limiting expression. The advantage of *in vivo* mutation systems is that phage are derived directly from mutated parent molecules without intervention, such as recloning and strains (F' mutD5-FIT) harbouring the F' pillus are available commercially, for direct phage infection. High titres of rescued phage can be achieved that are not possible with *in vitro* methods. These methods involve ligation and transformation of the mutated sequence and, therefore, the size of the mutated library will be limited by the transformation efficiency *E. coli*. Irving *et al.* (1996) used mutator cells to increase the affinity of an scFv isolated from a naïve library. The majority of mutations increased affinity 3-fold. However, a mutation introduced in CDR3 resulted in a 10^3 gain in affinity, but with associated antibody aggregation. Coia[A] *et al.* (2001) used F′ mutD5-FIT cells to enhance the poor expression levels of a hybridoma-derived scFv against hepatitis by a factor of 10.

1.12 *In Vitro* display and affinity maturation using ribosome display

Despite the progress represented by phage display in the field of scFv technology, it is a cell-dependant system, and, therefore, suffers from the limitations associated with *in vivo* systems. Namely, the library size is limited by the efficiency of the initial transformation step and selection disadvantages of host environments against particular scFv's may be encountered (Schaffitzel *et al.*, 1999). Ribosomal display is an entirely 'cell free' system, where transcription, translation and selection are all carried out *in vitro*. In addition to the selection and enrichment of functional binders (Hanes and Plückthun, 1997), ribosomal display also facilitates protein evolution and affinity maturation during the selection process when low fidelity DNA polymerases (e.g. T*aq*) are employed (Hanes *et al.*, 1998 and Hanes *et al.*, 2000).

Ribosome display, as detailed in Figure 1.14, 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 on a stop codon (Hanes and Plückthun, 1997 and He *et al.*, 1999). The ribosome stalls, forming a tertiary mRNA-Ribosome-scFv complex, which is stabilised by low temperature (4° C) and the magnesium ion content of the buffer (Schaffitzel *et al.*, 1999). The complex connects genotype (mRNA) to phenotype (protein) and therefore can be used directly for screening. This may be performed on tagged ligand immobilised on a surface (Hanes *et al.*, 1998) or in solution for its binding properties (Coia[B] *et al.*, 2001). Specific binders may be eluted with excess competing antigen (Hanes and Plückthun, 1997 and Schaffitzel *et al.*, 1999) or by using ethylenediaminetetracetic acid (EDTA) to dissociate mRNA-ribosome complex. The RNA can be used directly for further selection cycles, or alternatively the genetic information may be recovered in the form of cDNA, using RT-PCR, while the mRNA remains complexed to the ribosome (Hanes *et al.*, 1998). Transcription and translation may be performed as two separate reactions (Hanes and Plückthun, 1997; Hanes *et al.*, 1998 and Hanes *et al.*, 2000) or as a coupled procedure (He *et al.*, 1999). However, the dependence of T7 RNA polymerase on β-mercaptoethanol for stability may prevent disulphide bond formation of scFv's (Hanes and Plückthun, 1997 and Schaffitzel *et al*, 1999). The use of prokaryotic

(Hanes and Pluckthun, 1997) and eukaryotic (He *et al.*, 1999) translation systems has also been described.

The fundamental principle of ribosomal display requires the introduction of specific alterations in the initial mRNA sequence before each cycle. A T7 promoter is required at the 5′ untranslated end of the sequence, to ensure efficient transcription by T7 RNA polymerase. A Shine-Delgarno sequence is introduced to facilitate ribosome binding and initiation of translation. A stem loop structure is also added, to prevent the RNA complex from being degradated by RNAse E. At the 3′ end, the stop codon needs to be eliminated, in order to stall the ribosome on the mRNA. This facilitates the formation of tertiary complex. A second stem loop structure must also be added to protect mRNA from degradation by 3′ to 5′ endonucleases. A spacer region is also necessary to allow sufficient spatial separation of the polypeptide from the ribosome in order to facilitate correct protein folding for binding (Hanes and Plückthun, 1997 and Schaffitzel *et al.*, 1999). The presence of protein disulphide isomerase and molecular chaperones catalyses the formation and isomerisation of disulphide bonds on the ribosome complex (Ryabova *et al.*, 1997)

Using this technology, Hanes *et al.* (2000) have selected an scFv fragment of an antibody with picomolar affinity from a library of synthetic genes, representing a 40-fold maturation in affinity through point mutations introduced during PCR. However, if ribosome display is to be used solely as a screening tool, polymerases with proofreading capabilities may be employed. Diversification can be further improved by combining the technology of ribosome display with *in vitro* mutagenesis techniques such as DNA-shuffling. Jermutus *et al.* (2001) have used off-rate selection to assess affinity maturation with DNA shuffling at the end of each cycle to improve the affinity of an scFv 30-fold. Yau *et al.* (2003) successfully used ribosome display to isolate single domain, variable heavy chain fragments specific for the pesticide, picloram, from a naïve llama library.

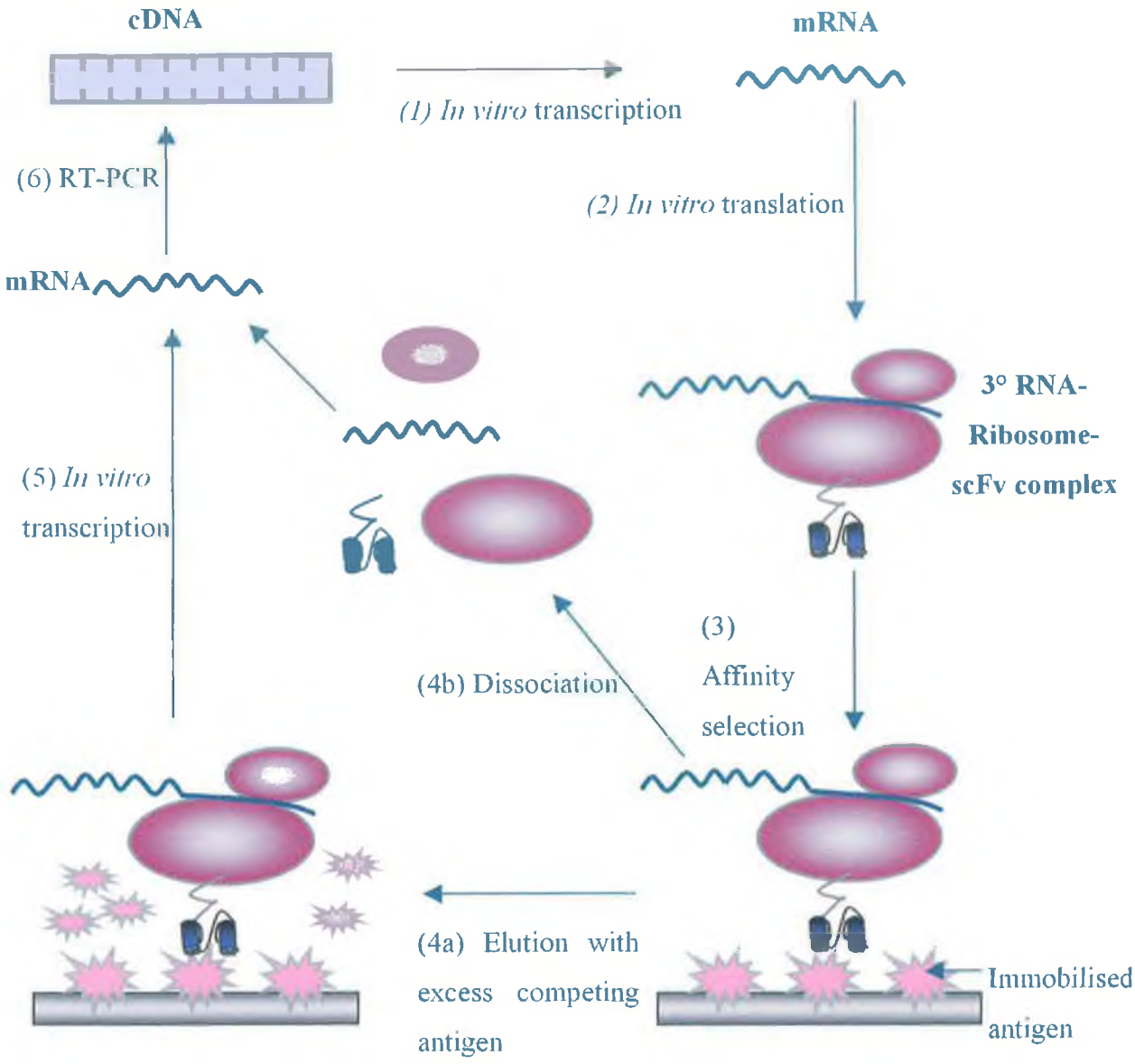


Figure 1.14: *Schematic illustration outlining the principle of ribosome display technology. (1) The DNA encoding the antibody library is amplified by PCR and transcribed in vitro to mRNA where a T7 promoter, ribosome-binding site and stem-loops are introduced. (2) The mRNA is then translated in vitro with factors that enhance the stability of the mRNA-ribosome-protein complex. (3) Antigen-specific complexes are selected on immobilised antigen. Non-specific complexes are removed by washing. (4) Affinity selected complexes are eluted using (4a) free antigen or (4b) EDTA. (5) The mRNA is isolated from the complex and (6) reverse transcribed into cDNA. The DNA is amplified by PCR and used in the next round of selection / enrichment (adapted from Hanes and Plückthun, 1997).*

1.13 Immunoassays

Immunoassays are a commonly used bioanalytical technique for the detection of illicit drugs. The fundamental principle of immunoassay technology relies on the specific recognition of the target compound by an antibody. A qualitative or quantitative measurement of analyte is then generated as a consequence of this antibody-antigen interaction. The signal is generated through labelling of either antibody or antigen. Commonly employed labels include enzymes, radioisotopes, fluorophores or chemiluminescent compounds. Immunoassays can be further divided into two categories, homogeneous or heterogeneous. Homogeneous assays are carried out in solution and do not involve the separation of reactants, whereas the more commonly used heterogeneous assay involves the immobilisation of one of the reagents and removal of unreacted reagents prior to measurement. Heterogeneous immunoassays are most commonly used for hapten detection (Dankwardt, 2000).

1.13.1 Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) is the most commonly used technique for measuring hapten-antibody interactions. It avoids the necessity of having to work with radioactive material and high levels of sensitivity can be achieved. The long term stability of the coloured end-product of the reaction make ELISA superior to fluorimetry or luminometry, even though these methods may achieve lower detection limits (Dankwardt, 2000). Simple and cheap photometers are also available with extremely rapid and sensitive measurement capabilities.

This type of immunoassay format involves the immobilisation of either the antigen or antibody onto a solid support matrix, for example 96-well plastic plates. Haptens cannot be directly immobilised onto a solid support. For this reason haptens are either linked via chemical means to a derivatised matrix or coupled to a protein molecule for adsorption. Proteins bind primarily to the surface of the wells of γ-irradiated plastic supports via hydrophobic interactions. An enzyme label is attached to a tracer molecule (either antigen or antibody) for the detection of bound antigen-antibody

complex. The most commonly used enzymes in ELISAs are horseradish peroxidase, β-galactosidase and alkaline phosphatase (Dankwardt, 2000).

Several different heterogeneous assay formats have been described, including competitive and non-competitive formats. Non-competitive immunoassays are normally used to determine the concentration of antibody in a sample (antibody titre). They can also be employed for the quantification of higher molecular weight analytes with multiple epitopes e.g. sandwich ELISA for determination of bacterial cells (Dunne, 2004). A competitive immunoassay involves creating competition for antibody binding through the introduction of free analyte into the sample for analysis. The signal measured is therefore inversely proportional to the concentration of analyte in the sample mixture.

Figure 1.15 illustrates the principles involved in an indirect non-competitive ELISA. A 96-well microtitre plate is coated with the antigen of interest, in this case a protein-hapten conjugate. Following a suitable incubation period (i.e. 1 hour at 37°C), the plate is washed and any unreacted sites are 'blocked' using a protein solution, which has a high protein concentration, such as BSA or a non-fat milk powder such as Marvel™. This eliminates any non-specific antibody binding. Following incubation and washing, varying dilutions of antibody are added to the plate and incubated for the desired period. Any unbound antibody is washed away and bound antibody is detected using a secondary enzyme-labelled antibody. This antibody may be specific for the particular species of antibody or for a tag encoded in the antibody sequence. The plate is then washed and a chromogenic enzyme substrate is added. The intensity of the colour developed is measured at the optimum wavelength in a spectrophotometer. The colour produced is directly proportional to the concentration of the specific antibody present (Tijssen, 1985).

Figure 1.16 illustrates a competitive ELISA format. This assay is carried out as for the indirect non-competitive ELISA except that varying concentrations of free antigen are added to the plate alongside the antibody. Free antigen in solution will compete with immobilised antigen for antibody binding. The colour produced with this assay format will be inversely proportional to the concentration of free antigen added. This yields a

sigmoidal dose-response curve, when signal is plotted against the logarithm of analyte concentration. This assay format can therefore be used for the quantitative detection of an antigen with reference to a standard dose-response curve (Tijssen, 1985).

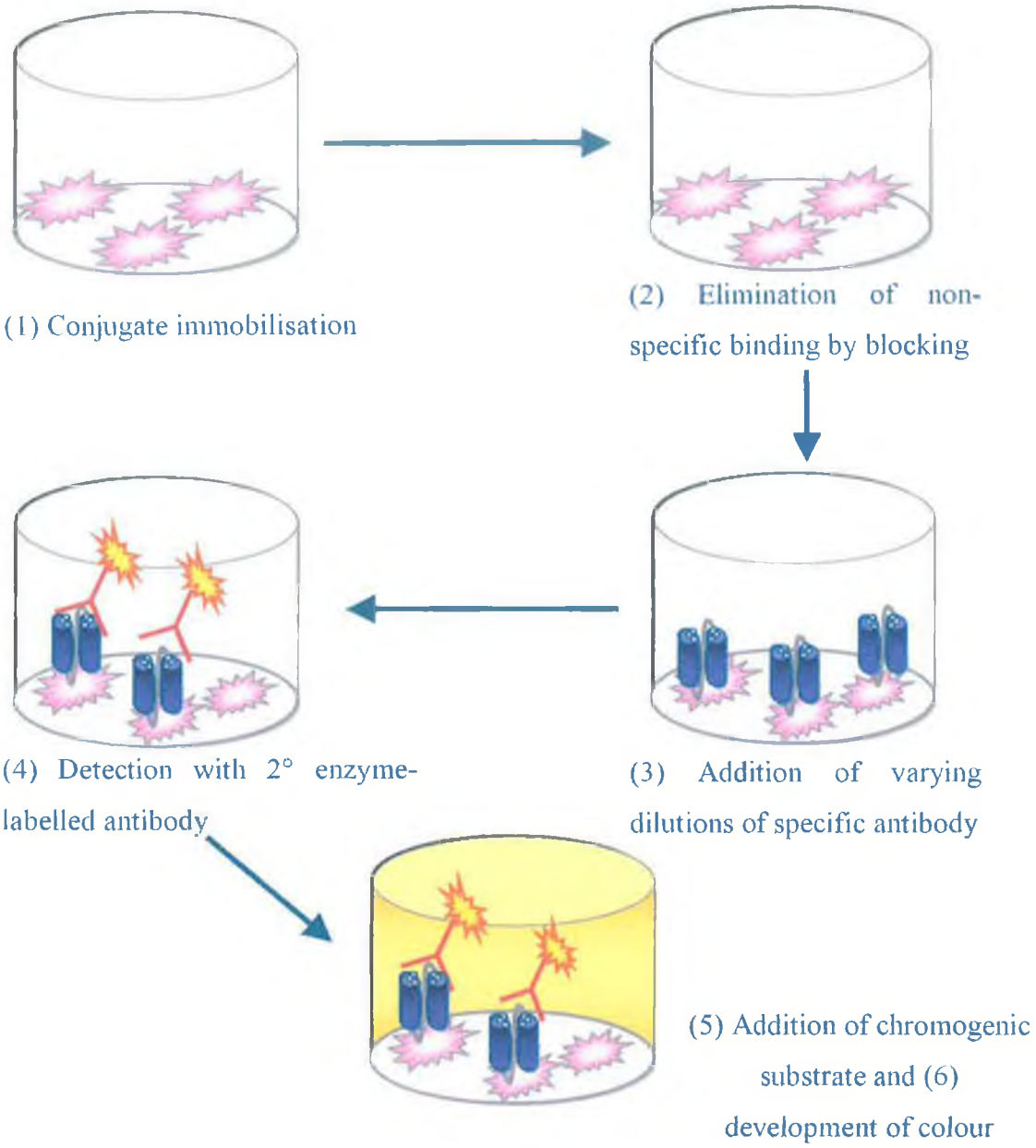


Figure 1.15: *Diagram of a typical non-competitive indirect ELISA mainly used for the determination of antibody titre. (1) The wells of an immunoplate are coated with a protein-hapten conjugate. (2) Wells are blocked with a suitable blocking solution like 2% (w/v) marvel™. (3) Dilutions of specific antibody are added to each well (4) Bound antibodies are detected using an enzyme-labelled secondary antibody. (5) A chromogenic substrate is added and the colour that develops is directly proportional to the amount of antibody present.*

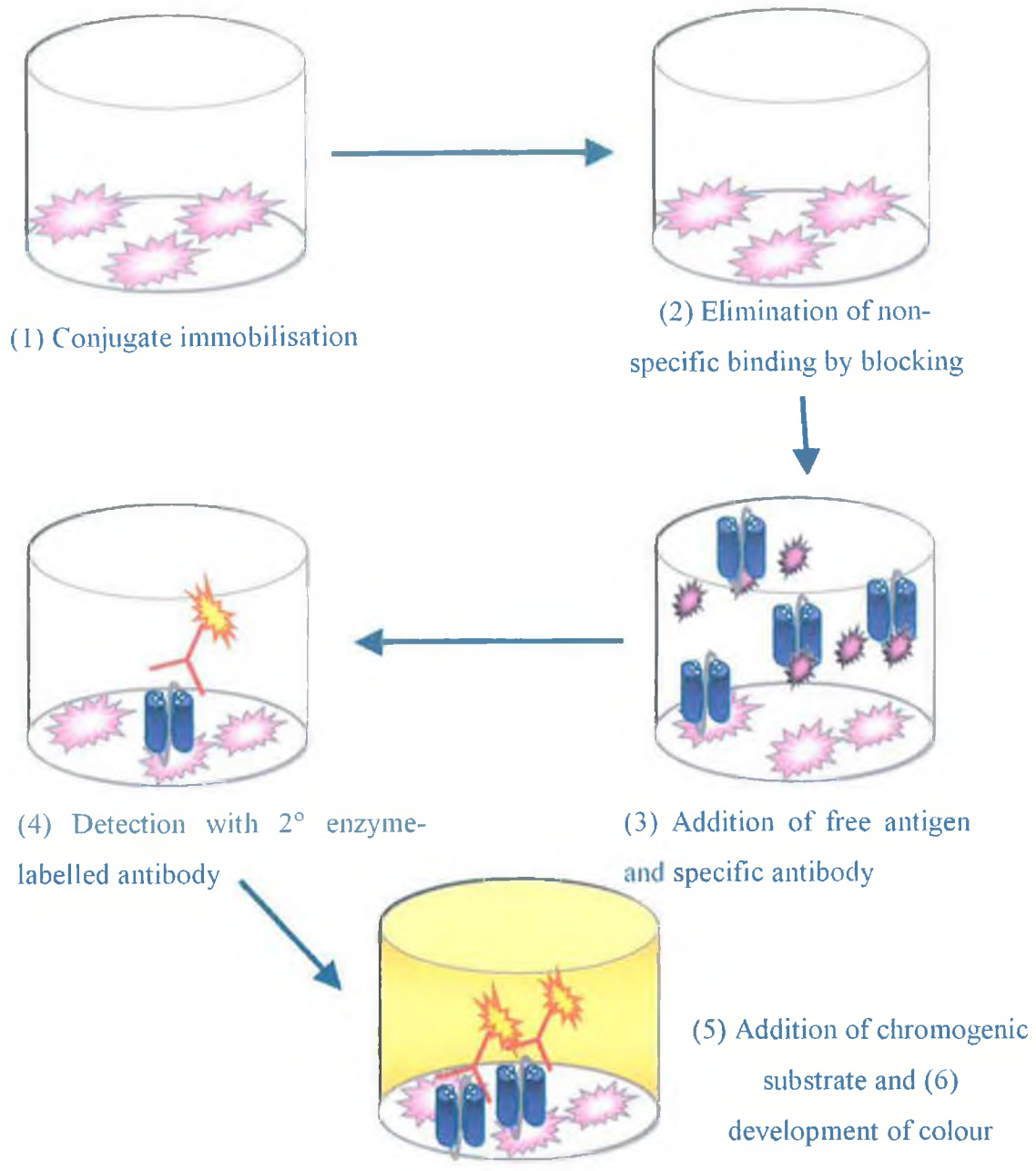


Figure 1.16: *Diagram of a typical competitive indirect ELISA that may be used for the determination illicit drugs (1) The wells of an immunoplate are coated with a protein-hapten conjugate. (2) Wells are blocked with a suitable blocking solution like 2% (w/v) marvel™. (3) Free and immobilised antigen compete for binding to the specific antibody. (4) Conjugate captured antibodies are detected using an enzyme-labelled secondary antibody. (5) A chromogenic substrate is added and the colour that develops is inversely proportional to the amount of free antigen.*

1.13.2 Lateral Flow Immunoassay

A lateral flow immunoassay (LFIA) combines the specificity of immuno-recognition with the separation of chromatography. The development and combination of specific antibodies, colloidal particles (carbon, silica, gold, latex etc.) as labels and lateral flow membrane devices have facilitated the development of robust, user friendly, portable LFIA devices, suitable for semi-quantitative analyte detection (O'Keeffe *et al.*, 2003). This technology offers reduced analysis time and does not require any elaborate instrumentation.

The principle of LFIA is outlined in Figure 1.17. In order to visualise the reaction, a labelled tracer is prepared by direct or indirect conjugation of colloidal particles with a specific antibody or antigen. The sample containing, the target analyte, is added to the membrane, along with this labelled-tracer molecule. Interactions between the target analyte and labelled-tracer occur as the sample flows along the nitrocellulose. In this manner the membrane serves to separate bound and unbound moieties. Bound tracer is focused to the capture area, an area that has been impregnated with a specific capture ligand that interacts with, and binds to the tracer molecule. This results in the generation of a visible narrow analyte test band, formed by a concentration of colloid-tracer in this area. A control line is always included in an LFIA directly above the test line to validate the assay. This ensures that the sample has indeed passed over the analyte capture area and that components are functioning correctly. Upon performing a LFIA, this control line must be visible otherwise any result is invalid. The control line signal is usually generated using an analyte specific for the labelled tracer used in the generation of the test line. This ensures that the component has indeed been included in the assay and that the sample has passed over the analyte capture zone.

LFIAs have been successfully developed for a variety of applications including the detection of human chorionic gonadotropin in urine (van Amerongen *et al.*, 1994), aflatoxins in peanuts, maize and milk (Niessen *et al.*, 1998 and Sibanda *et al.*, 1999), ricin (Shyu *et al.*, 2002) cannabinoids, cocaine and opiates in urine (Wenning *et al.*, 1998), sulphamethazine in swine urine (O'Keeffe *et al.*, 2003), the steroid hormone cortisol (Leung *et al.*, 2003), *Salmonella typhimurium* (Paek *et al.*, 2000), *Salmonella*

enteritis in raw eggs (Seo *et al.*, 2003), human heart type fatty acid binding protein (Chan *et al.*, 2003), *Mycobacterium bovis* in badgers (Greenwald *et al.*, 2003), antibodies to HIV 1 and 2 in serum (Soroka *et al.*, 2003) and verotoxin producing *E. coli* in food (Aldus *et al.*, 2003).

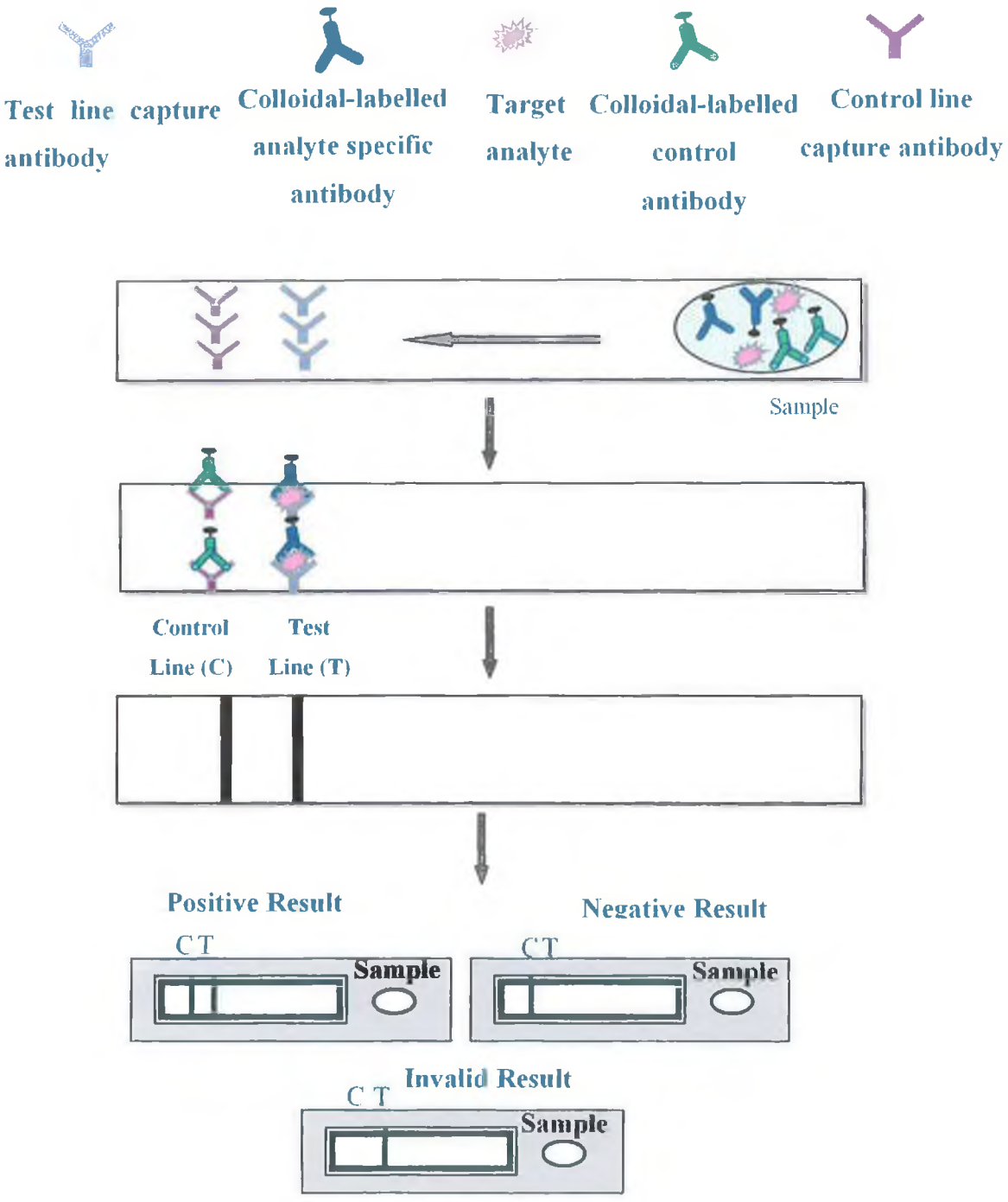


Figure 1.17: *Schematic representation of a lateral flow immunoassay device and the possible results. The presence of the control (C) line indicates that the test is valid. The appearance of a line below this (T) indicates an analyte positive result, whereas the absence of a test line is indicative of a negative result.*

1.13.3 BIAcore

BIAcore is an optical-based biosensor that measures specific biomolecular interactions in 'real-time'. The principles of BIAcore will be discussed in relation to its use as an immunosensor, although it can be used to monitor interactions between any receptor/ligand pair. BIAcore uses the phenomenon of surface plasmon resonance (SPR) to monitor binding interactions in 'real-time'. Immunoassays for a range of haptens have been successfully developed using BIAcore technology, including drugs, such as M3G (Dillon[A] *et al,* 2003), amphetamine, morphine and tetrahydrocannabinol (Fanning, 2002), warfarin (Fitzpatrick, 2001), fungal contaminants (Daly *et al.*, 2000) and microbial pathogens (Leonard *et al.*, 2004). The BIAcore system offers 'real-time' monitoring of interactions, without the use of labels for detection. The BIAcore system is a completely integrated analytical system consisting of an optical transducer, a biointerface, a LED detector and integrated microfluidics cartridge (Figure 1.18). The re-usable biointerface consists of a gold-coated sensor chip derivatised with a carboxymethylated dextran layer. The integrated microfluidics system offers automation and exact sample handling and with small sample consumption.

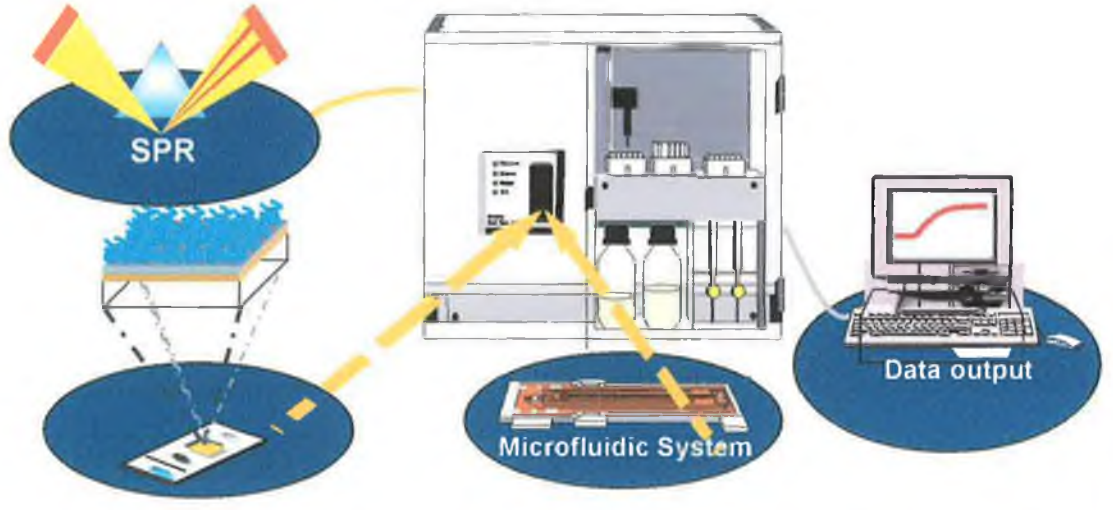


Figure 1.18: *Schematic representation of the BIAcore system consisting of optical transducer, gold-dextran biointerface, an integrated microfluidics cartridge and data acquisition system.*

Surface Plasmon Resonance (SPR) is an optical phenomenon that occurs under conditions of Total Internal Reflection (TIR). When a plane-polarised beam of light, at an angle greater than the critical angle, is incident on a medium of higher to lower refractive index, all of the light will undergo total internal reflection and propagate back into the medium of higher refractive index. Although the reflected beam does not lose any net energy across the interface, the beam leaks an electrical field intensity, called an evanescent wave, into the medium of lower refractive index. This decays exponentially from the surface over a distance of one wavelength. SPR occurs when the interface is coated with a metal layer (e.g. gold in the case of BIAcore). Under these conditions, the evanescent wave penetrates the metal, exciting the free electrons. This causes them to resonate as a collective wave of plasmons, parallel to the interface. This resonance absorbs energy from the incident light, causing a dip in the intensity of reflected light at a specific angle. The angle at which this occurs is known as the SPR angle. As the evanescent wave penetrates a short distance into the medium of lower refractive index, the SPR angle is sensitive to refractive index changes at the sensor chip surface. A linear correlation exists between resonance angle shift and surface protein concentration allowing 'real-tine' detection of mass changes without the need for labelling (Hutchinson, 1995). The response or resonance signal (RU) is displayed to the user as a function of time in the form of a sensorgram. The sensorgram provides quantitative information about the binding interaction in 'real-time', including binding specificity, affinity and kinetics (Quinn and O'Kennedy, 2001), in addition to the determination of analyte concentration (Dillon[A] *et al.*, 2003).

The BIAcore system employs a wedge-shaped beam of monochromatic, plane-polarised light to cause TIR at the sensor chip surface. The angle of minimum reflectance is measured using a two-dimensional detector array. In this system the medium of higher refractive index comprises a glass prism and the medium of lower refractive index constitutes the dextran coating of the chip and the surrounding sample buffer (ca 300 nm). SPR acts as a mass detector, irrespective of the type of protein or the type of interaction, as all proteins contribute to similar refractive indices (Hutchinson, 1995). An important feature of this system is that the area of low refractive index (i.e. sample) is only penetrated by the evanescent wave and not by the light itself. This facilitates the analysis of coloured, opaque or turbid samples (Quinn

et al., 1997). A resonance response of 1000 RU corresponds to a shift in SPR angle of 0.1°, which in turn correlates to a change of approximately 1 ng/mm^2 in surface protein concentration.

Figure 1.19 illustrates a diagrammatic representation of a competitive BIAcore assay involving immobilised antigen, antibody and free antigen in solution. In this assay, the antigen is immobilised on the sensor chip surface using the appropriate coupling chemistry e.g. carbodiimide coupling using EDC/NHS. The antibody and free analyte are then passed over the chip surface. This will result in a shift in SPR angle, which is expressed as a function of time and represented as a sensorgram. The resonance signal (RU) is proportional to the mass change at the surface and is, therefore, inversely proportional to the amount of analyte present in the sample. An alternative assay configuration may also be employed. This would involve immobilising the antibody at the sensor chip surface and monitoring the binding response of free antigen in solution. In this case, the binding response would be directly proportional to the concentration of free antigen in solution.

The sensor chip surface consists of a thin gold film deposited on a glass support. The gold is derivatised with a surface layer covalently attached to a surface matrix for ligand immobilisation. The CM5 sensor chip is the most commonly used sensor chip. The surface matrix consists of a carboxymethylated dextran hydrogel, which forms a flexible, hydrophilic environment for biomolecular interactions. The carboxyl groups facilitate ligand immobilisation and the open structure of the matrix facilitates protein adsorption. Dextran is stable in commonly used buffers and can withstand pH, salt and solvent extremes for short periods of time. An extensive range of other sensor chips are also available for specialised applications. These include a streptavidin derivatised chip, to capture biotinylated molecules, a NTA metal^{+2} chelating chip, which can be used to reversibly bind His-tagged biomolecules and sensor chip HPA, which enables the attachment of lipid monolayers for membrane biochemistry and the study of membrane-associated receptors. All chips are removable and interchangeable. It is possible to switch from detection of one analyte to another very easily, without extensive cleaning and equilibration of the system.

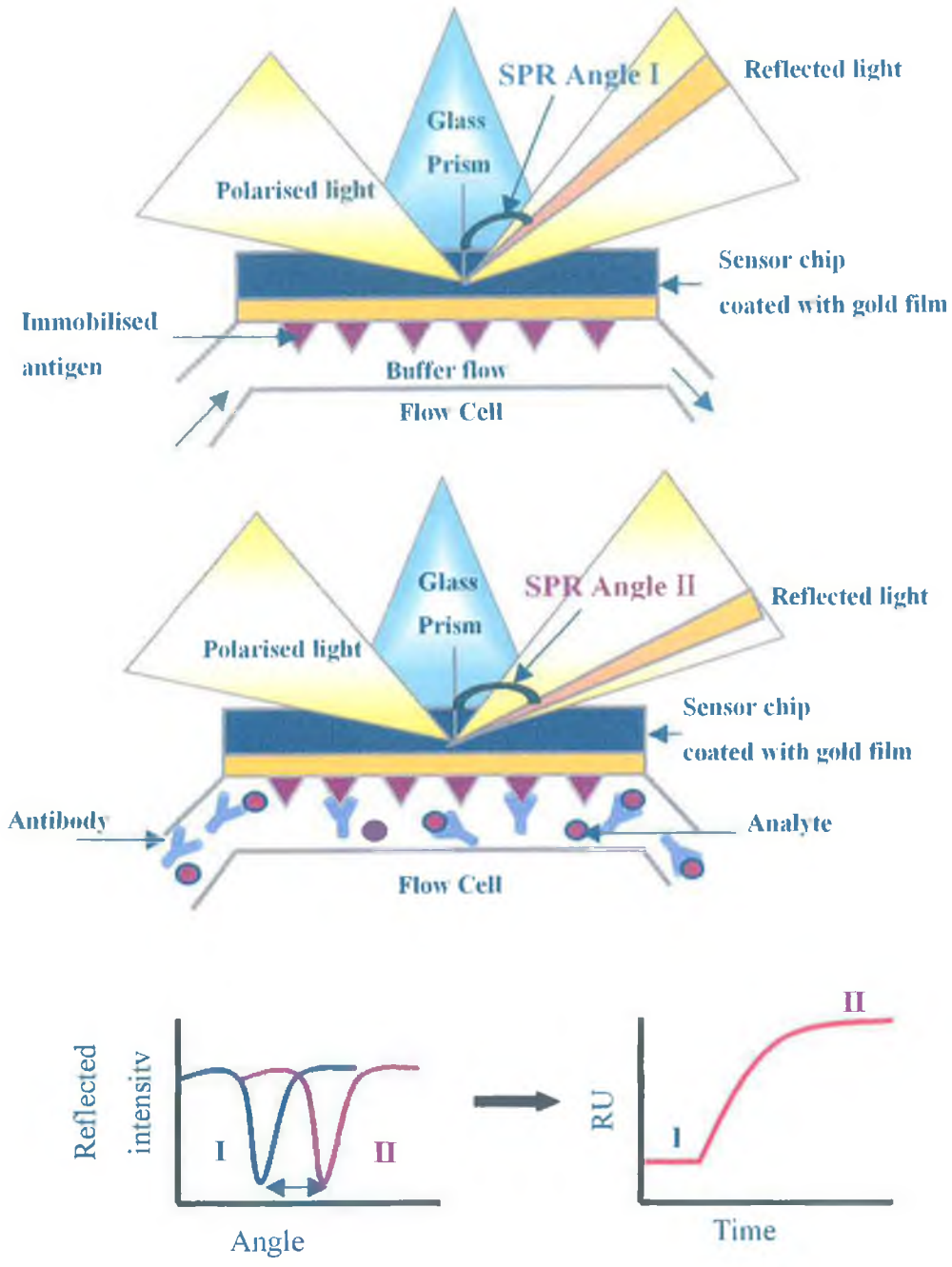


Figure 1.19: *Diagrammatic representation of competitive BIAcore immunoassay employing surface plasmon resonance. The sensor surface is immobilised with protein conjugate (▲) using EDC NHS chemistry. Under conditions of total internal reflection an evanescent wave propagates into the medium of lower refractive index, resulting in a dip in the intensity of the reflected light at a particular angle known as the SPR angle (I). When antibody is injected over the surface, the change in mass at the chip surface resulting from the binding interaction causes the SPR angle to shift (II). Changes in SPR angle are displayed as resonance units and expressed as a function of time in the form of a sensorgram, allowing binding interactions to be measured in 'real-time'.*

1.14 Commercial Immunoassay Tests for Drugs of Abuse in saliva

There are numerous commercial tests available for the detection of illicit drugs in urine and saliva. However, as different metabolites are present in each matrix at very different concentrations, these tests are only applicable to the matrix for which they were designed. The Roadside Testing Assessment (ROSITA) project, commissioned by the EU, was the first major study carried out to evaluate the performance of oral fluid tests. The aim of the study was to identify roadside testing equipment and make an internal comparative assessment of existing equipment or prototypes. The validity of results, equipment reliability, practicality and costs were also evaluated. Results of this study are available from www.rosita.org.

A market inventory carried out in 1999, revealed 19 different on-site tests for saliva analysis, representing 33 brand names and distributors. Of these 19 devices, 3 were designed for saliva analysis (Grönholm and Lillsunde, 2001). These include ORALscreen™ (Avitar Technologies Inc., USA), Rapiscan®, (Cozart Biosciences Ltd., UK) and Drugwipe® (Securetec GmbH, Germany), which is suitable for both saliva and sweat analysis.

ORALscreen™ is a lateral flow immunoassay-based test for the detection of opiates, cocaine, d-methamphetamine and cannabis. It combines an inventive saliva sampling system with a 'pipette on' sampling membrane and read device. The test takes 10 mins to perform, yielding two lines for a positive result (25 ng/ml cut-off for morphine) and just a control line for a negative result. The test exhibits 100 % cross reactivity with structurally related opiates (M3G, hydromorphone, hydrocodone, codeine and 6-MAM). Barrett *et al.* (2001) compared saliva analysis of drivers in the US, performed with ORALscreen™, to urine immunoanalysis and confirmation by GC/MS. The results showed very good correlation between results obtained for saliva and urine over a 2.5 to 3 day period for cocaine and opiates, and a 2-day period for THC.

Drugwipe® (Securetec GmbH) does not require the collection of a saliva sample and is also applicable to sweat. The test is a colorimetric-based lateral flow immunoassay.

Separate strips are available for opiates, cocaine and amphetamines, although no multi-panel test is available. The test is easy to apply, by wiping it over the tongue. Earlier versions of this test, included in the initial ROSITA report, did not include a built in control line and the interpretation of a positive result (i.e. a pink red product) was vague. The device has extremely high cut-off levels (300 ng/ml for opiates and amphetamines) and also shows high cross reactivity with structurally similar molecules (e.g. opiates: codeine 100%, morphine, 100%, dihydrocodeine 100%, ethylmorphine 100%, heroin 66%, hydrocone 66%, M3G 66%, thebaine 66% and hydromorphone 40%). The test has recently been updated to include a Drugread® (Securetec) hand photometer, that provides an electronic read out after two minutes. This newer version Drugwipe® was employed by Samyn *et al.* (2002), to detect drugs of abuse in drivers in Belguim. Drugwipe® results were compared to GC/MS analysis of saliva collected by spitting or using a Salivette® (Sarstedt). The accuracy of this system was 67% for opiates, 63% for cocaine and 79% for amphetamines. Cannabis samples were not compared due to the probability of sample contamination through hand to mouth contact with the Salivette® collection device.

The Rapiscan® device consists of three components, a saliva collection swab, a disposable 'pipette-on' cartridge, which houses the immunoassay, and an instrument, which gives a digital read-out of results. The device tests for amphetamines, cannabis, cocaine, opiates and benzodiazepines. The ROSITA evaluation found the Rapiscan® system to be the most objective system applicable for roadside testing. The digital read out system, yielded definitive quantitative results, however the instrument added considerable expense to the system (€3000) and required user expertise. Grönholm and Lillsunde (2001) evaluated both Rapiscan® and Drugwipe® devices and found Rapiscan® to be the most accurate compared to GC/MS analysis. Accuracies between 83% (opiates) and 99% (cannabis) were reported. Bennet *et al.* (2003) used Rapiscan® to compare oral fluid analysis to urinalysis, using a light photometric Enzyme Multiplied Immunoassay (EMIT) device, in a drug treatment setting. Sensitivity of the Rapiscan® device was found to be similar to urinalysis for opiates but with a higher specificity.

1.15 Summary of research

The main aim of this research project was the production of novel genetically-derived scFv antibody fragments to be employed in rapid immunoassays for the detection of illicit drugs of abuse in saliva.

Chapter 3 describes the production, purification and characterisation of a monomeric, a dimeric and an alkaline phosphatase-labelled scFv against M3G. The scFvs were applied to the development of competitive ELISAs and the cross reactivities of each antibody with structurally similar molecules were determined.

Chapter 4 describes application of monomeric scFv to morphine detection in saliva. The affinity constants of each scFv were determined and monomeric scFv was determined to have the best potential for saliva analysis. This antibody was used in a competitive immunoassay and an inhibition BIAcore assay for morphine detection in 'spiked' saliva samples. The competitive immunoassay was employed in a pilot study on the use of saliva for opiate analysis in a Drug Treatment setting. A rapid sol particle lateral flow immunoassay was also developed for the detection of M3G, using monomeric and dimeric scFvs.

Chapter 5 describes the production and characterisation scFv antibodies against tetrahydrocannabinol. Phage-displayed antibodies were isolated from a semi-synthetic human library. The antibodies were applied to the analysis of tetrahydrocannabinol in saliva by competitive phage ELISA.

Chapter 2: Materials and Methods

2.1 General Formulations

2.1.1 Reagents

All reagents were of analytical grade and purchased from Sigma-Aldrich Chemical Co. (Poole, Dorset, England), unless otherwise specified.

Reagents	Supplier
Bicinchoninic Acid Assay Kit Coomassie Protein Assay Kit Streptavidin Magna-Bind Beads Blue Ranger pre-stained molecular weight markers EZ-link NHS-LC-Biotin	Pierce Biotechnology Inc., Rockford, IL 61105, USA.
Tryptone Agar Technical Yeast Extract PBS tablets	Oxoid, Basingstoke, Hampshire, RG24 8PW, England.
Acetic acid Hydrochloric acid	Riedel de-Haen AG, Wunstorfer, Strasse 40, D-30926, Hannover, Germany (now Sigma-Aldrich, UK).
Amphetamine-BSA THC-BSA THC-BTG	Fitzgerald Industries International Inc., Concord, MA 01742-3049, USA.

Reagents	Supplier
Chelating Sepharose Fast Flow Peroxidase-labelled anti-M13 antibody	Amersham Pharmacia Biotech, Filial Sverige, Björkgatan 30, SE-751 25, Uppsala, Sweden.
Wizard Plus mini-prep kit Reverse Transcription Kit	Promega Corp., 2800 Wood Hollow Rd., Madison, WI 53711-5399, USA.
Eppendorf Perfectprep Gel clean up kit	Unitech, 11155 Knott Ave., Suite F, Cypress, CA 90630, USA.
Trizol T4 DNA Ligase M13 K07 Helper Phage	Gibco BRL, Renfew Rd., Paisley, PA49RF, Scotland.
PCR Optimiser Kit	Invitrogen, 9704-CH-Groningen, Netherlands.
Restriction Enzymes (*Sfi 1*, *Nco 1*, *Not 1*)	New England Biolabs, Hitchin, Herts., SG4 0TY, England.
Amphetamine-HCl	Lennox Chemicals, Naas Rd., Dublin 12.
VCSM13 Helper Phage	Stratagene, 11011 N. Torry Pines Rd., La Jolla, CA 92037, USA.
PCR primers	MWG Biotech Ltd., Milton Keyes, MK12 5RD, UK.
Colloidal Carbon	ATO BV, Postbus 17, NL-6700, AA, Wageningen, The Netherlands.

2.1.2 Equipment

Equipment	Supplier
BIAcore 3000™	Pharmacia Biosensor AB, Rapsgatan 7 SE-754 50, Uppsala, Sweden.
Heraeus Christ Labofuge 6000 Biofuge A Microfuge	Heraeus Instruments Inc., 111-a Corporate Boulevard, South Plainfield, New Jersey, USA.
Titertek Multiscan Plate Reader Titertek Twin Reader Plus SB 2 Blood Tube Rotator	Medical Supply Company, Damastown, Mulhuddart, Dublin 15.
3015 pH Meter	Jenway Ltd., Dunmow, Essex, UK.
UV 160A Spectrometer	Shimadzu Corp., Kyoto, Japan.
Atto dual minislab system AE-6450 Atto AE-6100 Atto mini trans blot cell	Atto, Bunyho-Kui, Tokyo 113, Japan.
Orbital Incubator	Gallenkamp, Monarch Way, Belton Park, Loughborough, Leicestershire, LE11 5XG, UK.
RM6 Lauda Waterbath T-Gradient BIOMETRA-PCR Millipore Filtration Apparatus	AGB Scientific Ltd., Dublin Industrial Estate, Glasnevin, Dublin 9.

Equipment	Supplier
UVP ImageStore 7500 gel documentation system	Ultra Violet Products, Upland, CA, USA.
Eppendorf centrifuge 5810R	Unitech, 11155 Knott Ave., Suite F, Cypress, CA 90630, USA.
CAMAG Linomat 5	CAMAG, Wilmington, US 28401, USA.
Stuart Platform Shaker STR6	Lennox Chemicals, Naas Rd., Dublin 12.

2.1.3 Consumables

Consumable	Supplier
BIAcore sensor chips	Pharmacia Biosensor AB, Rapsgatan 7 SE-754 50, Uppsala, Sweden.
General plastic consumables eg: eppendorfs, pipette tips etc.	Starstedt, Drinagh, Co. Wexford.
Maxisorb 96 well plates Maxisorb Immunotube Nunc Bio-Assay dish	Nunc, Kamstrup DK, Roskilde, Denmark

Consumable	Supplier
Prototype saliva collection device	Trinity Biotech, Southern Cross Business Park, Bray, Co. Wicklow.
Purabind A-FP Nitrocellulose	Whatman International Ltd., 20/20 Maidstone, Kent, ME16 0LS, UK

2.1.4 Antibodies

2.1.4.1 Morphine-3-glucuronide (M3G) scFv library

A pre-immunised antibody variable domain phage display library was obtained from Dr. Paul Dillon, Dublin City University (Dillon, 2001 and Dillon[B] *et al.*, 2003).

2.1.4.2 Amphetamine monoclonal antibody

A monoclonal antibody, clone 4EP18E, directed against amphetamine, was obtained from Dr. Lorna Fanning, Dublin City University (Fanning, 2002).

2.1.4.3 Naïve human antibody libraries

A natural naïve human scFv library was supplied by Cambridge Antibody Technology, The Milstein Building, Granta Park, Cambridge, CB1 6GH, UK.
The Griffin.1 library was kindly donated to the University of Aberdeen by MRC Geneservice, Medical Research Council, Hill Rd, Cambridge, CB2 2QH, UK.

2.1.5 General buffer formulations

2.1.5.1 Phosphate Buffered Saline (PBS)

Phosphate Buffered Saline containing 0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, pH 7.4, was prepared in distilled water. This buffer shall be referred to throughout as PBS.

2.1.5.2 Phosphate Bufferd Saline/Tween (PBST)

PBS, containing 0.05% (v/v) Tween 20 surfactant. This buffer shall be referred to throughout as PBST.

2.1.5.3 Marvel™ Phosphate Buffered Saline (MPBS)

PBS, containing non-fat milk powder (Marvel™). This was made to the required % (w/v) milk powder and shall be referred throughout as MPBS

2.1.5.4 Tris Buffered Saline (TBS)

Tris Buffered Saline (TBS) containing 0.05 M Tris, 0.15 M NaCl, pH 7.4, was prepared in distilled water.

2.1.5.5 Hepes Buffered Saline (HBS)

Hepes Buffered Saline (HBS) containing 10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA and 0.05% (v/v) Tween 20 was prepared in ultra pure water and the pH adjusted to pH 7.4 with NaOH. This was filtered (pore size 0.22 μm) and degassed using filtration apparatus (Millipore sintered glass filtration unit) immediately before use.

2.1.5.6 Tris–Acetate–EDTA buffer (TAE)

All agarose gels were run in 1 X TAE Buffer, containing 0.04 M Tris Acetate, pH 8.3, 0.01 M EDTA in ultra pure water.

2.1.5.7 Citric phosphate buffer

Citric Phosphate Buffer, containing 0.05 M sodium dihydrogen phosphate and 0.05 M citrate acid monohydrate, was prepared in ultra pure water and the pH adjusted to pH 5 with 5 N NaOH. Immediately before use 2 µl of 30% (v/v) hydrogen peroxide was added per 10 ml of buffer. A 10 ml volume of buffer was used to dissolve 1 TMB tablet (3,3′,5,5′-Tetramethylbenzidine hydrocloride) for use as a substrate in ELISA.

2.1.5.8 Tris-Sucrose fractionation buffer

Tris-sucrose fractionation buffer was prepared by dissolving 200 mM Tris-HCl, pH 7.5, 20% (w/v) sucrose and 1 mM EDTA in ultra pure water. Immediately before use 5% (w/v) lysozyme was added. The solution kept on ice thereafter.

2.1.5.9 20% (w/v) Sodium azide stock solution

Due to the toxicity of sodium azide, extreme care was taken to minimise inhalation or ingestion during handling. A face mask was worn when weighing out powder and gloves were used while handling any solutions of azide. A 20% (w/v) stock solution of sodium azide was prepared by dissolving 2 g of sodium azide in 10 ml of distilled water.

2.1.5.10 30% (w/v) Acrylamide stock solution

As acrylamide is a potent neurotoxin, a breathing apparatus was worn when in contact with powder and gloves were worn at all times during handling. A 30% (w/v) acrylamide stock solution was prepared by dissolving 29.2 g of acrylamide and 0.8 g of bis-acrylamide together in 100 mls of water. This stock solution was stored in the dark at 4°C.

2.1.5.11 2 mg/ml Ethidium bromide stock

Ethidium bromide is a DNA intercalating agent that causes frameshift mutations. It was therefore always handled with gloves. Extreme care was taken not to contaminate other laboratory areas, fixtures or equipment. All contaminated solid waste was incinerated prior to disposal. All liquid waste was decontaminated by activated charcoal filtration prior to disposal. One ethidium bromide tablet was dissolved in 10 mls of molecular grade water to yield a 20 mg/ml stock. This was further diluted 1 in 10 in molecular grade water to reduce the toxicity of the working stock.

2.1.6 Culture media formulations

Culture Media	Formulation	
2 x Tryptone and Yeast Extract (2 x TY) Medium	Tryptone	16 g/L
	Yeast Extract	10 g/L
	NaCl	5 g/l
Non–Expression Medium (NE)	Tryptone	16 g/L
	Yeast Extract	10 g/L
	NaCl	5 g/L
	Glucose	1% (w/v)
	Chloramphenicol	25 µg/ml
Low Expression Medium (LE)	Tryptone	16 g/L
	Yeast Extract	10 g/L
	NaCl	5 g/L
	Glucose	1% (w/v)
	Chloramphenicol	25 µg/ml
	IPTG	5 mM
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

Culture Media	Formulation	
Terrific Broth (TB)	Tryptone	12 g/L
	Yeast Extract	24 g/L
	Glycerol	4 ml/L
	KH_2PO_4	17 mM
	K_2HPO_4	72 mM
Luria-Bertani (LB) broth	Tryptone	10g/L
	Yeast Extract	5 g/L
	NaCl	10 g/L
Tryptone Yeast Extract (TYE) broth	Tryptone	8 g/L
	Yeast Extract	5 g/L
	NaCl	5 g/L
Luria-Bertani (LB) agar	Tryptone	10g/L
	Yeast Extract	5 g/L
	NaCl	10 g/L
	Agar	15 g/L
2 x TY agar	Tryptone	16 g/L
	Yeast Extract	10 g/L
	NaCl	5 g/L
	Agar	15 g/L
TYE agar	Tryptone	8 g/L
	Yeast Extract	5 g/L
	NaCl	5 g/L
	Agar	15 g/L

2.1.7 Bacterial strains used

Bacterial Strain		Genotype
E. coli XL-1 Blue	Stratagene	*recA1, endA1, gyrA96, thi-1, hsdR17 (r_K^-,m_K^+), supE44, relA1, λ^-, lac$^-$, [F' proAB, lacI^{q}ZΔM15, Tn10(Tet^r)].*
E. coli HB2151	Donated by University of Aberdeen	*K12 Δ (lac-pro), ara, nalr, thi/F', proAB, laqIq, lacZΔ-M15*
E. coli JM83	Donated by Prep Lab, DCU	*λ^-, araΔ (pro-lac,) rpsL, thi, Φ80 dlacZΔM15 λ^-,*
E. coli TG1	Stratagene	*K12Δ (lac-pro), supE, thi, hsdΔ5/F', [traD36, proAB, lacI^q, lacZΔM15]*

2.1.7.1 Maintenance of bacterial stocks

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

2.1.7.2 Amplification of Helper Phage

A single colony of XL-1 Blue was used to inoculate 500 ml 2 x TY, 30 μg/ml tetracycline. This was incubated at 37°C with shaking at 250 rpm until $O.D._{600nm}$=0.5-0.6. Helper phage were added at a multiciplicity of infection of 10:1

(phage to cells). The culture was incubated at 37°C stationary for 30 mins, followed by 30 mins with shaking at 150 rpm. The culture was centrifuged at 4,000 rpm for 20 mins. The cell pellet was resuspended in 500 ml of 2 x TY, 30 μg/ml tetracycline, 50 μg/ml kanamycin and incubated at 26°C overnight, with shaking at 200 rpm. The culture was spun at 6,000 rpm and the supernatant filtered through a 0.45 micrometre filter to remove any cell debris. The phage supernatant was titred as per section 2.5.3 and stored at 4°C short term or peg precipitated (section 2.5.2) and stored at –80°C.

2.2 Hapten-Protein Conjugate production

2.2.1 Production of drug-protein conjugates

2.2.1.1 Conjugation of morphine–3–glucuronide to protein

Morphine–3–glucuronide (M3G) was coupled to either BSA (bovine serum albumin) or OVA (ovalbumin) using standard carbodiimide coupling chemistry. Briefly, a 50 mg/ml solution of M3G was dissolved in 1 ml of a 20 mM HCl solution and made up to a volume of 5 ml with 0.2 M borate buffer, pH 9.0. NHS (N–hydroxysuccinimide) and EDC (N-ethyl–N-(dimethyl–aminopropyl) carbodiimide hydrochloride) were added to yield final concentrations of 0.1 M and 0.4 M, respectively. The solution was incubated at room temperature for 10 mins without agitation. Carrier protein (OVA or BSA), at a molar ratio of 1:100 to M3G, was prepared in 0.2 M borate buffer, pH 9.0, and added dropwise to the activated solution with stirring. The solution was incubated at room temperature under gentle rotation for 2 hours and dialysed against 100 volumes of PBS overnight at 4°C.

2.2.1.2 Conjugate concentration determination by Bicinchoninic Acid Assay (BCA)

BCA reagent was prepared by adding 1 part reagent B (4% (w/v) cupric sulphate) to 50 parts reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartate in 0.01 M sodium hydroxide). A 10 µl aliquot of conjugate dilution or protein standard was added to microtitre wells. The prepared BCA reagent was then added to the protein containing wells, at a volume of 200 µl per well. The plate was incubated in the dark, at room temperature for 30 mins. Absorbance was read at 562nm on a titertek twinreader plus.

2.2.1.3.Production of biotinylated-amphetamine

Due to the small molecular weight of amphetamine it proved difficult to separate from biotinylated amphetamine through normal means e.g. dialysis. For this reason it was decided to use a 10-fold excess of amphetamine in the biotinylation reaction. The biotinylated-amphetamine could then be pre-immobilised onto streptavidin-coated beads and free amphetamine washed away before use. In this manner it was possible to ensure all amphetamine used in the selection was biotin-conjugated. In order to give a 10-fold molar excess of amphetamine, 4 mg of amphetamine was reacted with 1 mg of EZ-Link NHS-biotin in dimethylformamide at room temperature for 2 hours, with stirring. The conjugate was then stored at 4°C prior to use.

2.2.1.4 Commercial conjugates

Amphetamine–bovine serum albumin (AMP–BSA) was obtained from Fitzgerald Industries International, MA, USA. The amphetamine was conjugated through the methylenedioxy group situated at the para–position of the phenyl ring.

Tetrahydrocannabinol–bovine thryoglobulin (THC–BTG) and Tetrahydrocannabinol–bovine serum albumin (THC–BSA) were obtained from Fitzgerald Industries International, MA, USA. THC-BTG was conjugated through the delta–9 position and THC-BSA was linked via the carboxy carbon at the delta-9 position.

2.3 Immunisation for Recombinant Antibody Production

2.3.1 Licensing for drug and animal use

The Department of Health and Children licensed the possession and use of heroin and its metabolites, morphine-3-glucuronide and morphine for analytical purposes. Licenses were also required for the possession of amphetamine and tetrahydrocannabinol. With the exception of amphetamine (Lennox), all of these drugs were obtained from Sigma-Aldrich (UK) and required a license for importation into the Republic of Ireland. All drugs were stored in a locked refrigerator in a controlled laboratory environment.

The Department of Health and Children also licensed all processes involving the use of live animals. Extreme care was taken to minimise stress to the animals involved. Experiments were carried out in accordance with the European Directive 86/609/EEC.

2.3.2 Immunisation schedule for the production of a recombinant library

Day 1:

6 week old Balb/c female mice were immunised by sub–cutaneous injection with an emulsion (250 µl) consisting of a 1 mg/ml solution of hapten–protein conjugate (THC–BSA) mixed 1:1 with Freund's Complete Adjuvant.

Day 21:

Mice were re-immunised intraperitoneally with an emulsion (250 µl) consisting of a 1 mg/ml solution of hapten–protein conjugate mixed 1:1 with Freund's Incomplete Adjuvant.

Day 28:

A blood sample was collected (by the minimally invasive tail bleed method) and the antibody titre determined against the respective antigen.

Day 56:

Mice were boosted intraperitoneally with Freund's Incomplete Adjuvant.

Animals were re–immunised at fortnightly intervals until an antibody titre in excess of 1 in 100,000 was obtained. One week after the last immunisation, the animal was sacrificed and the spleen removed.

2.3.2.1 Preparation of serum for estimation of antibody titre

For titre estimation the blood collected was allowed to clot at 4°C overnight. It was then centrifuged at 13,000 rpm for 15 minutes and the supernatant removed for antibody titre determination by direct ELISA.

2.3.2.2 Direct ELISA for antibody titre

A 10 μg/ml solution of THC-BSA was coated onto microtitre plates, at a volume of 100 μl per well and incubated overnight at 4°C. Plates were washed three times with PBST and three times with PBS. The plates were then blocked with 4% (w/v) MPBS for 1 hour at 37°C. Serial dilutions of serum in PBS, containing 1% (w/v) BSA, were added to the wells and allowed to bind at 37°C for 1 hour. This was followed by the addition of a 1 in 5000 dilution in PBS of HRP-labelled goat anti-mouse antibody and the plate was incubated for 1 hour at 37°C. Plates were again washed and chromogenic substrate (0.4 mg/ml *o*-phenylenediamine (o-PD), in 0.05 M phosphate citrate buffer, pH 5.0, and 0.4 mg/ml of urea hydrogen peroxidase) was added at a volume of 100 μl/well and incubated for 30 minutes at 37°C. Absorbance was read at 450nm on a Titertek twinreader plus.

2.4 Production of a Recombinant Antibody Library

2.4.1 RNA extraction

Total RNA, the genetic material encoding the antibodies of interest, was removed from the spleen of an immunised mouse or from a hybridoma cell line secreting a monoclonal antibody raised against the target antigen, using Trizol reagent. RNA was extracted from an anti–amphetamine secreting clone, 4EP18E, (Fanning, 2002) and used for cDNA synthesis. For the generation of an immunised recombinant antibody library, the spleen from a mouse immunised with THC–BSA was removed aseptically, weighed and homogenised in 1 ml of Trizol reagent per 50–100 mg tissue. The homogenate was centrifuged 14,000 rpm for 10 minutes at 4°C to remove insoluble material such as extracellular membranes, polysaccharides and high molecular weight DNA. The supernatant was removed and left at room temperature for 5 minutes before being supplemented with 0.2 ml of chloroform per ml of Trizol reagent used. Samples were shaken vigorously for 15 seconds and stored at room temperature for 15 minutes. The mixture was centrifuged at 14,000 rpm for 15 minutes at 4°C. Following centrifugation, three layers were observed, a lower red phenol/chloroform phase, a protein interphase and a colourless upper aqueous phase. The upper aqueous layer, containing RNA, was transferred to a clean tube and supplemented with 0.5 ml of isopropanol per ml Trizol reagent. The sample was incubated at room temperature for 10 minutes and centrifuged at 14,000 rpm for 8 minutes at 4°C. The supernatant was removed and the pellet washed with 1ml of 75% (v/v) ethanol. The RNA was then centrifuged at 10,000 rpm for 5 minutes at 4°C, the ethanol decanted and the pellet air–dried at room temperature for 5 minutes. The pellet was re-dissolved in 250 µl of 'nuclease-free' water.

2.4.2 Complementary DNA synthesis

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

Component	**Stock Concentration**	**Volume used**
$MgCl_2$	25 mM	4 µl
10 X reverse transcription buffer	10 X	2 µl
dNTPs	10 mM	2 µl
RNasin® ribonuclease inhibitor	40 U/µl	0.5 µl
Random Primers	0.5 µg/µl	0.4 µl
AMV RT	25 U/µl	0.6 µl

Approximately 7-10 µg of extracted RNA was added to the reaction mix and 'nuclease-free' water was used to bring the final volume up to 20 µl. The mixture was incubated at room temperature for 10 minutes to allow annealing of primers. The reaction was then incubated at 42°C for 1 hour and DNA analysed by agarose gel electrophoresis.

2.4.3 Agarose gel electrophoresis

DNA was analysed by electrophoresis on an agarose gel. Briefly, agarose was dissolved to the appropriate concentration (typically 0.7 – 1.2% (w/v)) in 1 X TAE buffer and boiled until the solution was clear. When cool, 0.5 µg/ml of ethidium bromide was added to the gel. This intercalating dye allowed the migration of DNA through the gel to be visualised. The gel was then cast on a horizontal gel apparatus (Atto AE–6100) and electrophoresed in 1 X TAE at 70V. Gels were visualised on a UV transluminator and photographed using a UV image analyser.

2.4.4 Amplification of antibody heavy and light chain genes

The primers listed below were obtained from MWG Biotech Ltd. and were used for assembling the mouse scFv fragment in the orientation V_L–$(Gly_4Ser)_4$–V_H, which is compatible with the pAK vector system as described by Krebber *et al.* (1997). The sequences are given using the standard IUPAC nomenclature of mixed bases where: R=A or G; Y=C or T; M=A or C; K= G or T; S=C or G; W=A or T; H=A or C or T; B=C or G or T; V=A or C or G; D=A or G or T. Primers, at a working concentration of 10 nM, were mixed according to the degree of degeneration. This gave a working stock of L_B and L_F, for variable light chain gene (V_L) amplification, and, H_B and H_F, for heavy chain amplification. For all non–degenerate primers (d=1), 1 µl was included in the mix; for d=2–4, 2 µl was included; for d=6–9, 3 µl was included and for d=12–16, 4 µl was included. For amplification of heavy and light chain genes derived from the anti–amphetamine monoclonal, the $L_F\lambda$ primer was omitted as the hybridoma clone secretes a kappa light chain. It has been shown that monoclonal antibodies of the kappa isotype may transcribe a non-functional lambda chain that competes with the kappa V_L gene for in–frame scFv antibody assembly (Krebber *et al., 1997*).

2.4.4.1 Primers for amplification of variable heavy and light chain genes

Variable light chain back primers		**Degeneracy**
L_B1	5′gccatggcggactacaaaGAYATCCAGCTGACTCAGCC3′	2
L_B2	5′gccatggcggactacaaaGAYATTGTTCTCWCCCAGTC3′	4
L_B3	5′gccatggcggactacaaaGAYATTGTGMTMACTCAGTC3′	8
L_B4	5′gccatggcggactacaaaGAYATTGTGYTRACACAGTC3′	8
L_B5	5′gccatggcggactacaaaGAYATTGTRATGACMCAGTC3′	8
L_B6	5′gccatggcggactacaaaGAYATTMAGATRAMCCAGTC3′	16
L_B7	5′gccatggcggactacaaaGAYATTCAGATGAYDCAGTC3′	12
L_B8	5′gccatggcggactacaaaGAYATYCAGATGACACAGAC3′	4
L_B9	5′gccatggcggactacaaaGAYATTGTTCTCAWCCAGTC3′	4
L_B10	5′gccatggcggactacaaaGAYATTGWGCTSACCCAATC3′	8
L_B11	5′gccatggcggactacaaaGAYATTSTRATGACCCARTC3′	16
L_B12	5′gccatggcggactacaaaGAYRTTKTGATGACCCARAC3′	16
L_B13	5′gccatggcggactacaaaGAYATTGTGATGACBCAGKC3′	12
L_B14	5′gccatggcggactacaaaGAYATTGTGATAACYCAGGA3′	4
L_B15	5′gccatggcggactacaaaGAYATTGTGATGACCCAGWT3′	4
L_B16	5′gccatggcggactacaaaGAYATTGTGATGACACAACC3′	2
L_B17	5′gccatggcggactacaaaGAYATTTTGCTGACTCAGTC3′	2
$L_B\lambda$	5′gccatggcggactacaaaGATGCTGTTGTGACTCAGGAATC3′	1

Variable light chain primers forward		**Degeneracy**
L_F1	5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTKATTTCCAGCTTGG3′	1
L_F4	5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTTATTTCCAACTTTG3′	1
L_F5	5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACGTTTCAGCTCCAGCTTGG3′	1
$L_F\lambda$	5′ggagccgccgccgcc(agaaccaccaccacc)$_2$ACCTAGGACAGTCAGTTTGG3′	1

Variable heavy chain back primers		Degeneracy
H_B1	5′ggcggcggcggctccggtggtggtggatccGAKGTRMAGCTTCAGGAGTC3′	8
H_B2	5′ggcggcggcggctccggtggtggtggatccGAGGTBCAGCTBCAGCAGTC3′	9
H_B3	5′ggcggcggcggctccggtggtggtggatccCAGGTGCAGCTGAAGSARTC3′	4
H_B4	5′ggcggcggcggctccggtggtggtggatccGAGGTCCARCTGCAACARTC3′	4
H_B5	5′ggcggcggcggctccggtggtggtggatccCAGGTYCAGCTBCAGCARTC3′	12
H_B6	5′ggcggcggcggctccggtggtggtggatccCAGGTYCARCTGCAGCARTC3′	4
H_B7	5′ggcggcggcggctccggtggtggtggatccCAGGTCCACGTGAAGCARTC3′	1
H_B8	5′ggcggcggcggctccggtggtggtggatccGAGGTGAASSTGGTGGARTC3′	4
H_B9	5′ggcggcggcggctccggtggtggtggatccGAVGTGAWGSTGGTGGAGTC3′	12
H_B10	5′ggcggcggcggctccggtggtggtggatccGAGGTGCAGSTGGTGGARTC3′	4
H_B11	5′ggcggcggcggctccggtggtggtggatccGAKGTGCAMCTGGTGGARTC3′	4
H_B12	5′ggcggcggcggctccggtggtggtggatccGAGGTGAAGCTGATGGARTC3′	2
H_B13	5′ggcggcggcggctccggtggtggtggatccGAGGTGCARCTTGTTGARTC3′	2
H_B14	5′ggcggcggcggctccggtggtggtggatccGARGTRAAGCTTCTCGARTC3′	4
H_B15	5′ggcggcggcggctccggtggtggtggatccGAAGTGAARSTTGAGGARTC3′	4
H_B16	5′ggcggcggcggctccggtggtggtggatccCAGGTTACTCTRAAASARTC3′	8
H_B17	5′ggcggcggcggctccggtggtggtggatccCAGGTCCAACTVCAGCARCC3′	6
H_B18	5′ggcggcggcggctccggtggtggtggatccGATGTGAACTTGGAASARTC3′	1
H_B19	5′ggcggcggcggctccggtggtggtggatccGAGGTGAAGGTCATCGARTC3′	1

Variable heavy chain forward primers		Degeneracy
H_F1	5′ggaattcggcccccgaggcCGAGGAAACGGTGACCGTGGT3′	1
H_F2	5′ggaattcggcccccgaggcCGAGGAGACTGTGAGAGTGGT3′	1
H_F3	5′ggaattcggcccccgaggcCGCAGAGACAGTGACCAGAGT3′	1
H_F4	5′ggaattcggcccccgaggcCGAGGAGACGGTGACTGAGGT3′	1

2.4.4.2 PCR amplification of variable heavy and light chain genes

Component	**Stock Concentration**	**Volume used**
dNTPs	10 mM	2 µl
5 X reverse PCR buffer	5 X	10 µl
Forward primer mix	0.1 mM	1 µl
Reverse primer mix	0.1 mM	1 µl
T*aq* polymerase	5 U/µl	2 µl

A 2 µl volume of cDNA was added to each reaction and 'nuclease-free' water was used to bring the final volume up to 50 µl. For amplification of light chain genes the final buffer composition (1 X) was optimised to contain 60 mM Tris-HCl, 15 mM ammonium sulphate and 2.5 mM $MgCl_2$, pH 8.5. A buffer composition of 60 mM Tris-HCl, 15 mM ammonium sulphate and 2.5 mM $MgCl_2$, pH 9.0, supplemented with 2.5% (v/v) DMSO, was found to be optimal for the amplification of heavy chain genes.

2.4.4.3 PCR conditions for amplification of antibody light and heavy chain genes

94°C x 5 min 'Hot Start' * *Addition of T*aq* polymerase
94°C x 1 min
63°C x 30 sec
58°C x 50 sec
72°C x 1 min Repeat X 7 cycles

94°C x 1 min
63°C x 30 sec
72°C x 1 min Repeat X 24 cycles

72°C x 10 min
All ramping rates were at 4°C / sec

2.4.4.4 Purification of PCR reaction products

Purification of PCR reaction products was performed using an Eppendorf Perfectprep Gel clean-up kit. The PCR products were separated by agarose gel electrophoresis. The appropriate bands were excised using a clean scalpel, transferred to a clean collection tube and weighed. Binding buffer was added to a volume of three times the weight of the agarose slice. The samples were inverted three times and incubated at 55°C for approx. 10 minutes, until the agarose had melted. Isopropanol was added at a volume equal to the weight of the agarose slice. The mixture was poured into a spin column and centrifuged for 2 minutes at 14,000 rpm. The flow through was discarded and the spin column washed with 750 µl of wash buffer. The column was centrifuged at 14,000 rpm for 1 min and the flow through discarded. The column was centrifuged as before to remove any residual wash buffer. The spin column was transferred to a clean tube, 30 µl of 'nuclease-free' water was added and the column centrifuged at 14,000 rpm for 1 minute to elute DNA. Purified PCR products were stored at – 20°C until required.

2.4.5 Splice by Overlap Extension PCR

Variable heavy and light chains were annealed and amplified using a Splice by Overlap Extension (SOE) to produce an 800 bp fragment.

2.4.5.1 SOE PCR primers

SOE Primers

Single chain forward (scfor) '5 ttactcgcggcccagccggccggccatggcggactaccccg 3'

Single chain back (scback) '5 ggaattcggcccccgag 3

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

Component	**Stock Concentration**	**Volume used**
dNTPs	10 mM	2 µl
5 X reverse PCR buffer	5 X	10 µl
scfor	1 nM	1 µl
scback	1 nM	1 µl
T*aq* polymerase	5 U/µl	2 µl

Approximately 10 ng of amplified V_H and V_L genes were used in the SOE PCR reaction and 'nuclease-free' water was used to bring the final reaction volume up to 50 µl. The final concentration of the buffer present in the reaction was optimised to contain 60 mM Tris-HCl, 15 mM ammonium sulphate and 1.5 mM $MgCl_2$, pH 9.

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

92°C X 1 min	
45°C X 50 min	
72°C X 1 min	Repeat X 5 cycles – Addition of scfor and scback primers and *Taq* polymerase
92°C x 1 min	
68°C x 30 sec	
72°C x 1 min	Repeat X 25 cycles

All ramping rates were at 4°C / sec.

2.4.6 Recombinant antibody library construction

2.4.6.1 Isolation of pAK 100 vector

A single colony of *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) containing the pAK 100 vector (all pAK vectors were kindly donated by Prof. A. Plückthun (Universität Zürich, Switzerland)) was grown overnight in 10 ml of LB media, supplemented with 30 μg/ml tetracycline, at 37°C with vigorous shaking. The plasmid was isolated and purified using a Wizard Plus Miniprep kit, according to manufacturer's instructions. Briefly, the culture was centrifuged at 4,000 rpm for 10 minutes, the supernatant discarded and the pellet resuspended in 250 μl of cell resuspension solution (50 mM Tris–HCl, pH 7.5, 10 mM EDTA, 100 μg/ml RNase A). The cell contents are released by the addition of 250 μl of cell lysis solution (0.2 M NaOH, 1% (w/v) SDS) and the mixture inverted four times. Once the lysate began to clear, 10 μl of alkaline protease was added to the mixture. The tube was inverted four times and incubated at room temperature for 5 minutes. The pH and salt concentration of the lysate was adjusted using 350 μl of neutralisation solution (1.32 M potassium acetate, pH 4.8), the sample was inverted four times and centrifuged at 14,000 rpm for 10 minutes. The supernatant was transferred to a spin column and re–centrifuged at 14,000 rpm for 1 minute. The flow through was discarded and the column was washed with 750 μl of column wash solution (80 mM potassium acetate, 8.3 mM Tris–HCl, pH 7.5, 40 μM EDTA 55% (v/v) ethanol). The column was centrifuged at 14,000 rpm for 1 minute and the flow through discarded. The column was washed as before with 250 μl of wash solution, transferred to a clean tube and 100 μl of 'nuclease-free' water was added. The plasmid was eluted from the column by centrifugation at 14,000 rpm for 1 minute and stored at – 20 °C.

2.4.6.2 Sfi 1 Digest of scFv insert and pAK 100 vector

The SOE product and the phage display vector pAK 100 were digested using *Sfi 1* in New England Biolabs, Buffer 2 (10 mM Tris-Cl, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT).

SOE Fragment Digestion

NEB Buffer 2	13 µl
100 X BSA solution (10 mg/ml)	1.3 µl
Sfi I	2 µl
'Nuclease-free' water	73.7 µl
SOE Product	40 µl

pAK 100 Plasmid Digestion

NEB Buffer 2	5 µl
100 X BSA solution (10 mg/ml)	0.5 µl
Sfi I	1 µl
'Nuclease-free' water	34 µl
Plasmid (50-100 ng)	10 µl

The restriction was incubated at 50°C overnight. The scFv fragment and vector minus 'stuffer' gene were purified and quantified by agarose gel electrophoresis prior to ligation.

2.4.6.3 Ligation of scFv gene into pAK 100

Purified digests of SOE product (20 ng) and pAK 100 vector were ligated at a ratio of 1.5:1 using T4 DNA ligase at 16°C overnight.

2.4.6.4 Production of electrocompetent E. coli XL – 1 Blues with high transformation efficiency

For initial library construction, electroporation was the chosen method for transformation to yield the highest transformation efficiency. For all other cloning and sub-cloning procedures heat shock competent cells were used as described in Sections 2.4.6.6 and 2.4.6.7. A 5 ml aliquot of 2 x TY, 30 μg/ml tetracycline, was inoculated with a single XL-1 Blue colony and incubated at 37°C overnight with shaking at 200 rpm. The overnight culture was used to inoculate a 500 ml volume of 2 x TY broth and this was incubated at 37°C with shaking until $OD_{600} = 0.6$. The cells were left on ice for 10 minutes and kept cold and sterile from this point. Cells were centrifuged at 4,000 rpm at 4°C for 20 mins. The supernatant was decanted and the pellet washed with 500 mls of ice-cold sterile water. The water was decanted from the tube and the pellet resuspended in 50 ml ice-cold sterile water. The mixture was incubated on ice for 10 mins and the cells pelleted by centrifugation at 4,000 rpm at 4°C for 20 mins. The pellet was resuspended in 50 mls of ice-cold 10% (v/v) DMSO and incubated on ice for 10 mins. Cells were pelleted as before, resuspended in 25 ml 10% (v/v) DMSO and incubated on ice for 10 mins. Cells were re-centrifuged and resuspended in 2.5 ml 10% (v/v) DMSO. Cells were aliquoted into 100 μl volumes, flash frozen in liquid nitrogen and stored at – 80°C until required.

2.4.6.5 Transformation of E. coli XL – 1 Blues with pAK 100 vector

The ligation mixture was desalted by ethanol precipitation prior to electroporation. Briefly, one-tenth the volume of 3 M sodium acetate, pH 5.2, and 3 volumes of ethanol were added to the ligation mixture and incubated at – 20°C for 2 hours. The

DNA was pelleted by centrifugation at 14,000 rpm, 4°C for 20 mins. The pellet was washed in 1 ml 70% (v/v) ethanol and centrifuged at 14,000 rpm, 4°C for 10 mins. The pellet was air-dried and resuspended in 5 µl of 'nuclease-free' water. The competent cells were thawed on ice and 100 µl was dispensed into a polypropylene tube. A 1 µl volume of the ligation reaction was added to the cells and the mixture immediately transferred to a pre-chilled 2 mm electroporation cuvette. The cells were pulsed at 2500V, which gave a time constant of $\geq$ 5.2 msec. Immediately after electroporation, 900 µl of SOC media was added to the cells and the culture incubated at 37°C for one hour with gentle shaking. Cells were plated out on NE agar plates and incubated at 37°C overnight. Transformants were also titred, using 2 x TY as a diluent, on NE plates. Plates were scraped using 2 x TY and library stocks containing 15% (v/v) glycerol were prepared. Library stocks were flash frozen in liquid nitrogen and stored at – 80°C.

2.4.6.6 Production of competent E. coli: Transformation and storage of bacterial cells in the same solution (TSS)

Heat shock competent *E. coli* (XL-1 Blue or JM83) were prepared as described by Chung *et al.* (1989). Briefly, 5 ml of LB media, containing appropriate antibiotic, was inoculated with a single bacterial colony and grown overnight at 37°C. A 1 ml volume of this overnight culture was used to seed 100 ml of LB media and this was incubated at 37°C, 220 rpm until $O.D._{600nm}$ = 0.5. Cells were pelleted by centrifugation in pre-chilled tubes at 4,000 rpm for 20 mins and resuspended in 10% (v/v) of their original volume in ice-cold transformation and storage solution (TSS, which consists of LB broth with 10% (w/v) PEG 3350, 5% (v/v) dimethylsulphoxide and 50 mM $MgCl_2$). Cells were aliquoted into 250 µl volumes, flash frozen in liquid nitrogen and stored at – 80°C, until required.

2.4.6.7 Transformation of TSS competent E. coli

E coli cells (XL-1 Blue or JM83) were defrosted on ice and 100 µl added to 5 µl of ice cold plasmid DNA. This was incubated on ice for 20 mins. Cells were heat shocked at 42°C for 90 seconds exactly before being placed back on ice for 2 mins. After this time 200 µl of LB broth was added to the transformed cells and the mixture was incubated at 37°C, with shaking at 200 rpm for 1 hour. The transformation was spread on TYE agar plates, containing 1% (w/v) glucose and appropriate antibiotic, and incubated at 37°C overnight.

2.5 Selection and Characterisation of Specific scFv Antibodies from an Immunised Murine Library

2.5.1 Rescue of scFv-displaying phage

A 50 ml volume of NE media was inoculated with 10^9 cells from the glycerol library stock. The culture was then incubated at 37°C, with shaking at 225-250 rpm until $O.D._{600nm}$ = 0.4-0.6 was reached. The culture was infected with 10^{11} cfu VCSM13 helper phage and 25 µl 1 M IPTG solution was added. The culture was incubated for 30 minutes at 37°C without agitation. The culture was then diluted in 100 mls of LE media in a 2 L baffle flask. The culture was shaken at 200 rpm for 2 hours at 26°C for phage production, after which time 30 µg/ml kanamycin was added. Phage production was allowed to proceed at 26°C, with vigorous aeration overnight.

2.5.2 Concentration of phage by PEG/ NaCl precipitation

The library (150 ml) was transferred to a sterile sorvall tube and centrifuged at 10,000 rpm, 4°C for 10 minutes. One-fifth the volume (30 mls) of PEG/NaCl (20% (w/v) polyethylene glycol 10,000, 0.25 M NaCl prepared in ultra pure water) was added to the supernatant, mixed and incubated on ice for at least 1 hour. The mixture was centrifuged at 10,000 rpm, for 30 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 40 mls ultra pure water plus 8 mls PEG/NaCl. This was incubated on ice for 20 minutes and centrifuged at 10,000 rpm, for 20 minutes at 4°C. The supernatant was removed and the pellet was re-centrifuged at 7,000 rpm, for 10 minutes at 4°C. Any remaining supernatant was removed and the pellet was resuspended in 2 ml sterile filtered PBS. Phage were stored at 4°C (short-term) / - 80°C (long- term) in 15% (v/v) glycerol.

2.5.3 Phage titre

A 4 ml aliquot of 2 x TY, 30 μg/ml tetracycline was inoculated with a single colony of XL-1 blue and incubated overnight at 37°C with shaking at 225-250 rpm. This culture was used to seed 5 ml of 2 x TY, 30 μg/ml tetracycline at a 1% (v/v) cell density. The culture was incubated at 37°C with shaking at 225-250 rpm until $O.D._{600nm}$ = 0.4-0.6. Serial dilutions of phage were prepared in the bacterial culture (10^{-1}-10^{-11}). The dilutions were allowed to sit at 37°C for 30 minutes without agitation and 100 μl of each dilution was spread on NE agar plates. All plates were incubated overnight at 37°C.

2.5.4 Selection of antigen binders by panning

2.5.4.1 Selection of antigen binders using acidic elution

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

2.5.4.2 Selection of antigen binders using bacterial elution

Phage were subject to selection as per Section 2.5.4.1 except bound phage were eluted using 4 ml of exponentially growing XL-1 Blue (O.D. $_{600nm}$ = 0.5) and incubated for 30 mins at 37°C. Any remaining phage were eluted using 1 ml 100 mM triethylamine, pH 12, under gentle rotation, for ten minutes. The solution was immediately neutralised with 0.5 ml 1 M Tris, pH 7.4. The phage were then re-infected and titred as before.

2.5.5 Re-infection of E. coli XL–1 Blue cells with eluted phage

A 5 ml volume of 2 x TY, 30 µg/ml tetracycline was inoculated with a single colony of XL-1 Blue and grown overnight with shaking (200-250 rpm) at 37°C. This was used to inoculate 5 ml of 2 x TY, 30 µg/ml tetracycline at 1% (v/v) cell density. The culture was grown with shaking at 37°C until O.D. $_{600nm}$ = 0.4-0.6. Cells were then incubated at 37°C without agitation for 10 minutes. A 700 µl aliquot of phage rescued from the panning step was added to the culture and allowed to stand at 37°C for 30 minutes without agitation. The culture was then centrifuged at 4,000 rpm for 2 minutes and resuspended in 600 µl of 2 x TY. This was plated out on three NE agar plates and incubated overnight at 37°C. The following day infected cells were scraped from the solid media using 2 x TY, concentrated by centrifugation at 4,000 rpm for 2 minutes and resuspended in 2 ml 2 x TY, 15% (v/v) glycerol. Stocks were aliquoted into 500 µl volumes, flash frozen and stored at – 80°C

2.5.6 Master plate construction

A 96 well sterile culture plate was filled with 200 µl per well of 2 x TY, 1% (w/v) glucose, 25 µg/ml chloramphenicol and 30 µg/ml tetracycline. Each well was inoculated with a single colony from the phage titre plates, leaving one or two wells blank as non-inoculated controls. The plate was incubated at 37°C with shaking at 150 rpm overnight. This was known as the master plate and frozen at - 20°C with

15% (v/v) glycerol. Another plate was prepared with 180 µl /well 2 x TY containing 1% (w/v) glucose, 25 µg/ml chloramphenicol. This plate was inoculated with 20 µl from each corresponding well on the master plate and incubated at 37°C with shaking at 150 rpm for approx 5 hours. After 5 hours, 25 µl 2 x TY, 1% (w/v) glucose, 25 µg/ml chloramphenicol, 9.5 mM IPTG and 5 x 10^9 VCS M13 helper phage/ml was added to each well. The culture was left to stand for 30 minutes at 37°C followed by shaking at 26°C, 150 rpm for 2 hours. The plate was centrifuged at 4,000 rpm for 10 minutes. The supernatant was removed by inversion and the pellets resuspended in 200 µl of 2 x TY, 1% (w/v) glucose, 25 µg/ml chloramphenicol, 1 mM IPTG and 30 µg/ml kanamycin. The plate was incubated overnight at 26°C, shaking at 150 rpm. The plate was centrifuged at 4,000 rpm for 10 minutes and 75 µl of the supernatant used for analysis in phage ELISA.

2.5.7 Phage ELISA

A microtitre plate was coated with 100 µl of between 1 and 10 µg/ml of appropriate conjugate and incubated overnight at 4°C. The plate was washed three times with PBST and three times with PBS. The plate was then blocked with 150 µl 4% (w/v) milk marvel™ in PBS for 1 hour at 37°C. A 75 µl aliquot of supernatant from the working phage plate and 25 µl of 4% (w/v) MPBS was added to the corresponding well in each ELISA plate, mixed gently and incubated for 2 hours at 37°C. The plates were washed as before and 100 µl of anti-fd bacteriophage antibody at a 1 in 1000 dilution, in 1% (w/v) MPBS, was added to each well. This was incubated for 1 hour at 37°C. The plate was washed as before and 100 µl of peroxidase-labelled anti-rabbit antibody [at a 1 in 5000 dilution in 1% (w/v) MPBS] was added and incubated for 1 hour at 37°C. The plate was washed and 100 µl of *o*–phenylenediamine (*o*-PD) substrate was added and incubated for 30 minutes at 37°C. The substrate reaction was stopped by addition of 25 µl of 2 M H_2SO_4 per well and absorbance read at 492nm. These steps are repeated until positive clones were identified.

2.5.8 Preparation of positive clones for ELISA

A 5 ml volume of NE media was inoculated with a single colony of any positive clones identified from the master plate and this was incubated at 37°C with 250 rpm shaking overnight. This was used to inoculate 5 ml of NE media at a 1% (v/v) cell density. The culture was allowed to grow at 37°C with shaking (250 rpm) until $O.D._{550nm}$ had reached 0.5 A.U.. Cells were infected with 5 x 10^9 VCS M13 helper phage and 0.5 mM IPTG were added and the culture was incubated at 26°C (150 rpm) for two hours. After this time, kanamycin was added to a final concentration of 30 μg/ml, and phage production was allowed to continue overnight. The culture was centrifuged at 4,000 rpm for 10 minutes and the supernatant removed for analysis by competitive phage ELISA. This was performed as per Section 2.5.7 except 50 μl of free analyte standard was mixed with 50 μl of phage before addition to the blocked wells of the immunoplate.

2.5.9 Production of soluble scFv

2.5.9.1 Isolation of scFv gene

Once positive clones were identified, the pAK 100 plasmid was isolated using a Wizard Miniprep kit as per Section 2.4.6.1. The scFv gene insert was isolated by *Sfi* I restriction as per Section 2.4.6.2 and ligated into a previously restricted expression vector using T4 DNA ligase, at a vector to insert molar ratio of 1.5:1. Expression plasmids used included: pAK 400, for large-scale soluble scFv expression; pAK 500, for soluble dimeric scFv expression and pAK 600 for expression of soluble dimeric alkaline phosphatase–labelled scFv. Plasmids were transformed into a non-suppressor *E. coli* strain (e.g. JM83) (prepared using TSS method, as per section 2.4.6.6). Chloramphenicol resistance was used to select for recombinant bacteria.

2.5.9.2 Soluble expression of scFv Fragments

For periplasmic expression of scFv antibody fragments; 500 ml of 2 x TY media, supplemented with 25 μg/ml chloramphenicol and 1% (w/v) glucose, was inoculated with an overnight culture of *E. coli* JM83, harbouring the expression plasmid pAK 400, 500 or 600 (containing the scFv insert), at a 1% (v/v) cell density. This was incubated with vigorous aeration at 37°C. Expression was induced with 1 mM isopropylthiogalactopyranoside (IPTG) when $O.D._{600nm}$ reached 0.5–0.6. Expression was allowed to continue for 4 hours at 26°C with vigorous aeration (6 hours for pAK 500 and 600 expression). The cells were collected by centrifugation and resuspended in 5% (v/v) of the initial culture volume TES (200 mM Tris-HCl, pH 8, 0.5 M sucrose, 0.5 mM EDTA). The mixture was incubated for 1 hour on ice and centrifuged at 4,000 g for 20 minutes to remove cell debris. The supernatant was dialysed against 50 volumes PBS, 0.02% (w/v) sodium azide, overnight at 4°C.

2.5.10 Purification of soluble scFv

Monomeric scFv could be purified by immunoaffinity chromatography using M3G-BSA-Sepharose Affinity Column.

2.5.10.1 Preparation of immunoaffinity matrix

Cyanogen bromide-activated sepharose (1.2g) was swollen with 200 ml of 1 mM HCl for 20 minutes and sucked dry under vacuum (Millipore sintered glass filtration unit with 0.22 μm pore size filter). Hapten–carrier conjugate was dissolved to concentration of 5 mg/ml in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and added dropwise to the gel with stirring. This was incubated overnight with stirring, washed with coupling buffer and incubated with 10 mls of 1 M ethanolamine, pH 8, for 2 hours under gentle rotation to block any unreacted sites. The gel was washed six times alternating between 0.1 M acetate buffer, 0.5 M NaCl, pH 4, and

coupling buffer (20 mls each). The gel was then washed with PBS, 0.02% (w/v) azide and stored in this solution at 4°C.

2.5.10.2 Purification using M3G-BSA-Sepharose affinity column

The affinity column was equilibrated with PBS and 2 column volumes of pAK 400 soluble periplasmic extract were loaded onto the gel. Contaminants were removed by washing with 4 column volumes of PBST and 4 column volumes of PBS. Bound antibody fragments were eluted using 0.1 M glycine/HCl, pH 2.2. Fractions were immediately neutralised with 200 µl 2 M Tris, pH 8.6, pooled and dialysed against 50 volumes PBS, overnight at 4°C.

2.5.11 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed under reducing conditions according to Laemmli (1970) to assess antibody purity. This technique enables protein separation on the basis of size. This is achieved through two factors: free radical polymerisation of acrylamide, and a reducing environment created by SDS. The polymerised acrylamide gives rise to the cross-linked gel of defined pore-size, while SDS disrupts secondary, tertiary and quartenary protein structure, wrapping the protein in a uniform negative charge. This masks the native charge of the protein, resulting in a constant charge/mass ratio and uniform shape, allowing electrophoretic mobility to be influenced almost entirely by size. Ammonium persulphate is the used to initiate free radial formation. N,N,N′,N′-tetramethylethylenediamine (TEMED) is included as a catalyst to speed up the reaction. The free radicals formed, react with acrylamide to form long acrylamide polymer chains, which are viscous but do not form gels. N, N'-methylene-bis-acrylamide causes cross-linking of the polymers, producing a large network of acrylamide chains. The pore size of the gel depends on amount of acrylamide and degree of crosslinking. The compositions of gels, electrophoresis buffer and sample buffer are shown in Table 2.1. Samples and protein markers were prepared in sample

loading buffer (4:1, sample:buffer) and boiled for 5 minutes. Samples were run at 50 mA per gel on an Atto AE-6450 minigel gel until samples had reached the base of the stacking gel, and then at 20 mA per gel until the dye had reached the base of the resolving gel. Coomassie blue was used to stain the gel or the gel was used for western blotting.

Table: 2.1 *Composition of stacking gel, resolving gel, electrophoresis buffer and sample loading buffer for SDS-PAGE.*

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

2.5.11.1 Coomassie blue staining for SDS-PAGE gel

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

2.5.11.2 Western blotting of scFv antibodies

Alternatively, proteins were transferred to a nitrocellulose membrane by electrophoresis using a Biorad wet blotter at 70V for 1 hour. The membrane was blocked using 4% (w/v) non-fat milk powder in Tris Buffered Saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4) overnight at 4°C, washed five times with TBST and five times with TBS. Bound antibody was detected using monoclonal anti-flag M1 antibody (at a 1 in 1000 antibody dilution) in TBS, with 1% (w/v) non-fat milk powder and 1 mM $CaCl_2$. The nitrocellulose was then probed with an alkaline phosphatase-labelled anti-mouse IgG antibody (1 in 2000 dilution) in TBS, containing 1% (w/v) non-fat milk powder. Antibody bands were visualised using 5–bromo–4–chloro–3–indolyphosphate/nitro blue tetrazolium chloride (BCIP/NBT) substrate. The reaction was allowed to proceed until bands could be clearly visualised and the reaction was then stopped by the addition of 2 mM EDTA.

2.5.12 ELISA analysis of scFvs

2.5.12.1 Immunoblot analysis

A nitrocellulose membrane was sprayed with 10 µl of M3G-OVA conjugate using the Linomat 5 and allowed to dry. The membrane was cut into strips and each strip was blocked with 4% (w/v) non - fat milk powder in PBS. Following blocking each strip was washed three times with PBS. Decreasing concentrations of M3G were added to each nitrocellulose strip with 10 µl of alkaline phosphatase-labelled scFv in 1% (w/v) MPBS. This was incubated for 2 hours at room temperature. The membrane was washed as before and bound phosphatase activity was visualised using BCIP/NBT chromogenic substrate.

2.5.12.2 Standard Enzyme-Linked Immunosorbent Assay (ELISA)

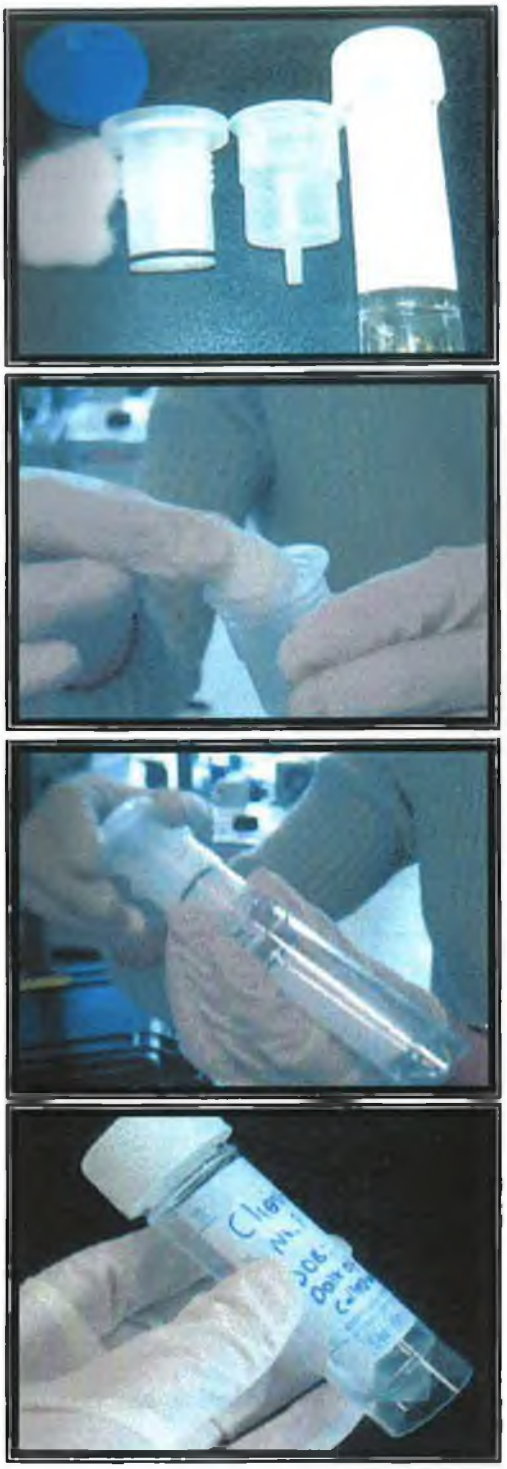
Nunc maxisorb Microtitre plates were coated with 100 µl of appropriate concentration of antigen conjugate diluted in PBS at 4°C overnight. Plates were blocked with 150 µl/well 4% (w/v) MPBS for 1 hour at 37°C. Wash steps were carried out 3 times with PBST and three times with PBS. All antibody dilutions were made in sterile filtered PBS, 1% (w/v) non-fat milk powder at a volume of 100 µl/well and incubations were performed at 37°C for 1 hour. Decreasing concentrations of antibody were added to each well, followed by 100 µl per well of monoclonal anti-flag M1 antibody, at a 1 in 400 dilution. This was detected using 100 µl per well of peroxidase–labelled anti-mouse IgG antibody, at a 1 in 2000 dilution. Bound antibody was detected using *o*–phenylenediamine (*o*-PD). Absorbances were read after 30 minutes at 450nm.

2.5.12.3 Indirect ELISA for determination of optimal antibody dilution and optimal coating concentration of protein conjugates

Ranges of drug protein conjugates were coated onto microtitre plates and incubated overnight at 4°C. Plates were washed three times with PBST and three times with PBS. The plates were then blocked with 150 µl of 4% (w/v) MPBS solution for 1 hour at 37°C. Wash steps were carried out 3 times with PBST and three times with PBS. All antibody dilutions were made in sterile filtered PBS, 1% (w/v) MPBS at a volume of 100 µl/well and incubations were performed at 37°C for 1 hour. Serial dilutions of scFv were added to the wells of each conjugate concentration, followed by monoclonal anti-flag M1 antibody, peroxidase–labelled anti-mouse IgG antibody and detected using *o*-PD. Absorbances were read after 30 minutes at 450nm.

2.5.12.4 Competitive studies

These were performed in the same manner as for standard ELISA except decreasing concentrations of free analyte (50 µl) were incubated with a constant concentration of scFv (50 µl) on the microtitre plate. In the case of saliva analysis, the sample was subjected to a freeze-thaw cycle and then a brief 30 second centrifugation step to remove any endothelial cells or foodstuffs. For competitive studies, the volume of sample analyte and antibody had to be reduced to 25 µl each, due to the small volumes of saliva samples obtained. Saliva samples were collected as described in Figure 2.1.



A: Saliva collection device. The absorbent pad is removed and placed in the individual's mouth for a few minutes until it becomes saturated

B: The pad is placed into the plastic 'syringe-like' component of the device

C: The 'plunger-like' component is screwed into the 'syringe-like' component to squeeze the saliva from the pad. The saliva is collected in a suitable container

D: The sample is labelled with a number and initials of the individual

Figure 2.1: *Collection of saliva samples. Saliva samples were collected using a prototype saliva collection device from Trinity Biotech. The absorbent pad was removed and placed in the individual's mouth for a period of a few minutes, until it became saturated. The pad was removed and place into the plastic 'syringe-like' component. The 'plunger-like' component was then screwed into this container. The pressure applied effectively squeezes the saliva from the pad, and this can be collected in a suitable container.*

2.5.12.5 Direct detection of pAK 600 alkaline phosphatase-labelled antibody

Microtitre plates were coated with 100 µl of appropriate hapten-conjugate and blocked as for standard ELISA. Alkaline phosphatase-labelled scFv was incubated with serial dilutions of free drug for 1 hour at 37°C prior to addition to the microtitre plate. Bound antibody was detected using para-nitrophenyl phosphate (pNPP) substrate and absorbance at 405nm read after 45 minutes at 37°C.

2.5.12.6 Cross reactivity studies

Cross reactivities of monomeric, dimeric and enzyme-labelled scFvs to morphine, methadone, heroin, codeine, norcodeine, dihydrocodeine and 6-monoacetylmorphine were analysed in ELISA. The assays were carried out as for the competitive ELISAs (Sections 2.5.12.4 and 2.5.12.5) with standards of cross reactant in place of M3G.

2.5.12.7 Affinity analysis in ELISA-Friguet method

Affinity analysis was carried out by ELISA according to the method developed by Friguet *et al.* (1985). Firstly, a microtitre plate was coated with 1 µg/ml M3G-OVA and blocked at 4°C, overnight. A series of nominal antibodies concentrations was added to the plate and used to construct a standard curve of nominal antibody concentration versus absorbance @ 450nm. Prior to performing the assay, a series of antibody-antigen mixtures were incubated in eppendorfs and allowed to reach equilibrium overnight. Each of these solutions contained a constant, nominal dilution of antibody, referred to as '1', but each had a different concentration of antigen. Absorbance readings at 450nm of the antibody:antigen mixtures were related to nominal antibody concentration values by reference to the standard curve generated. The fraction of total antibody bound by the antigen (V) was calculated for each of the antibody:antigen mixtures. The dissociation constant for each antibody was determined by the slope of the plot of 1/V versus 1/[A].

2.5.13 Sequence analysis of genes encoding specific scFvs

In order to generate high quality DNA for sequencing, each clone was transformed into XL-1 Blue cells. A single colony of each clone was grown overnight in LB media. The expression vector, containing scFv gene of interest, was isolated by miniprep, as described in Section 2.4.6.1. Plasmid DNA from the Griffin. 1 Library was sent to the Institute of Medical Sciences, University of Aberdeen for sequencing. All other clones were sequenced by MWG-Biotech. Raw DNA sequences in each direction were analysed using CHROMAS sequence analyser software. The DNA sequences were exported and analysed using a variety of web-based bioinformatics tools listed below.

Table 2.2: *Source of web-based bioinformatics tools used for sequence analysis of antibody fragments.*

Tool	Source
Chromas	www.technelysium.com.au/chromas.html
Translate Tool	www.biolundberg.gu.se/edu/translat.html
BLAST	www.expasy.org
ClustalW	www.ch.embnet.org/softward/ClustalW.html
Geneodoc	www.psc.edu.biomed/gendoc
Swiss Model	http://swissmodel.expasy.org
DS Viewer Pro	www.accelrys.com/dstudio/ds_viewer/
Kabat Rules	www://acrmwww.biochem.ucl.ac.uk/abs

2.6 Biopanning of a Naïve Human Antibody Library against Amphetamine and Tetrahydrocannabinol

The Griffin.1 library was accessed in the University of Aberdeen. The library was kindly donated by Greg Winter's Laboratory, Centre for Protein Engineering, Medical Research Council, Cambridge. The Library was produced from synthetic human V-gene segments, with a diversity of 1.2×10^9 clones. The library utilises the phagemid vector pHEN 2, encoding scFv genes flanked by *Nco 1* and *Not 1* restriction sites.

2.6.1 Rescue of scFv displaying phage

For initial library rescue 50 ml of 2 x TY, containing 100 µg/ml ampicillin, 1% (w/v) glucose, was inoculated with 1×10^{10} clones (200 µl of library stock) and grown at 37°C with shaking at 250 rpm until O.D. $_{600nm}$ = 0.4. The culture was then infected with 6×10^{11} cfu/ml of M13K07 helper phage. This yields a ratio of helper phage:bacteria of 20:1 (O.D. $_{600nm}$ = 1 is equivalent to approx. 1×10^8 cfu/ml bacteria). Infection was allowed to proceed at 37°C for 30 mins without agitation. The infected culture was centrifuged at 4,000 rpm for 10 mins and the pellet resuspended in 30 ml 2 x TY, 100 µg/ml ampicillin and 30 µg/ml kanamycin. This was diluted in 470 ml of pre-warmed 2 x TY, 100 µg/ml ampicillin and 30 µg/ml kanamycin in a 2 L baffled flask. Phage production was allowed to continue while shaking at 250 rpm, at 30°C, overnight.

2.6.2 Concentration of Phage by PEG/NaCl precipitation

The overnight culture was spun at 8,000 rpm, for 30 mins at 4°C. One fifth the volume of PEG/NaCl (20% PEG 6000, 2.5 M NaCl) was added to the supernatant and incubated on ice for at least an hour. The mixture was centrifuged at 8,000 rpm, for 30 mins at 4°C. The pellet was resuspended in 40 ml sterile water and 8 ml PEG/NaCl and incubated on ice for 20 mins. The mixture was centrifuged at 6,000

rpm, for 30 mins at 4°C. The supernatant was removed by aspiration and the pellet re-centrifuged briefly to remove any traces of PEG/NaCl. The pellet was resuspended in 5 ml of sterile PBS and stored at 4°C short-term or in 15% (v/v) glycerol at –80°C long-term.

2.6.3 Selection of specific scFv-displaying phage by biopanning

2.6.3.1 Selection on an antigen-coated immunotube

An immunotube was coated at 4°C overnight with appropriate concentrations of the antigen of interest (for initial rounds of panning a concentration of 10 µg/ml was used; this was decreased to 5 µg/ml and to 1 µg/ml in subsequent rounds). The immunotube was washed three times with PBS and blocked with 5 ml 4% (w/v) MBPS for two hours at 37°C. The immunotube was washed as before and 1 ml of PEG precipitated phage (approx 10^{12} to 10^{13} cfu/ml) was added to the immunotube in 4 ml of 2% (w/v) MPBS. Phage were allowed to bind for 90 mins at room temperature under gentle rotation and for a further 60 mins while stationary at room temperature. The immunotube was washed 10 times with PBST and 10 times with PBS (For second and subsequent rounds washing stringency was increased to 20 times with PBST and 20 times with PBS). Bound phage were eluted using 700 µl of 100 mM Triethylamine, pH 12, and rotated end-over-end continuously for 10 mins. Eluted phage were then added to 500 µl of 1 M Tris, pH 7.4, for quick neutralisation. A 9.25 ml volume of exponentially growing TG1 (O.D. $_{600nm}$ = 0.4-0.6) was added to 750 µl of eluted phage and the remainder of the eluted phage were stored at 4°C. Another 200 µl of 1 M Tris, pH 7.4, was added to the immunotube to neutralise any remaining phage. Any remaining phage were eluted by the addition of 4 ml of exponentially growing TG1 to the immunotube and both cultures were incubated in a 37°C waterbath for 30 mins without agitation. Both cultures were pooled and 100 µl was used to make five 100-fold serial dilutions. These were plated on TYE plates, containing 100 µg/ml ampicillin and 1% (w/v) glucose. The remaining infected TG1 culture was centrifuged at 4,000 rpm for 10 mins and resuspended in 1 ml of 2 x TY.

This 1 ml culture was plated on a large Nunc Bio-Assay Dish of TYE plates, containing 100 μg/ml ampicillin and 1% (w/v) glucose.

2.6.3.2 Free antigen elution of specific phage

Selection was performed as per Section 2.6.3.1 except that bound phage were eluted using 4 ml of free antigen for 1 hour at room temperature prior to the addition of triethylamine. The concentration of free antigen used was decreased from 100 μM to 1 nM during rounds of selection. Both elutions were reinfected separately.

2.6.3.3 Selection on streptavidin-coated magnetic beads

A 1 ml aliquot of streptavidin-coated magnetic beads was washed three times with PBS. The beads were then incubated with 2 μg of biotinylated amphetamine for 30 mins at room temperature. Captured amphetamine was separated from unbiotinylated amphetamine by three washes with PBS followed by magnetic separation and 150 μl of PEG-precipitated phage were added to the beads in 1 ml of PBS. Phage were allowed to bind at room temperature with gentle agitation for 90 mins. The beads were then washed 10 times with PBST and 10 times with PBS. Bound phage were eluted by a 10 min incubation with 200 μl of 100 mM triethylamine. Eluted phage were isolated via magnetic separation from the beads. Phage were immediately added to 100 μl of 1 M Tris, pH 7.4, for neutralisation and used to infect exponentially growing TG1 cells as for immunotube biopanning.

2.6.4 Further rounds of selection

Reinfected cells from the previous round of selection were scraped from the Bioassay dish (Nunc) using 6 ml of 2 x TY, 15% (v/v) glycerol to loosen cells with a spreader. Stocks were aliquoted into 500 μl volumes and stored at –80°C. A 100 μl aliquot of

this glycerol stock was used to inoculate 100 ml of 2 x TY, containing 100 μg/ml ampicillin and 1% (w/v) glucose. This was grown at 37°C with shaking at 250 rpm until O.D. $_{600nm}$ =0.4. A 10 ml volume of this culture was removed and infected with 6 x 10^{11} cfu/ml of M13K07 helper phage. Infection was allowed to proceed at 37°C for 30 mins without agitation. The infected culture was centrifuged at 4,000 rpm for 10 mins and the pellet resuspended in 50 ml 2 x TY, 100 μg/ml ampicillin and 30 μg/ml kanamycin. Phage production was allowed to continue overnight at 30°C, with shaking at 250 rpm overnight. A 40 ml volume of overnight culture used for PEG precipitation. The culture was centrifuged at 6,000 rpm, 4°C for 30 mins and one fifth the volume, i.e. 8 ml of PEG/NaCl, was added to the supernatant. This was incubated on ice for at least 1 hour. The mixture was centrifuged at 6,000 rpm, for 30 mins at 4°C. The supernatant was removed by aspiration and the pellet briefly re-centrifuged to remove any remaining traces of PEG/NaCl. The pellet was resuspended in 2 ml of sterile PBS. A 1 ml volume of this PEG precipitated phage was used for the next round of selection. The biopanning selection process was repeated at least three times until positive scFvs were identified.

2.6.5 Preparation of monoclonal phage for analysis by phage ELISA

A 96 well sterile culture plate was filled with 200 μl of 2 x TY, 1 % (w/v) glucose and 100 μg/ml ampicillin. Each well was inoculated with a single colony from the phage titre plates, leaving one or two wells as non-inoculated controls. The plate was incubated at 37°C with shaking at 150 rpm overnight. This was known as the master plate and was frozen at - 80°C with 15% (v/v) glycerol. Another plate was prepared with 200 μl /well 2 x TY containing 1% (w/v) glucose and 100 μg/ml ampicillin. A small volume (1-2 μl) from each well on the master plate was transferred to the corresponding well on the second plate and incubated at 37°C with shaking at 150 rpm for approx 2.5 hours until O.D.$_{600nm}$ = 0.5. After this time 25 μl of 2 x TY, 100 μg/ml ampicillin, 1% (w/v) glucose and 10^9 cfu/ml M13K07 was added to each well. The culture was left to stand for 30 minutes at 37°C followed by shaking at 37°C, 150 rpm for 1 hour. The plate was centrifuged at 2,500 rpm for 10 minutes. The supernatant was removed by inversion and the pellets resuspended in 200 μl of 2 x

TY, 1% (w/v) glucose, 100 μg/ml ampicillin and 30 μg/ml kanamycin. The plate was incubated overnight at 30°C while shaking at 150 rpm. The plate was centrifuged at 2,500 rpm for 10 minutes and 50 μl of the supernatant used for analysis in phage ELISA.

2.6.6 Screening of phage by monoclonal phage ELISA

A microtitre plate was coated with 100 μl of between 1 and 10 μg/ml of appropriate conjugate and incubated overnight at 4°C. The plate was washed three times with PBST and three times with PBS. The plate was then blocked with 150 μl of 4% (w/v) MPBS for 1 hour at 37°C. Supernatant from the monoclonal phage plate (50 μl) and 50 μl of 2% (w/v) MPBS was added to the corresponding well in each ELISA plate and incubated for 1 hour at room temperature. The plates were washed as before and 100 μl of peroxidase-labelled anti-M13 bacteriophage antibody at a 1 in 1000 dilution, in 1% (w/v) MPBS, was added to each well. This was incubated for 1 hour at room temperature. The plates were washed and 100 μl of 3,3',5,5'-Tetramethylbenzidine dihydrochloride (TMB) substrate diluted to 1 mg/ml in 0.05 M citric phosphate buffer, pH 5, was added. The reaction was allowed to proceed for 5 to 10 mins until a blue colour developed. The reaction was stopped by addition of 50 μl of 1 M H_2SO_4 per well. Absorbance read at 450nm, with reference subtraction at 620nm, i.e. 450-620nm.

2.6.7 Production of soluble antibody Fragments

Monoclonal phage (20 μl) were used to infect 180 μl of exponentially growing HB2151 and incubated at 37°C for 30 mins without agitation. Infected cells were then streaked onto TYE plates, containing 100 μg/ml ampicillin and 1% (w/v) glucose. A single colony was used to inoculate 5 ml of 2 x TY containing 100 μg/ml ampicillin and 1% (w/v) glucose. The culture was grown at 37°C, with shaking at 250 rpm, until $O.D._{600nm} = 0.9$. The infected cells were centrifuged at 4,000 rpm for 10 mins and the

pellet resuspended in 5 ml 2 x TY, 100 μg/ml ampicillin and 1 mM IPTG. Antibody production was allowed to proceed at 30°C overnight. The cells were pelleted by centrifugation, resuspended in 500 μl of fractionation buffer and incubated on ice for 15 mins. 5 μl of 1 M $MgCl_2$ was added and the culture incubated on ice for a further 15 mins. Bacterial debris was removed by centrifugation at 13,000 rpm for 5 mins and the supernatant used directly in ELISA.

2.6.8 ELISA on small scale soluble scFv from pHEN 2 vector

Plates were coated and blocked as per Section 2.5.12.2. Bound antibody was detected using a 1 in 1000 dilution of peroxidase-labelled anti-c-myc monoclonal antibody and developed using TMB. The reaction was stopped using 1 M H_2SO_4. Absorbance was read at 450-620nm.

2.6.9 Cloning into pIMS 147 for expression as single chain antibody (scAb)

The pIMS 147 vector is an enhanced expression vector, encoding the constant region of the human kappa antibody, produced by The Molecular and Cell Biology Group, University of Aberdeen, UK. The scFv gene of interest was extracted from the pHEN 2 vector using *Nco 1* and *Not 1* restriction sites flanking the region. The pHEN 2 vector was isolated from HB 2151 cells using a Wizard Plus Miniprep kit as per Section 2.4.6.1. Approximately 2 μg of plasmid DNA was restricted using 20 Units of *Not 1* and 10 units of *Nco 1* in 1 x NEB buffer 3, diluted in 'nuclease-free' water, at 37°C for 5 hours. The pIMS 147 vector was isolated from XL-1 Blue cells and restricted in the same manner. The scFv gene was ligated with digested pIMS 147 at a 1.5:1 vector to insert ratio as per Section 2.4.6.3. The ligated plasmid was then transformed into heat shock competent XL-1 Blue cells as per Section 2.4.6.7, using ampicillin resistance to select for transformants.

2.6.10 Large scale expression in pIMS 147

A single colony of XL-1 Blue harbouring pIMS 147 and scFv insert, was used to inoculate 5 ml of TB_{GAT} (TB, containing, 1% (w/v) glucose, 100 μg/ml ampicillin and 30 μg/ml tetracycline). This was used to seed 12 x 50 ml of TB_{GAT} at a 1% (v/v) cell density. The cultures were incubated all day at 37°C with shaking at 250 rpm and then centrifuged at 4,000 rpm for 20 mins. The pellets were resuspended in 50 ml fresh TB_{GAT} and incubated overnight at 25°C and 250 rpm. The following day, the cultures were centrifuged at 4,000 rpm for 20 mins, resuspended in TB containing 100 μg/ml ampicillin and allowed to recover for 1 hour at 25°C with shaking at 250 rpm. After this time, each culture was induced with 1 mM IPTG and incubated for 4 hours at 25°C and 250 rpm. The cultures were centrifuged at 4,000 rpm for 30 mins and the pellets subjected to osmotic shock with fractionation buffer to release the periplasm of cells. This involved incubating the cell pellet with 10% (v/v) the original culture volume of fractionation buffer on ice for 15 mins with gentle shaking, at 100 rpm. After this time 1 M $MgCl_2$, to a final concentration of 0.05% (v/v) of original culture volume, was added and the mixture incubated on ice for a further 15 mins. The lysate was then centrifuged at 4,000 rpm for 20 mins to remove bacterial debris. The supernatant was sterile filtered through a 0.45 micrometre filter prior to purification.

2.6.11 Purification by IMAC

2.6.11.1 Preparation of resin

A 250 μl aliquot of chelating sepharose fast-flow was used for every 100 ml of culture volume used. The resin had a binding capacity of 5 mg of 6-His protein/ml of slurry. The gel was sedimented by centrifugation, washed using 5 gel volumes of distilled water and incubated in a Biorad minicolumn with end-over-end rotation for 10 mins. The water was drained from the gel, 5 gel volumes of 1 M $NiSO_4$ was added to the column and incubated for 1 hour at room temperature with end-over-end rotation. The column was drained and washed three times with 5 gel volumes of PBS. The

column was now ready for use or suitable for storage at 4°C in 5 ml of PBS for 1 month.

2.6.11.2 Binding of 6-His tagged scAb

ScAb released from bacterial periplasm was added to the resin and incubated overnight at 4°C with end-over-end rotation. The flow through was collected on ice and put back through the column a total of three times. The column was washed with 5 gel volumes of 10 mM imidazole, followed by 5 gel volumes of 25 mM imidazole. The scAb-containing fraction was eluted using 250 mM imidazole and collected in 500 µl aliquots on ice. A total of 20 aliquots were collected and analysed by ELISA as per Section 2.6.13.1. Antibody containing aliquots were pooled and dialysed against 100 volumes of PBS at 4°C overnight. Once dialysed, the scAb could be stored at –20°C. The purity of the scAb was monitored by SDS-PAGE electrophoresis as described in Section 2.5.11.

2.6.12 Quantification of scAb expression

2.6.12.1 Quantification of scAb expression by capture ELISA

An immunoplate was coated with 1 µg/ml anti-human kappa light chain antibody overnight at 4°C. The plate was blocked with 4% (w/v) MPBS for 1 hour at 37°C. The plate was washed three times with PBS. A total of twelve doubling dilutions of human kappa IgG, ranging in concentrations from 1,250 to 0 ng/ml, were prepared and 100 µl of each antibody dilution was added in duplicate to the individual wells of the plate. Twelve doubling dilutions of scAb were also prepared and added in duplicate to the immunoplate. The plate was incubated for 1 hour at 37°C and then washed three times with PBST and three times with PBS. Bound antibody was detected using 100 µl of a 1 in 1000 dilution of peroxidase-labelled anti-human kappa light chain antibody was added to each well and incubated at 37°C for 1 hour. The

plate was washed as before and developed using 100 µl/well of TMB substrate in 0.05 M citric phosphate buffer, pH 5. The reaction was allowed to proceed to 10 minutes until a blue colour developed and was then stopped using 50 µl/well 1 M H_2SO_4. Absorbance was read at 450-620nm. A standard curve of human IgG of known concentrations was plotted against absorbance observed. This could then be corrected for differences in molecular weight between whole IgG (150 kDa) and scAb (40 kDa).

2.6.12.2 Quantification of scAb expression by SDS-PAGE Electrophoresis

Purified scAb was quantified by including a sample of previously purified, characterised and quantified scAb on the gel. Electrophoresis was carried out as described in Section 2.5.11. The protein band observed for the scAb could then be compared with that of the scAb of known concentration.

2.6.12.3 Bradford assay quantification of total protein

Varying dilutions of protein/antibody standards were prepared, ranging in concentration from 100 to 1000 µg/ml and 5 µl of each dilution was added to each well of a microtitre plate. The protein in each well was detected by the addition of 250 µl of Coomassie reagent (containing, Coomassie G-250 dye, methanol, phosphoric acid and stabilising agents in water) to each well. The plate was incubated at room temperature for 10 mins and absorbance read at 595nm.

2.6.12.4 Determination of antibody aggregation by Native Polyacrylamide Gel Electrophoresis (PAGE)

To access the degree of antibody aggregation, a purified scAb fraction was run on a native polyacrylamide gel. The composition of the gel used was as described in Table 2.1 with SDS and mercaptoethanol omitted from all formulations. This maintained the integrity of the protein in its native form, allowing any protein aggregation to be visualised.

2.6.13 ELISA analysis of scAbs

2.6.13.1 Direct ELISA

Plates were coated and blocked as per Section 2.5.12.2. Bound antibody was detected using a 1 in 1000 dilution of peroxidase-labelled anti-human kappa light chain antibody. The plate was washed and developed using 100 µl/well of TMB substrate in 0.05 M citric phosphate buffer, pH 5. The reaction was allowed to proceed to 10 minutes until a blue colour developed and was then stopped using 50 µl/well 1 M H_2SO_4. Absorbance was read at 450-620nm.

2.6.13.2 Competitive ELISA

Plates were coated and blocked as per Section 2.5.12.2. Free analyte standards were prepared and 50 µl of each concentration was added to each well along with 50 µl of scAb. The plate was incubated for 1 hour at 37°C. Bound antibody was detected using peroxidase-labelled anti-human kappa light chain antibody at a 1 in 1000 dilution. This plate was developed using TMB, and stopped using 1 M H_2SO_4. Absorbance was read at 450-620nm.

2.7 BIAcore Analysis of scFvs

Analysis was performed using a Biacore 3000® optical-based biosensor. All data analysis was performed using BIAevaluation 3.0 (BIAcore AB, St Albans, Hertfordshire, England). Research grade CM5 sensor chips were employed and Hepes Buffered Saline (HBS) (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA and 0.025% (v/v) Tween 20) was used as running buffer. This was filtered (pore size 0.22 micrometre) and degassed using filtration apparatus (Millipore sintered glass filtration unit) immediately before use. All samples were syringe-filtered (0.45 micrometre pore size) to remove any particulate matter.

2.7.1 Preconcentration studies

In order to optimise immobilisation of the hapten-carrier to the sensor chip surface, a pre-concentration step was carried out to identify the optimal pH conditions. At pH values above pH 3, the carboxymethylated surface of the CM5 sensor chip is negatively charged. The desired pH conditions for electrostatic immobilisation will result in the ligand possessing a net positive charge. This is achieved by using a buffer with a pH below the pI of the ligand to be immobilised. Preconcentration studies involved preparing solutions of 100 μg/ml hapten-protein conjugate in 10 mM sodium acetate solution at a range of varying pHs. Sodium acetate buffer was chosen due to its low ionic strength so as to maximise ionic interactions. The samples were then passed over an underivatised chip surface in random order. The surface response was normalised to zero following each injection. The amount of electrostatic interaction monitored by measuring the binding response at each pH. The pH, which gave the highest degree of electrostatic interaction, i.e. highest, binding, was chosen for to perform immobilisations.

2.7.2 Immobilisation of hapten-carrier on sensor surface

Immobilisation of conjugate was carried out according to standard amine coupling. Briefly, the carboxymethylated dextran surface was activated by mixing equal volumes of 0.1 M NHS (N–hydroxysuccinimide) and 0.4 M EDC (N-ethyl–N-(dimethyl–aminopropyl) carbodiimide hydrochloride) and injecting the mixture over the sensor chip surface for 7 minutes at a flow rate of 5 µl/min. A 100 µg/ml solution of M3G – OVA prepared in 10 mM acetate buffer, pH 4.9, was injected over the activated surface, at a flow rate of 5 µl per minute, for 40 minutes. Unreacted sites were capped by an injection of 1 M ethanolamine, pH 8.5, for 7 minutes. The surface was regenerated five times with 10 mM NaOH prior to use.

2.7.3 Regeneration studies

To assess the stability of the immobilised drug-protein conjugate surfaces, a known concentration of antibody was passed over the chip surface, and the surface regenerated by passing over various concentrations of NaOH and HCl ranging from 1-100 mM or 1 M ethanolamine, pH 13.6. This cycle of binding and regeneration was usually completed for greater than 50 cycles, and the binding signal measured to assess the stability and suitability of the immobilised surface for assay purposes. A regeneration solution of 10 mM NaOH proved optimal for removal of monomeric and dimeric scFvs.

2.7.4 Non-specific binding studies

Purified scFv antibody solutions, at the optimal assay dilution, were passed over a 'capped' dextran surface and a surface with the protein of interest immobilised on the dextran matrix to rule out any non–specific binding of the antibody to these surfaces.

2.7.5 BIAcore inhibition immunoassay

Standards of free analyte were prepared at varying concentration ranges. All further additions of reagents and incubation steps were automated. Each sample was incubated with an equal volume of antibody, allowed to equilibrate for a specific time interval (2 hours at room temperature), and then passed over the sensor surface. A 20 µl aliquot of each sample was injected over the surface, at a flow rate of 5 µl per minute for 4 minutes, in random order. The surface was regenerated with a 1-minute pulse of 10 mM NaOH prior to further injections. A calibration curve was constructed by plotting the change in response (RU) for each standard against the log of concentration, and sample concentrations were determined from this curve. The intra-day variability of the assay was investigated by running a set of standards across the linear range, three times in one day, and determining the coefficient of variation (C.V.) between the response units measured for each analyte concentration. The inter-day variability of the assay was assessed by running standards across the linear range on three different days. This proves sufficient reproducibility for an automated analysis system (Wong *et al.*, 1997).

2.7.6 Equilibrium dissociation constant determination by solution affinity analysis using BIAcore

Approximately 8,000 RU of M3G-OVA conjugate was immobilised on the sensor chip surface as described in Section 2.7.2. Serial dilutions of purified scFv of known concentration were passed over the surface in random order. A calibration curve of mass bound in response units versus scFv concentration (M) was constructed. A known concentration of scFv, within the linear range of the calibration curve, was incubated with varying concentrations of analyte and allowed to reach equilibrium for 2 hours at 37°C. Each mixture was injected over the surface, in random order, at a flow rate of 5 µl/min for 2 mins and the binding response monitored. This response value was used to calculate the concentration of free scFv, present in each of the equilibrium mixtures, from the calibration curve. Analyte concentration (M) was plotted against free scFv concentration (M) and a solution phase affinity interaction model was fitted using

BIAevaluation 3.1 software. This was used to calculate equilibrium dissociation constant.

2.8 Lateral Flow Immunoassay (LFIA) Development

Table 2.3: *Composition of Buffers used in Lateral Flow Development*

Buffer	Composition
Colloidal carbon Suspension	1% colloidal carbon (w/v) in demineralised water
100 mM Borate buffer	100 mM boric Acid adjusted to pH 8.5 with 100 mM borax solution.
Carbon conjugate washing solution	5 mM borate buffer, pH 8.5, 1% (w/v) BSA, 0.02% (w/v) NaN_3.
Carbon conjugate storage solution	100 mM borate buffer, pH 8.5, 1% (w/v) BSA, 0.02% (w/v) NaN_3.
LFIA Running Buffer	100 mM borate buffer, pH 8.5, 1% (w/v) BSA, 0.05% (v/v) Tween 20, 0.02% (v/v) Trition-X-100, 0.02% (w/v) NaN_3.

2.8.1 Physical adsorption of protein onto colloidal carbon particles

Buffers used in the development of the LFIA were prepared according to Table 2.3. A 1% (w/v) carbon stock suspension was diluted 5 times in 5 mM borate buffer, pH 8.5 to give a working concentration of 0.2% (w/v). This was sonicated for 30 secs and the pH adjusted to approximately 1 pH unit above the pI of the protein so it has a slight negative charge. Approximately 300-400 μg of protein was added to the 1 ml suspension and this was gently stirred at 4°C overnight. The suspension was centrifuged at 14,000 rpm for 15 mins at 4°C and washed with 1.5 ml of conjugate wash solution. This was repeated four times. After washing, the pellet was resuspended in 1 ml of conjugate storage solution and stored in a glass vial at 4°C in the dark.

2.8.2 Spraying of nitrocellulose strips

A piece of nitrocellulose was cut to 10 cm wide and 4.5 cm long. This generated 10 lateral flow strips. A 70 mm line was sprayed with 10 μl of capture antibody at 450 nl/secs using the Linomat 5 instrument. The control line was sprayed 3 cm from the bottom of the strip and the test line sprayed 0.3 cm beneath this (i.e. 2.7 cm from the bottom of the strip). Strips were dried at 37°C overnight and cut into 10 strips 7 mm wide. This was equivalent to 1 μl of capture antibody per strip. Dried strips were stored in a sealed laminated foiled pouch containing a 0.25 g sachet of silica minipax at room temperature.

2.8.3 Running of LFIA strips

Nitrocellulose strips were stuck to the perspex bridge using double-sided sticky tape as in Figure 2.2. A small piece of Whatman 3 mm chromatography paper was stuck to the top of the strip to facilitate sample migration and allow adsorption of excess test solution. A piece of parafilm was laid under the perspex bridge for sample application. The sample was prepared in 100 μl of running buffer containing the

desired amount of antibody/carbon conjugate and free analyte was added beneath LFIA strip and allowed to migrate up the strip for 10-15 mins. At this stage a signal line should be observed at the top of the strip. The strip was then washed with 100 µl of running buffer to remove any carbon aggregates.

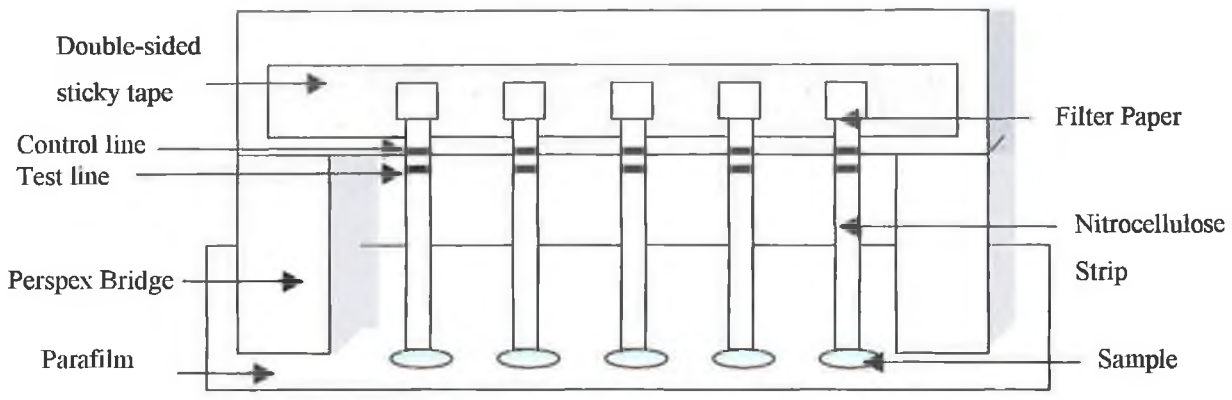


Figure 2.2: *Schematic showing set-up of a lateral flow immunoassay system (LFIA). Prepared nitrocellulose strips were stuck to the bridge using double-sided sticky tape. The perspex bridge was placed on a sheet of parafilm containing samples of interest. Samples were allowed to run along the nitrocellulose strips for approx. 15 mins, until the control line signal was generated.*

Chapter 3: The Production of Genetically-Derived scFvs against Morphine-3-Glucuronide

3.1 Introduction

3.1.1 Introduction to heroin and the opiates

Opiates are a class of drugs that are structurally similar to morphine and are derived from opium extracted from the seed pod the opium poppy (*Papavar somniferum*) (Braitwaite *et al.*, 1995). Opiates are used clinically for their analgesic properties in the treatment of severe pain but opiate abuse arises from their psychotropic properties. They have a highly addictive nature due to their high physical and psychological dependence. Opiates are similar in structure to endorphins, the body's natural painkillers. Endorphins work by binding to opiate receptors in the brain, presynaptically inhibiting the release of 'substance P'. This is a neurotransmitter responsible for the perception of pain. By blocking its release, endorphins play a role as a natural analgesic. The structural similarity between opiates and endorphins mean that they will both compete for binding to the opiate receptors. Opiate abuse leads to a reduction in the levels of endorphin production in the body. This causes opiate tolerance and leads to the extreme symptoms observed following opiate withdrawal. These symptoms are a major factor in dependency on this class of drug and will persist until the body can restore normal endorphin production.

3.1.1.1 Morphine

Morphine gets its name from *Morpheus*, the Greek god of sleep. It was the first of the opiates to be extracted from the opium plant in a pure form, by Sertürner in 1806 (Simini, 1995). Although it has been in clinical use as a powerful analgesic for over a century, it remains a medication of choice for the treatment of severe pain (Gilson *et al.*, 2004). It is also commonly administered to terminally ill patients to alleviate suffering.

3.1.1.2 Heroin

Heroin or 'smack' is probably the best known of the opiates due to its highly addictive nature and prevalence of abuse worldwide. It is estimated to have caused economic losses of $21.9 billion in the USA alone in 1996 (Mark *et al.*, 2001). Heroin is a chemically synthesised, diacetyl derivative of morphine. It is produced by the 'Lime Method,' which involves the one-step acetylation of morphine in a large excess of acetic anhydride (Dams *et al.*, 2001). It is typically sold as a white or brownish powder or as the black sticky substance known on the streets as "black tar heroin." Heroin bought on the street is diluted or 'cut' with other substances, such as sugar, chalk or starch. Local anaesthetics, like procaine for example, may be added to relieve the local pain of IV administration (Janhunen and Cole, 1999, Dams *et al.*, 2001). Quinine may also be added to imitate the bitter taste of heroin, in an attempt to increase the apparent strength of the preparation. The purity of heroin on the streets has increased with improving refinement technologies. Its addictive nature is associated with its high lipophilicity, allowing it to pass more rapidly across the blood/brain barrier than morphine. This gives heroin its euphoric properties, making it one of the most commonly abused drugs worldwide.

3.1.1.3 Codeine

Codeine is a less potent and 'non–controlled' opiate, small quantities of which are found in combination analgesic preparations, such as with paracetamol and in cough remedies for its anti-tussive properties (e.g. Benylin). Codeine may also be found in poppy–seeds, which are used to flavour speciality breads and crackers. Codeine is metabolised to morphine and nor–codeine, with morphine being the most predominant metabolite detectable in urine after a two–day period (Braitwaite *et al.*, 1995).

3.1.1.4 Dihydrocodeine

Dihydrocodeine is another opiate found in combination analgesics like Calpol, for example. It is less potent than morphine but more potent than codeine (Braithwaite *et al.*, 1995). Unlike codeine however, it is not metabolised to morphine.

3.1.1.5 The opioids

The opioids are a class of compound that display opiate-like pharmacological activity but are structurally unrelated to morphine (Braithwaite *et al.*, 1995). Methadone is a good example of a commonly used opioid, as it exhibits narcotic-analgesic effects, while being structurally unrelated to morphine. Methadone is used as a substitution withdrawal therapy for heroin addiction as it is can be taken orally and is absorbed more slowly than heroin. The affects are, therefore, longer lasting helping to break the cycle of addiction.

3.1.2 Heroin administration and metabolism

Heroin may be taken orally, intravenously, intranasally ('snorted') or by heating the powder and inhaling the vapours ('chasing the dragon'). With increases in the level of purity of illicit heroin available, many users have switched from intravenous to intranasal administration (Cone, 1998). Heroin for oral, intranasal or 'chasing the dragon' administration is generally the base product formed through the 'Lime method'. Heroin for intravenous administration, needs to be converted to a hydrochloride salt powder by acidification in the presence of acetone (Dams *et al.*, 2001).

The metabolism of heroin is outlined in Figure 3.1.1. It is rapidly metabolised to 6–monacetylmorphine (6-MAM), the metabolite responsible for the characteristic euphoric and 'rush' feelings (Beike *et al.*, 1999). 6-MAM is the only metabolite that is specific to heroin use. This is subsequently hydrolysed to morphine by enzymes in the liver. Morphine is a short–lived species, undergoing glucuronidation, mainly at the 3–phenolic position, to morphine-3-glucuronide (M3G). Glucuronidation also occurs at the 6–alcoholic position, to a lesser extent, producing morphine-6-glucuronide (M6G). Urinary recoveries of M3G and M6G account for 55% and 10% of the administered does, respectively (Zheng *et al.*, 1998). Other minor metabolites include morphine–3-diglucuronide, nor–morphine and nor–morphine–3–ethereal sulphate (Braitwaite *et al.*, 1995). Morphine, morphine-3-glucuronide and morphine-6-

glucuronide are all metabolites observed following heroin and/or morphine administration.

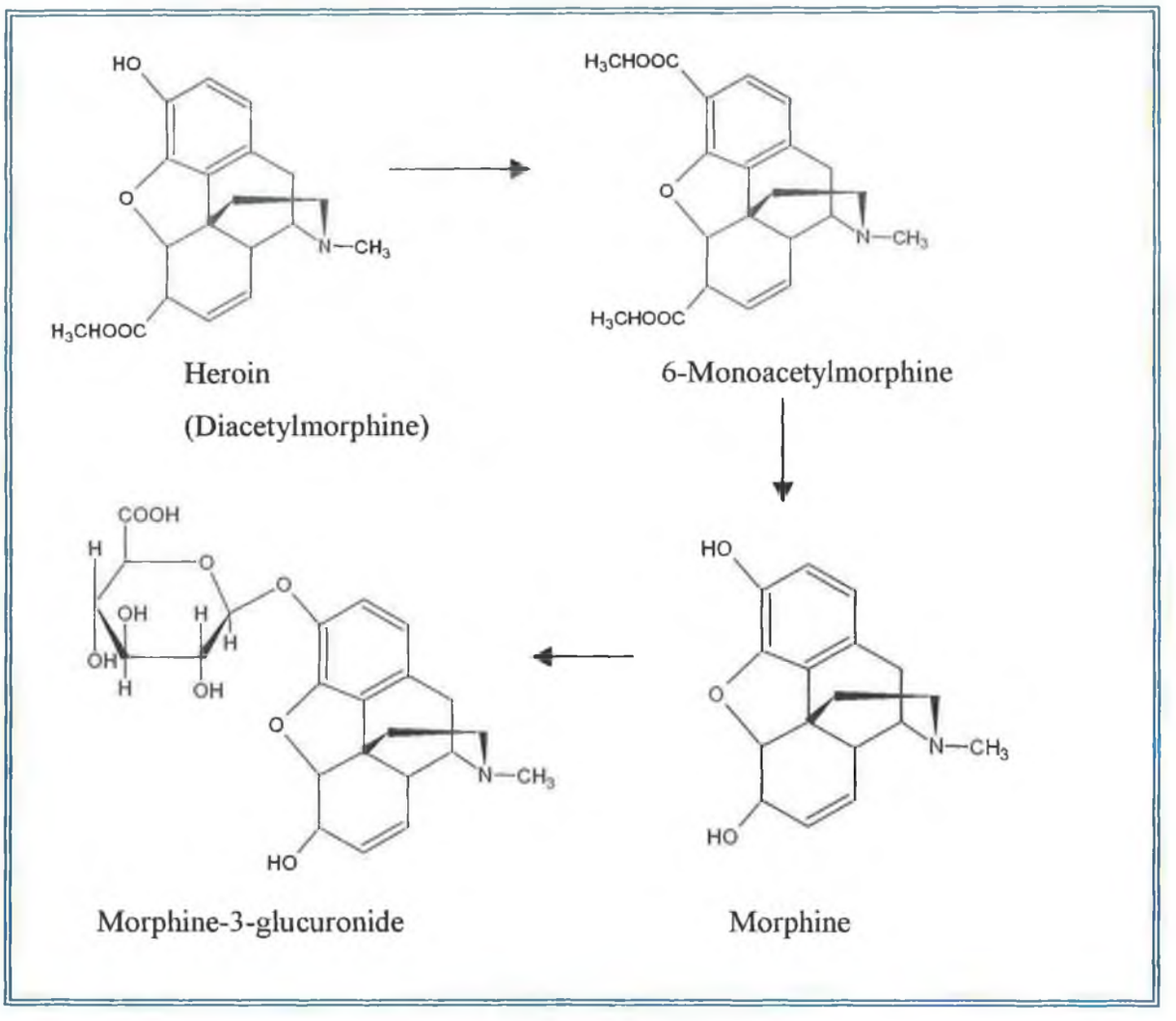


Figure 3.1.1: *Metabolism of heroin to morphine-3-glucuronide.*

3.1.3 Sample analysis for heroin abuse

3.1.3.1 Matrices for detection

Heroin abuse has been detected in a variety of biological matrices including, urine, plasma, hair, sweat and saliva (Cone *et al.*, 1996; Kidwell *et al.*, 1998; Moeller *et al.*, 1998; Beike *et al.*, 1999; Nakahara, 1999 and Spiehler, 2000). The choice of matrix will determine which metabolite will be targeted in the analysis to detect heroin use. Heroin itself is rarely found in any bodily fluid (Braithwaite *et al.*, 1995), except perhaps for an extremely brief period following administration (Cone, 1998). 6-Monoacetylmorphine is the only heroin-specific metabolite that can definitively determine illicit heroin use, but it has a very short window of detection (Cone *et al.*, 1996). Morphine-3-glucuronide is the metabolite of choice for detection in urine and plasma, due to its high concentrations and long persistence. Morphine is also detected in plasma and urine but for shorter periods of time. Heroin, 6-MAM and morphine have all been isolated from saliva, following heroin use. Concentrations were generally found to be lower than those in urine and the window of detection for 6-MAM and heroin was found to be extremely short (Kidwell *et al.*, 1998).

Plasma is the obvious method of choice in a forensic setting. In reality, the procedure for sample collection is invasive, requiring the involvement of a healthcare professional, who may be exposed to a potential health risk from syringes. Sample extraction from plasma is also extensive. Urine is routinely used for drug analysis in a clinical setting. Sample volumes are typically large and metabolites can be detected for a prolonged period of time. This is useful if substance misuse is the aim of the analysis, however, the result bears no correlation to behavioural impairment due to substance use. Urinalysis, suffers from some major drawbacks. Sample collection proves invasive, making it unsuitable for 'on-site' monitoring without private facilities. Sample collection is required to be supervised by an official in authority, to eliminate any possibility of sample adulteration. This in itself is an invasion of privacy. Sweat is also gaining increased recognition as an alternative matrix for detection of illicit drug residues. Recent draft guidelines by the Substance Abuse and Mental Health Services Administration (SAMHSA), in the USA, include the

requirement for testing for heroin use in alternative matrices such as saliva and sweat (Presley *et al.*, 2003). Saliva collection is non-invasive and can be easily supervised. It can be easily collected from subjects in any type of field setting, from the workplace to on the roadside. Sample volume is adequate to yield a number of aliquots for multiple analyses. Sample extraction is minimal and drug metabolite concentrations give a good indication of recent drug use and/or impairment (Kidwell *et al.*, 1998). This makes saliva monitoring, particularly suited to drug testing for law enforcement purposes. Bennet *et al.* (2003), reported oral fluid analysis to be as accurate as urinanalysis for the detection of opiates. Saliva, as a matrix for drug detection, will be discussed in more detail in Chapter 4. Sweat wipes have been successfully used for opiate detection in drivers in Belguim. The accuracy of the wipes was only 70% when compared with plasma analysis by GC-MS (Samyn *et al.*, 2002). Sweat analysis for drugs of abuse, as reviewed by Kidwell *et al.* (1998), was found to lack a correlation to intoxication and suffer from the limited sample volumes obtained. Hair sample analysis has also been used to determine heroin abuse. Hair analysis, however, is mainly carried out for forensic toxicology to monitor long-term patterns of drug use (Uhl, 1997; Nakahara, 1999 and Spiehler, 2000).

3.1.3.2 Methods of detection

Opiate detection can be divided into two groups, the first, which comprises traditional analytical detection methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC) and mass spectrometry (MS), and the second, which involves immunoanalysis. Immunoanalysis is routinely used to carry out initial screening of samples for possible opiate presence and positive results are then confirmed and quantified using analytical detection systems. Analytical detection is sensitive and specific for opiate detection in biological samples and multiple analytes can be determined simultaneously. The complex nature of biological samples, however, requires them to undergo an initial sample clean-up step through either solid phase extraction (Gerostamoulos and Drummer, 1995) or immunoaffinity extraction (Beike *et al.*, 1999).

Zheng *et al.*, (1998) used HPLC-MS-MS to analyse the pharmacokinetic of morphine and metabolites in Sprague Dawley rats. The assay required a plasma sample volume of 100 µl and had limits of detection of 3.8 ng/ml for morphine and 5.5 ng/ml for morphine-3-glucuronide. Naidong *et al.* (1999) could simultaneously detect morphine, M3G and M6G in human plasma using LC-MS-MS. The range of detection was from 0.5-50 ng/ml for morphine, 10-1,000 ng/ml for M3G and 1 to 100 ng/ml for M6G, with excellent relative recoveries. A novel amperometric opiate assay for heroin and morphine detection, is reported by Holt *et al.* (1995). The method utilises two enzymes specific for heroin and morphine. Acetylmorphine carboxyesterase, hydrolyses heroin to morphine, and morphine dehydrogenase, oxidises the C-6 of morphine, releasing NADPH, which can then be detected amperometrically. The assay requires 100 µl of sample and can be carried out in a couple of minutes. Detection limits of 6.8 µg/ml for morphine and 28 µg/ml for heroin could be achieved. The same enzymes were subsequently employed in a more sensitive assay for heroin and its metabolites (Holt *et al.*, 1996). The assay was based on bioluminescence and was sensitive to 89 ng/ml heroin and 2 ng/ml morphine.

Immunoanalysis is performed as a preliminary screening technique as it is rapid and easy to perform, does not require costly equipment for analysis and does not involve complex sample extraction procedures, prior to analysis. A variety of sensitive immunoassays have been developed for detection of opiate residues. An early immunoassay for morphine in serum developed by Spector (1971) proved extremely sensitive. The radioimmunoassay was capable of detecting between 50 and 100 pg morphine, in a final assay mixture of 0.5 ml. Steiner and Spratt (1978), report a sensitivity of 500 ng of morphine per litre of serum using a polyclonal anti-morphine antibody in a radioimmunoassay. Although radioimmunoassays offer increased levels of sensitivity, the use of radioactive isotopes as tracer labels is undesirable, due to safety concerns. A rapid immunochromatographic test for the presence of opiates in urine was developed by Wenning *et al.* (1998). The threshold opiate level was set at 200 ng/ml. The rapid test had a sensitivity of 99%, when compared with enzyme multiplied immunoassay technique or EMIT assays. Polyclonal antibodies were employed in a biosensor-based assay using BIAcore to detect M3G in urine, with a detection limit of 762 pg/ml. ELISA analysis, however, proved more sensitive, with a

detection limit of 30 pg/ml for M3G in urine (Dillon[A] *et al.*, 2003). Another ELISA assay using a polyclonal for the detection of morphine in saliva had a limit of detection of 24.4 ng/ml (Fanning, 2002). Recombinant antibodies have more recently been employed for the detection of M3G in urine using ELISA and BIAcore technologies (Dillon[B] *et al.*, 2003). BIAcore analysis offered a detection limit of 3 ng/ml. Moghaddam *et al.* (2003), utilised phage display, antibody chain shuffling and selective biopanning to generate an scFvs that specifically detects 6-MAM, with no cross reactivity to morphine. The antibodies were capable of detecting 50 to 100 ng/ml 6-MAM. Although this is above the sensitivity of 10 ng/ml, required for most drug screens, the antibody's specificity for 6-MAM makes it an ideal analytical tool for a rapid, sensitive and specific immunoassay that could be definitive for abuse of heroin.

3.1.4 Chapter outline

This chapter focuses on the production and characterisation of three genetically-derived scFv antibody fragments directed against morphine-3-glucuronide (M3G). A monomeric, dimeric and alkaline-phosphatase conjugated scFv were produced. Expression conditions were optimised for each antibody and monomeric and dimeric antibodies were purified to a high degree of purity. The scFvs were used to develop three competitive ELISA assays for the detection of M3G. The specificity of each antibody was also characterised.

3.2 Results

A recombinant antibody clone, clone number G12, directed against morphine-3-glucuronide was donated by, Dr. Paul Dillon, DCU (Dillon, 2001: Dillon[B] *et al.*, 2003). The recombinant antibody was isolated from a pre-immunised murine library produced using the Krebber system of phage display (Krebber *et al.*, 1997). Briefly, Balb/C mice were immunised with an M3G-BSA conjugate. When sufficient titres were obtained the splenomic RNA was isolated and reverse transcribed into cDNA. The murine heavy and light chain genes were then amplified by multiplex PCR and annealed together using a splice by overlap extension PCR (SOE-PCR). The 800 base pair SOE-PCR product was then ligated into the phage display vector, pAK100, and transformed into supercompetent XL-1 Blue *E. coli*, yielding a library of 5 x 10^3 clones. The resulting phage display library was subjected to two rounds of biopanning and a large proportion of scFvs were isolated that showed recognition of the conjugate. Clone G12 was chosen as the most sensitive scFv for M3G detection and was chosen for subsequent soluble expression with the pAK 400 vector.

3.2.1 Genetic analysis of scFv genes encoding clone G12

In order to generate high quality DNA for sequencing, plasmid DNA was transformed into XL-1 Blue cells. A single colony of clone G12 was grown overnight in LB media. The pAK 100 phage display vector, containing the scFv gene of interest, was isolated by miniprep, as described in Section 2.4.6.1. Plasmid DNA was sent to MWG Biotech (Germany), for sequencing. Comfort reads were performed in both directions using the primers scfor ('5 ttactcgcggcccagccggccggccatggcggactaccccg 3') and scback ('5 ggaattcggcccccgag 3'). The DNA sequences were exported and translated using the DNA to protein translation package on the Bio.lundberg sequence analysis server. The antibody complementarity determining regions (CDR's) were identified using the Kabat rules and the amino acid sequence was exported to Genedoc for manipulation (Figure 3.2.1). Antibodies were modelled using Swiss-Model, a fully automated protein structure homology-modelling server (Schwede *et al.*, 2003). The server operates by comparing the given amino acid sequence to all

experimentally solved 3D protein structures held at the Protein Data Bank (PDB). This identifies a solved protein structure with the greatest sequence similarity to the target sequence. The degree of identity between sequences is calculated and a theoretical model for the target sequence extrapolated. Modelling results were exported to Swiss-PDB Viewer and DS Viewer Pro for visualisation (Figure 3.2.2 and Figure 3.2.3). CDR regions are highlighted as blue or red, depending on whether they are of heavy or light chain origin, with the linker region shown in yellow.

Figure 3.2.2, shows a ribbon illustration of clone G12 modelled using DS Viewer Pro software. Heavy chain CDRs are shown in red and light chain CDRs are shown in blue. The linker region is shown in yellow. Figure 3.2.3, is a schematic representation of clone G12 generated using Swiss-PDB Viewer software. In this model, the arrows pointing in the direction of the C-terminus represent beta sheets and coils are represented by a tubular structure. Each variable domain consists of nine beta sheets connected via a synthetic Gly_4Ser linker, which is clearly visible as a yellow tube. Panel A illustrates a front view of the antibody and Panel B illustrates an enlarged view of the antigen-binding site. The deep antigen-binding pocket, characteristic of 'anti-hapten' antibodies, is clearly visible.

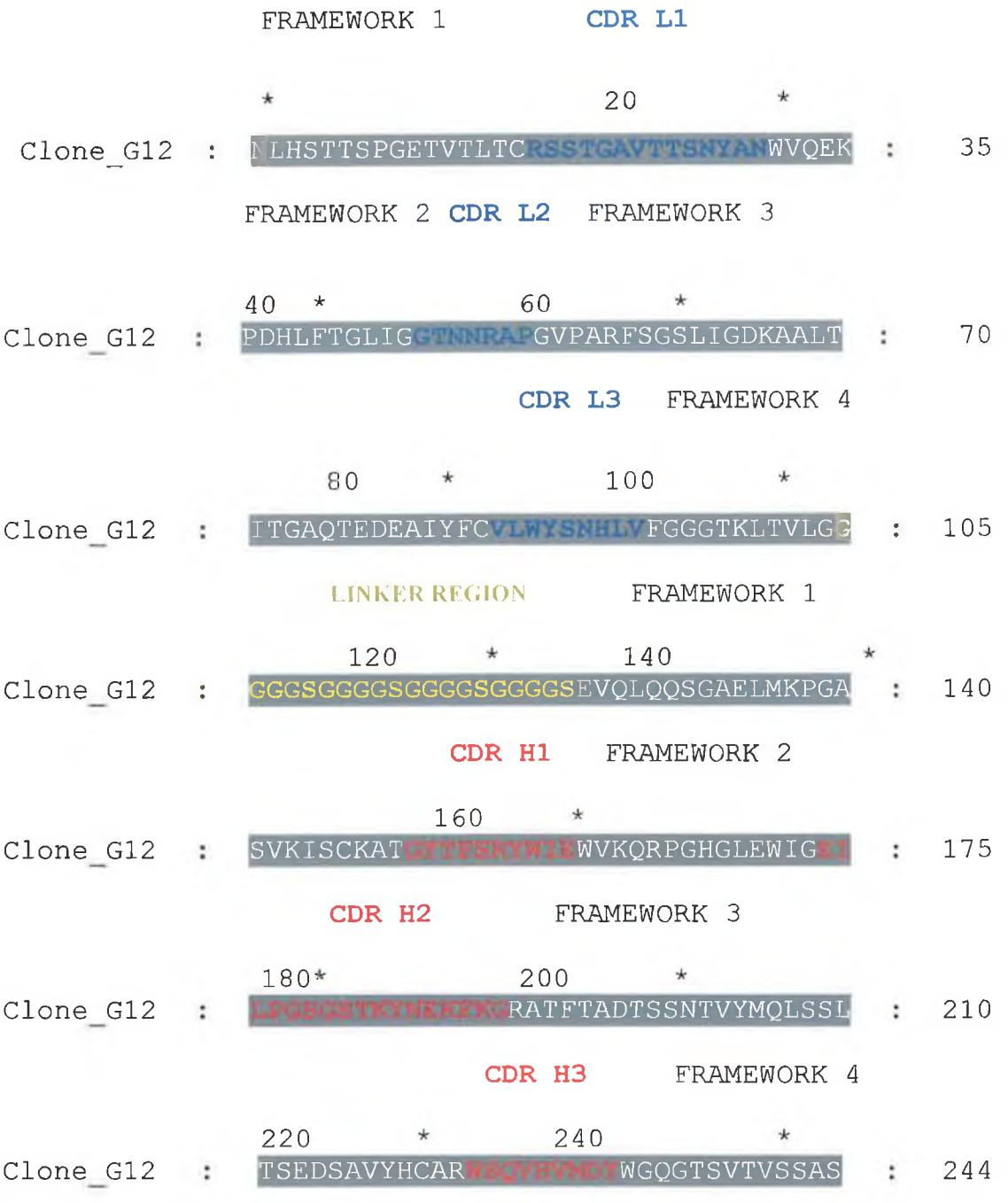


Figure 3.2.1: *Amino acid sequence analysis of clone G12, capable of specifically detecting illicit heroin residues. Light chain CDR regions are shown in blue font and heavy chain CDRs are shown in red font. The* $(Gly_4Ser)_4$ *linker region is shown in yellow.*

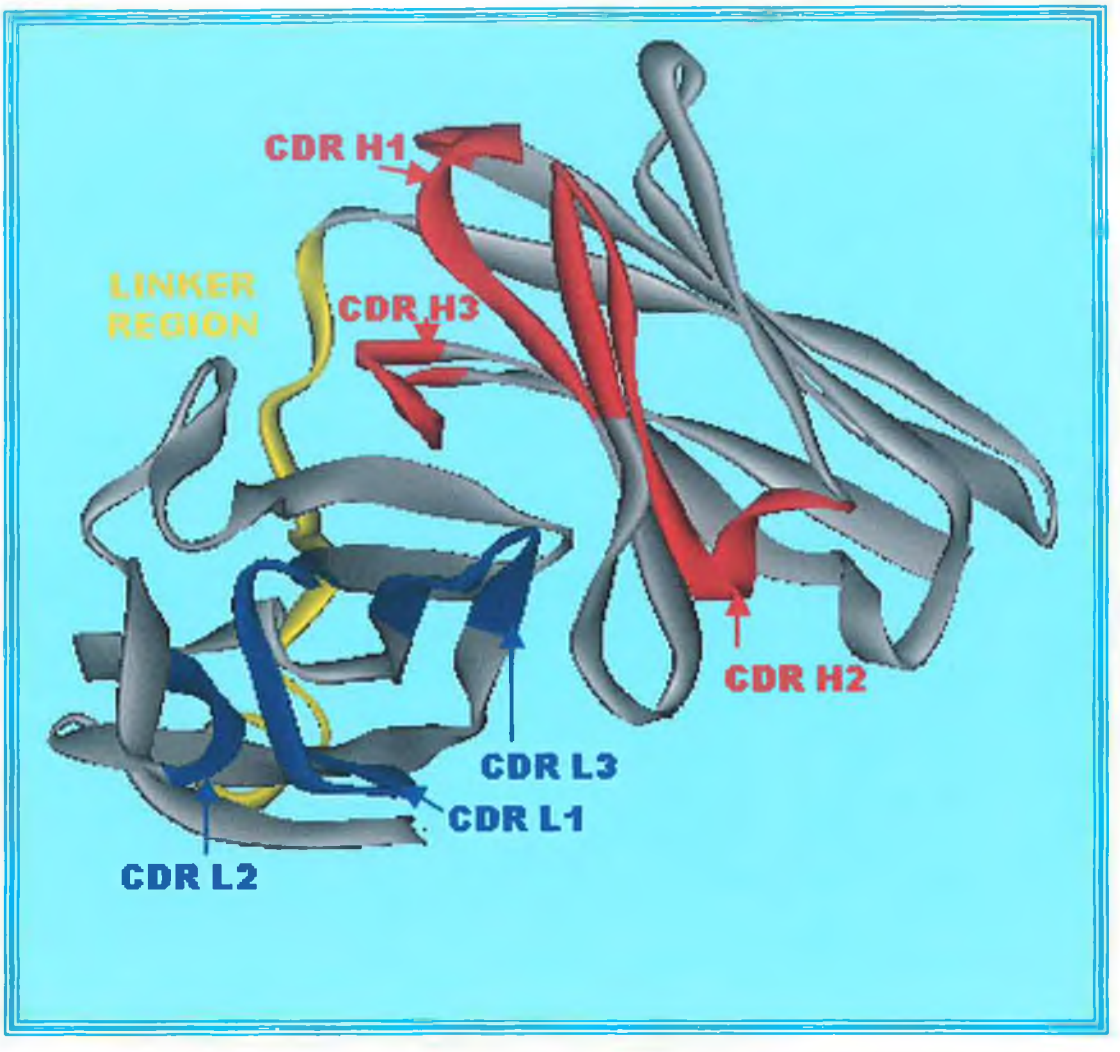


Figure 3.2.2: *Ribbon illustration of anti-M3G clone G12 scFv antibody. The antibody structure was modelled using DS ViewerPro modelling software. Heavy chain CDRs are shown in red and light chain CDRs are shown in blue. The linker region is shown in yellow.*

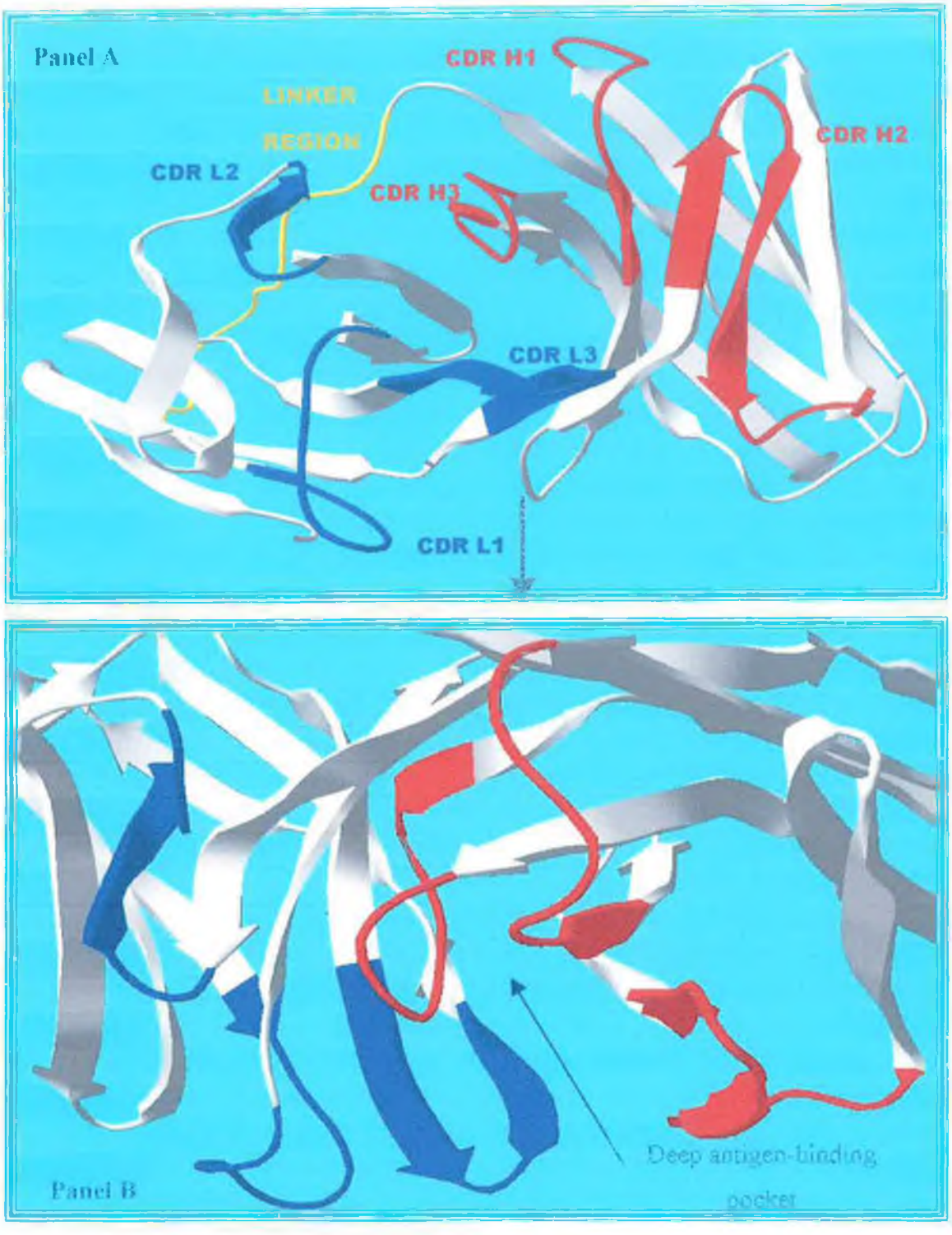


Figure 3.2.3: *Schematic homology model of scFv antibody derived from clone G12. Panel A illustrates a front view of the antibody, looking into the antigen-binding pocket and panel B represents an enlarged view, looking into the antigen-binding pocket. Beta sheets are shown as arrows and coils are represented as tubes. Heavy chain CDRs are shown in red and light chain CDRs are shown in blue. The linker region is shown in yellow.*

3.2.2 Subcloning of scFv gene into pAK, 500 and 600

The pAK 500 vector encodes a double helix for dimerisation and the pAK 600 vector encodes the bacterial alkaline phosphatase gene. The pAK 400 vector, harbouring the scFv gene of interest, was isolated from XL-1 Blues, as detailed in Section 2.4.6.1. The 800-base pair insert, encoding the variable heavy and light chain regions, connected via a $(gly_4ser)_4$, was isolated from the expression plasmid, using the *Sfi 1* restriction sites, flanking the region. This was ligated into previously digested plasmids, pAK 500 (4,094 base pairs) and pAK 600 (5,269 base pairs). Figure 3.2.4 demonstrates restriction analysis of each plasmid. Lane 2 shows undigested pAK 400 plasmid, the higher band representing the supercoiled form of plasmid DNA and the lower band representing the open circular form of plasmid DNA. Lane 3 illustrates an *Sfi* 1 digestion of the pAK 400 plasmid with the 800 bp scFv gene removed. Lane 4 contains undigested pAK 500 plasmid (6,195 bp), which is only visible in its open circular form. Although an undigested plasmid never migrates exactly according to its size, opencircular DNA will migrate more slowly than supercolied DNA in an agarose gel. Lane 5 illustrates an *Sfi 1* digestion of the pAK 500 plasmid, the 4,094 bp band corresponding to the restricted vector and the 2,101 bp band corresponding to the tetracycline 'stuffer' gene. Lane 6 contains undigested pAK 600 plasmid (7,370 bp). The plasmid DNA is only visible in its supercolied form, which migrates at a faster rate through the gel. Lane 7 illustrates an *Sfi 1* digestion of the pAK 600 plasmid, the 5,269 bp band corresponding to the restricted vector and the 2,101 bp band corresponding to the tetracycline 'stuffer' gene. The 'stuffer genes' was removed from both pAK 500 and 600 vectors and were replaced by the 800 base pair insert, encoding the scFv from pAK 400. Each ligated plasmid was transformed into competent *E. coli* JM83 cells, as described in Section 2.4.6.7, and recombinant bacteria were selected using chloramphenicol resistance to identify transformants.

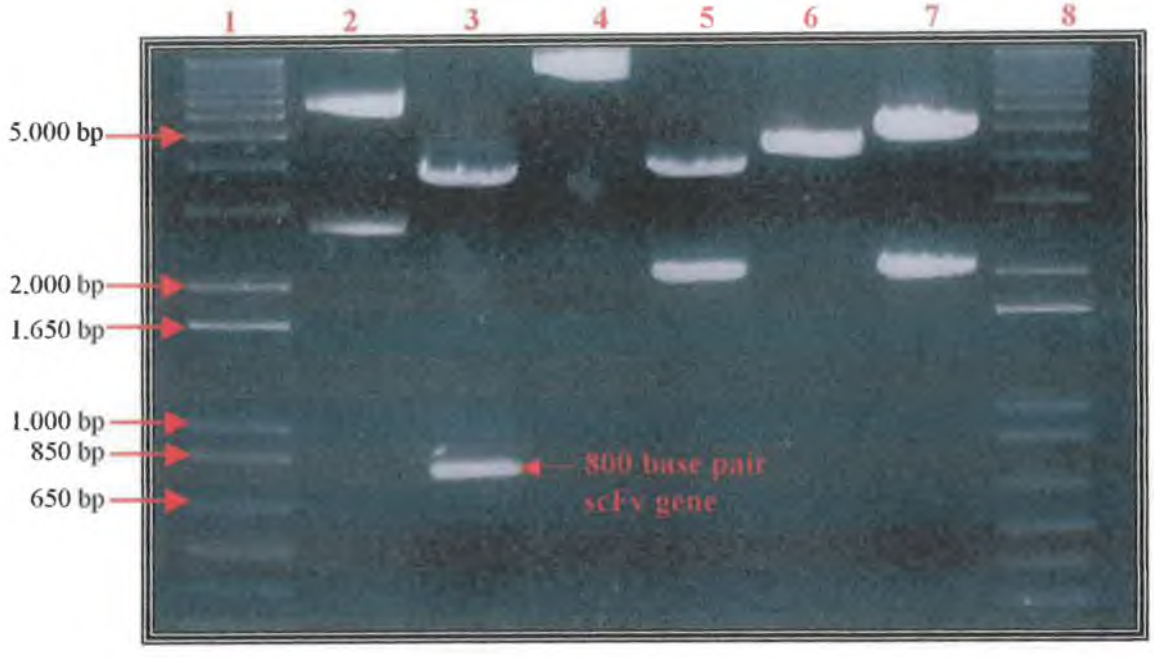


Figure 3.2.4: *Restriction enzyme digest on pAK 400, 500 and 600 using SfiI. Lane 1: 1 Kb Plus DNA ladder (Gibco-BRL); Lane 2: Unrestricted pAK 400 plasmid DNA, containing scFv gene; Lane 3: SfiI restricted pAK 400 with 800 bp scFv insert removed; Lane 4: Unrestricted pAK 500 plasmid DNA; Lane 5: SfiI restricted pAK 500 with 2101 bp tetracycline insert removed; Lane 6: Unrestricted pAK 600 plasmid DNA; Lane 7: SfiI restricted pAK 600 with 2101 base pair tetracycline insert removed; Lane 8: 1 Kb Plus DNA ladder.*

3.2.3 Soluble expression of monomeric, dimeric and dimeric alkaline phosphatase-labelled scFvs

Single colonies of pAK 400, 500 and 600 transformants were grown in 2 x TY until cells reached the exponential phase of growth i.e. $O.D._{600nm} = 0.5$. At this stage antibody expression was induced with IPTG. Expression was allowed to proceed for 4 hours for monomeric antibodies. However, induction time needed to be increased to 8 hours for successful expression of larger dimeric antibodies (data not shown). The antibody was secreted into the periplasm without any significant cell lysis, as only trace amounts were isolated from the culture supernatant (as demonstrated in Figure 3.2.5 and 3.2.6). For this reason, the antibody was isolated from the periplasmic space of bacteria using osmotic shock for all subsequent expressions. Once the

location of expressed antibodies had been identified, the inducer concentration was optimised. For expression of monomeric scFv, an IPTG concentration of 0.1 mM proved to yield the highest expression levels (Figure 3.2.7). The strength of inducer had to be increased for expression of larger, more complex proteins. Dimeric antibodies, expressed with the pAK 500 vector required 0.5 mM IPTG for optimal expression levels and dimeric alkaline phosphatase-labelled scFv, produced with the pAK 600 expression vector, required 1 mM IPTG for optimal expression (Figures 3.2.8 and 3.2.9).

The bifunctional nature of scFv produced using pAK 600 vector was analysed by immunoblot analysis (Figure 3.2.10). 125 µg/ml of M3G-OVA conjugate was sprayed onto nitrocellulose and allowed to dry. The membrane was cut into strips and each strip was blocked with 4% (w/v) MPBS. A 10 µl volume of crude periplasmic lysate was added to each strip in 1 ml of 1% (w/v) MPBS, containing increasing concentrations of free M3G. Bound antibody was detected using BCIP/NBT chromogenic substrate. This demonstrates the bifunctional nature of the scFv-AP fusion protein. The antibody can be seen to specifically bind to M3G, while retaining alkaline phosphatase enzymatic activity

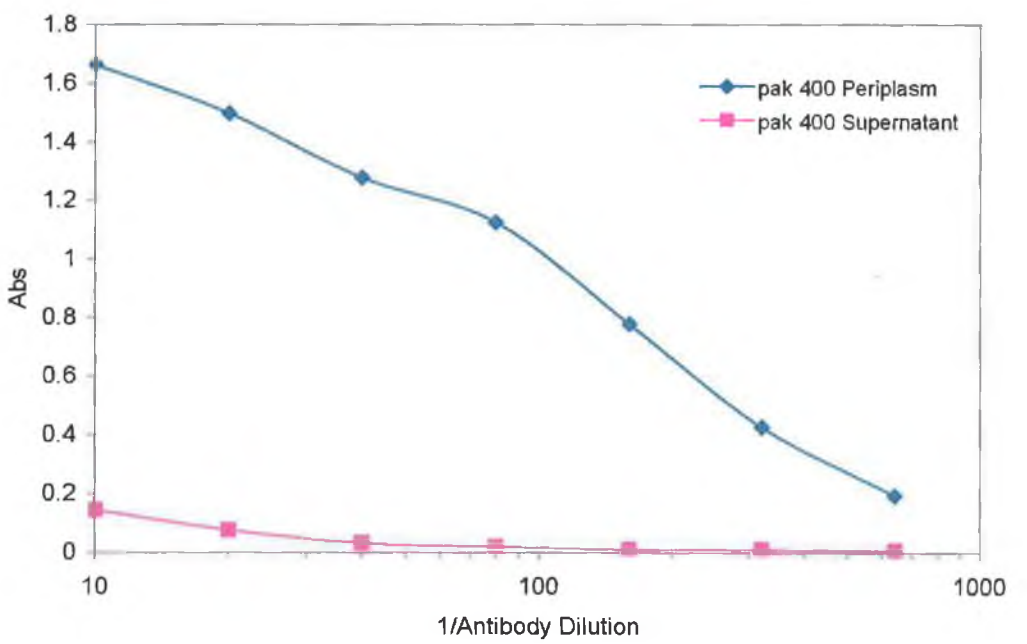


Figure 3.2.5: *ELISA analysis of culture supernatant and bacterial periplasm, following soluble expression of the monomeric scFv. Monomeric scFv was found to be located in the periplasm of bacterial cells.*

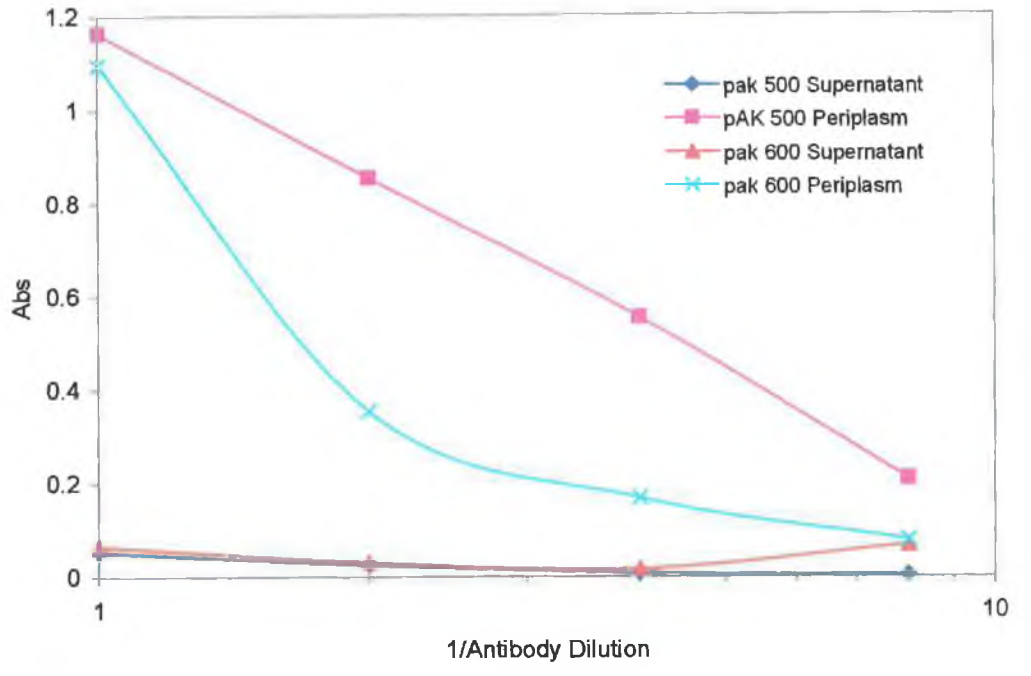


Figure 3.2.6: *ELISA analysis of culture supernatant and bacterial periplasm, following soluble expression of the dimeric and dimeric enzyme-labelled scFvs. Both scFv's were found to be expressed into the periplasm of bacterial cells*

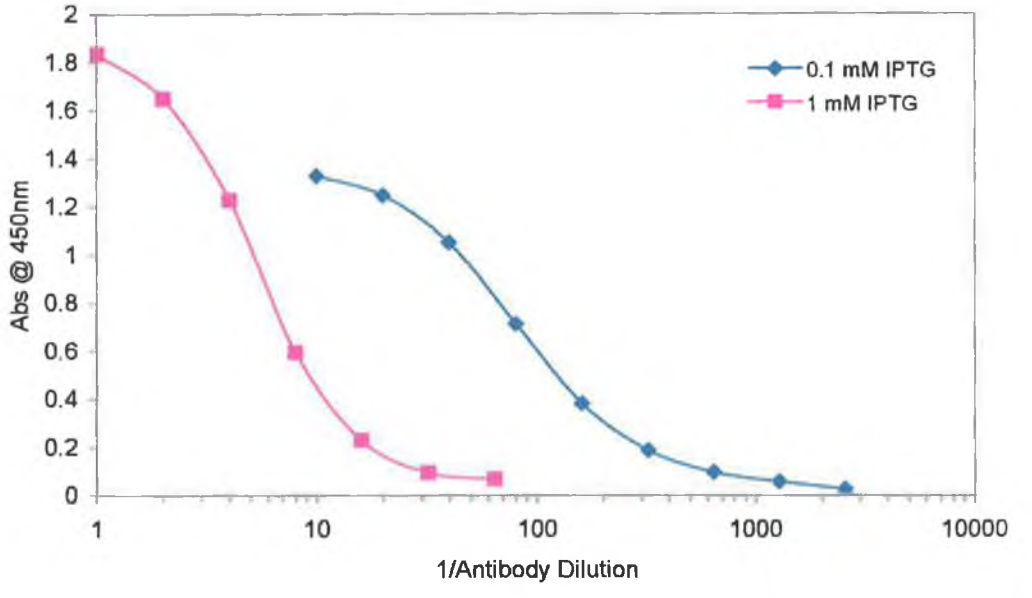


Figure 3.2.7: *Optimisation of IPTG concentration for monomeric scFv, expressed with pAK 400 vector. Results show ELISA analysis of antibody present in the periplasmic lysate fraction following induction with 1 mM and 0.1 mM IPTG. The optimal inducer concentration was found to be 0.1 mM.*

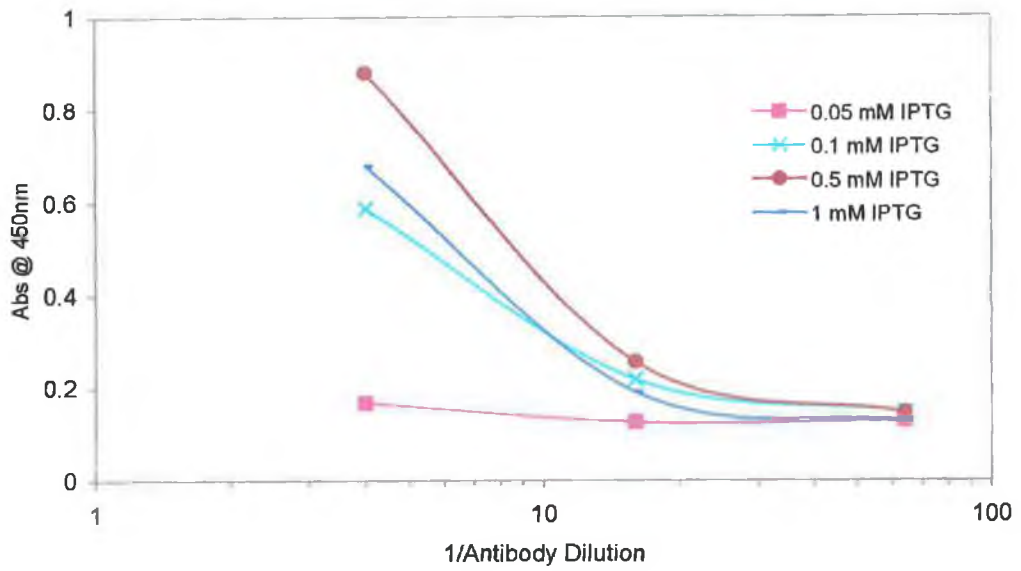


Figure 3.2.8: *ELISA analysis of the periplasmic lysate fraction for antibody activity, following induction with 1, 0.5, 0.1 and 0.05 mM IPTG. The optimal inducer concentration was found to be 0.05 mM IPTG, followed by an 8 hour induction period at 26°C.*

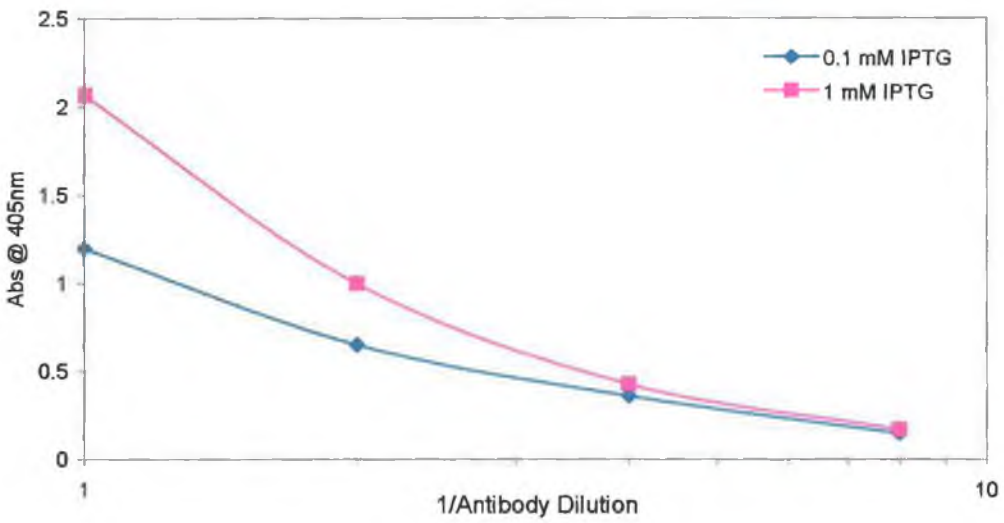


Figure 3.2.9: *Optimisation of inducer concentration for expression of alkaline phosphatase-labelled dimeric scFv. ELISA analysis was carried out to determine antibody titre in the periplasmic lysate fraction following induction with 1 and 0.1 mM IPTG. The optimal inducer concentration was found to be 1 mM IPTG.*

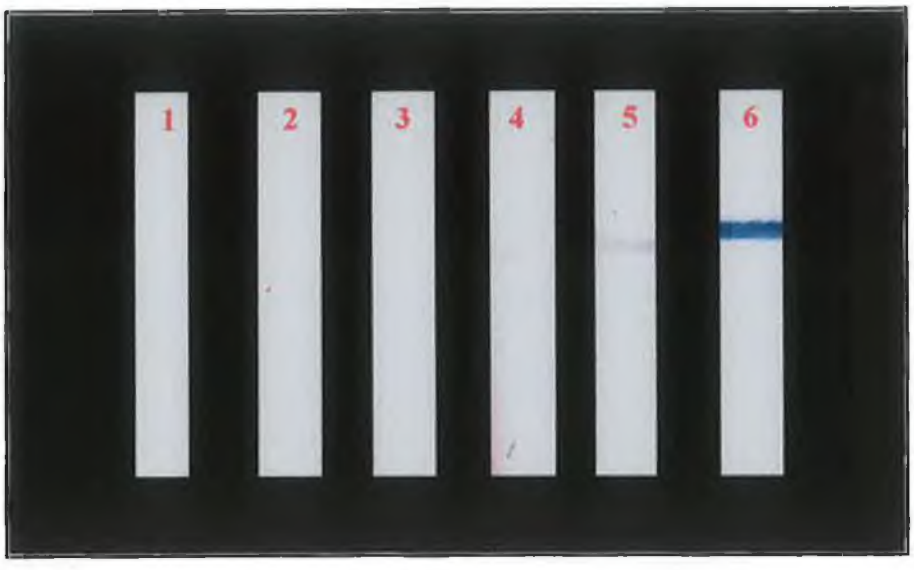


Figure 3.2.10: *Immunoblot analysis demonstrating the bifunctional nature of alkaline phosphatase-labelled dimeric scFv produced using pAK 600 expression vector. The antibody is capable of specifically binding M3G, while retaining alkaline phosphatase enzymatic activity. Blot 1 was sprayed with PBS and incubated with enzyme-labelled dimeric scFv and developed using BCIP/NBT. This illustrates that the antibody does not bind non-specifically to the membrane. Blot 2 shows monomeric scFv, incubated on a strip sprayed with 125 µg/ml M3G-OVA and developed with BCIP/NBT. This illustrates that monomeric scFv is not bifunctional. Blot 3-6 were sprayed with 125 µg/ml M3G-OVA and each one incubated with 10 µl of bifunctional scFv in 1% (w/v) MPBS, and standard concentrations of M3G. A concentration of 100 µg/ml M3G was incubated with blot 3. This was decreased to a concentration of 10 µg/ml in blot 4, 1 µg/ml in blot 5 and 0 µg/ml in blot 6. The signal intensity increases as the concentration of M3G decreases. In blot 3 and 4, no signal is seen. A faint band can be seen in blot 5, incubated with 1 µg/ml M3G and an intense signal can be seen in the absence of M3G in blot 6. This illustrates that the antibody is in fact bifunctional and is capable of specifically detecting M3G, while retaining the enzymatic activity of alkaline phosphatase. As the strips were incubated with unpurified antibody, the blot also illustrates that the antibody can be specifically detected directly from crude E. coli cell lysate.*

3.2.4 Purification of monomeric dimeric and bifunctional scFvs

Initial attempts to purify monomeric scFv by IMAC, via the hexahistidine tail, proved unsuccessful. Problems were also encountered with this method of purification for scFvs produced in a similar manner by Dillon[B] *et al.* (2003). It was thought this was either due to low expression yields, in relation to expression levels of other *E. coli* proteins or through denaturation of the histidine tag. For this reason it was decided to employ antigen-affinity chromatography to purify the antibody. This involved the immobilisation of an M3G-BSA conjugate to a sepharose column. The periplasmic fraction of bacterial cells was applied to the column and contaminating proteins removed through several washes with PBST and PBS. The antibody was then released from the column using an acidic shock. The specificity of the antibody for its target was therefore exploited to achieve a high degree of purity, as demonstrated by SDS PAGE (Figure 3.2.11). The only drawback from this method was the dilution factor of the sample during the purification process. Western blot analysis (Figure 3.2.12) revealed a single band at 32 kDa for purified and unpurified monomeric scFv. This demonstrates that both secondary and tertiary antibodies are capable of specifically detecting the antibody, even when present in the crude bacterial lysate preparation.

The dimeric scFv produced had a significantly higher avidity for the antigen of interest, and therefore, could not be eluted from the affinity resin with an acidic shock. Increasing the stringency of the elution conditions was found to severely affect the antibody's activity. For this reason, the pentahistidine tag on the secreted antibody was employed as a handle for purification on a Ni-NTA resin. The dimer showed increased affinity for the resin compared to the monomeric scFv, due to the presence of two histidine tags on each scFv (data not shown). Although this offered a simple clean-up step of the lysate fraction, the purified antibody fraction still contained a large proportion of impurities. Western blot analysis of the dimeric scFv yielded three bands, one at 32 kDa, representing monomeric scFv, a predominant band at 36 kDa, representing the reduced monomeric fusion protein incorporating the scFv attached to the helix and a faint band at and 77 kDa representing the dimeric version of the scFv. This demonstrates the stability of the aliphatic helix as a dimerisation

domain, as a small proportion still remains intact, even under the denaturing conditions of SDS–PAGE. From this analysis, it was concluded that the secondary antibody to the flag tag, was capable of specifically detecting the scFv as no background was observed from contaminating *E. coli* proteins (Figure 3.2.12). The antibody could be used in this form for ELISA analysis as no interference from contaminating proteins was observed.

Several attempts were made to purify pAK 600 lysate on an anti–flag M1 agarose resin. However, the affinity of the resin for the flag tag of the scFv proved too weak to bind the antibody sufficiently. There are several possibilities for this. Firstly, the flag tag may have been inefficiently processed or cleaved during lysis of the periplasm. Secondly, the antibody recognition site may be inaccessible for binding due to steric hindrance by the enzyme. However, this is not the case as the anti-flag M1 antibody was capable of binding the scFv fusion protein in an ELISA situation (data not shown). Using the flag protein as a standard it was observed that there were variations in the binding capabilities of the resin supplied and, therefore, it was not possible to further optimise it for purification. From immunoblot analysis, as shown in Figure 3.2.10, it was demonstrated that the antibody could be specifically detected from crude *E. coli* lysate, as a band is only observed following detection of the M3G-OVA conjugate. *E. coli* lysate from cells lacking the pAK 600 vector gave no detectable colour change when mixed with pNPP substrate. This proved that the *E. coli* lysate itself contained no contaminating proteins that possessed alkaline phosphatase activity (data not shown). For this reason the antibody could be used in this form in subsequent ELISA analysis. Western blot analysis of the bifunctional scFv (Figure 3.2.12) yielded 3 bands, at sizes of 85 kDa, 34 kDa and 32 kDa. The band at 85 kDa represents the reduced, monomeric form, of the alkaline phosphatase-scFv fusion protein and the band at 32 kDa, represents monomeric scFv. The band at 34 kDa represents a minor degradation product.

In order to increase expression levels of these antibodies various expression protocols were investigated. The fed-batch method of expression, described for use in the Griffin.1 Library (Section 2.6.10), proved the most effective, with dramatically increased expression levels. This method of expression prevented any degradation of proteins through cell lysis as only viable cells were harvested. The use of Terrific

Broth media was also chosen over 2 x TY, as it has been reported to improve the yield of plasmid-bearing *E. coli* by extending the period of time the cells remain in log-phase growth. The high concentrations of tryptone and yeast extract provide the necessary nutrients and cofactors for cell growth, while the increased concentration of yeast extract facilitates elevated cell yields. Glycerol acts as a source of carbon and energy, which unlike glucose, is not fermented to acetic acid. Potassium phosphate salts provide potassium for cellular systems and act as a buffer, preventing cell death due to a decrease in pH. IMAC chromatography was subsequently employed to purify monomeric and dimeric antibodies. Chelating fast flow sepharose coupled to iminodiacetic acid by a stable ether linkage, via a seven-atom spacer was employed for this purpose. This was charged with nickel ions for successful capture of histidine tags. Both monomeric (hexahistidine) and dimeric (pentahistidine) scFvs could be eluted in a pure form and free from contaminating proteins using 250 mM imidazole (Figure 3.2.13). The success of this purification was attributed to the spacer arm separating the Ni-IDA complex from the resin, which facilitated binding of the histidine tag located at the carboxy terminus of the antibody. Using the one-step expression protocol, described earlier, expression levels of scFv were below levels of quantification. Using the fed-batch method of expression however, high levels of pure scFvs could be produced in our laboratory (Table 3.2.1). The successful use of IMAC chromatography for scFv purification, also served as a useful means of increasing the concentration of scFvs in a single-step. Western Blot analysis (Figure 3.2.14), demonstrates that the intensity of scFv bands, following purification, is more than 10 times greater than that of unpurified antibodies.

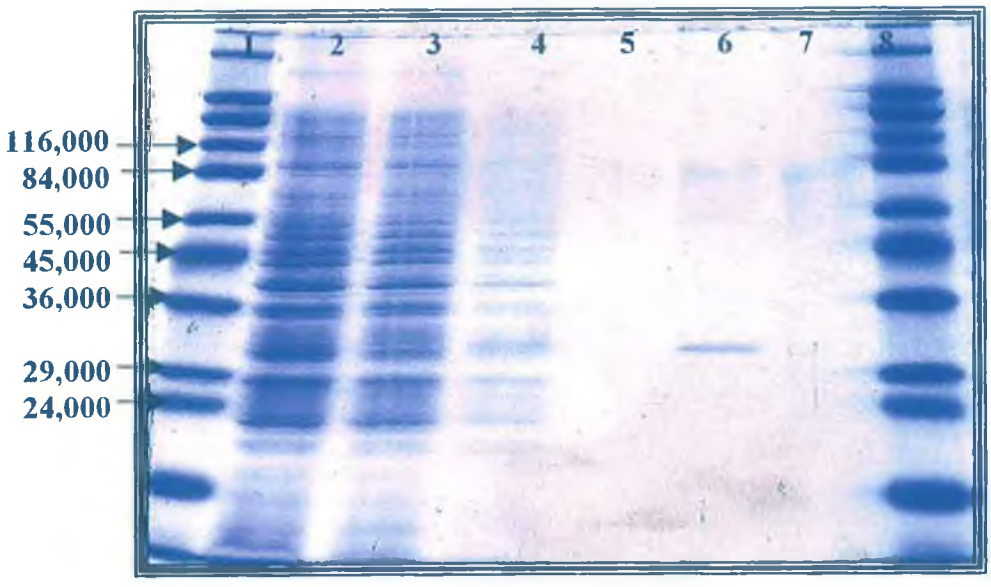


Figure 3.2.11: *SDS-PAGE analysis of purified monomeric scFv. Lane 1: Molecular weight markers, Lane 2: Periplasmic lysate; Lane 3: Flow through; Lane 4: Wash 1; Lane 5: Wash 2; Lane 6: Eluted purified monomeric scFv; Lane 7: Blank; Lane 8: Molecular weight markers.*

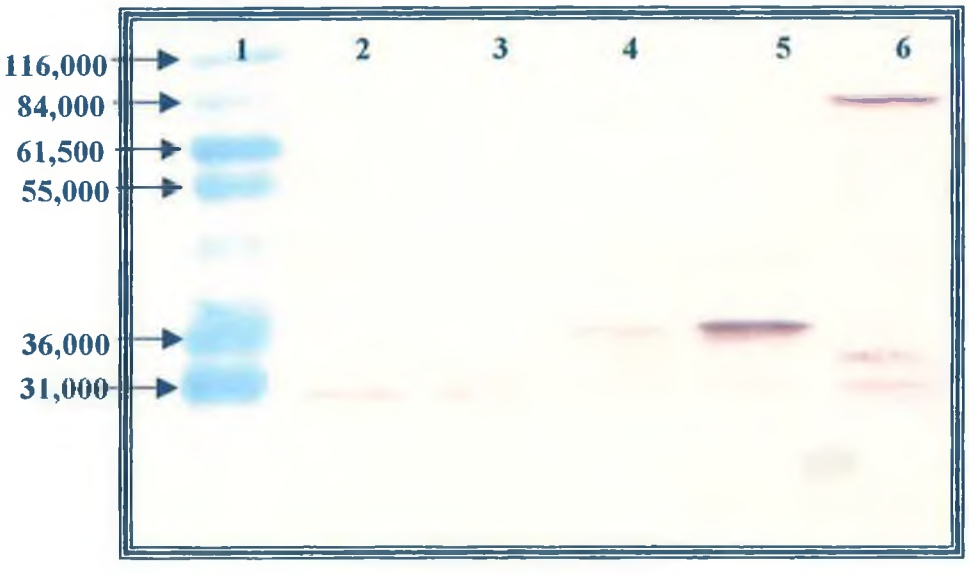


Figure 3.2.12: *Western blot analysis on the monomeric, dimeric and bifunctional scFvs. The membrane containing the transferred scFvs was probed using the anti-flag monoclonal M1 antibody followed by an alkaline phosphatase-labelled anti-mouse antibody and visualised with BCIP/NBT substrate. Lane 1: Molecular weight markers; Lane 2: Unpurified monomeric scFv; Lane 3: Purified monomeric scFv; Lane 4: Unpurified dimeric scFv; Lane 5: Purified dimeric scFv; Lane 6: Bifunctional scFv.*

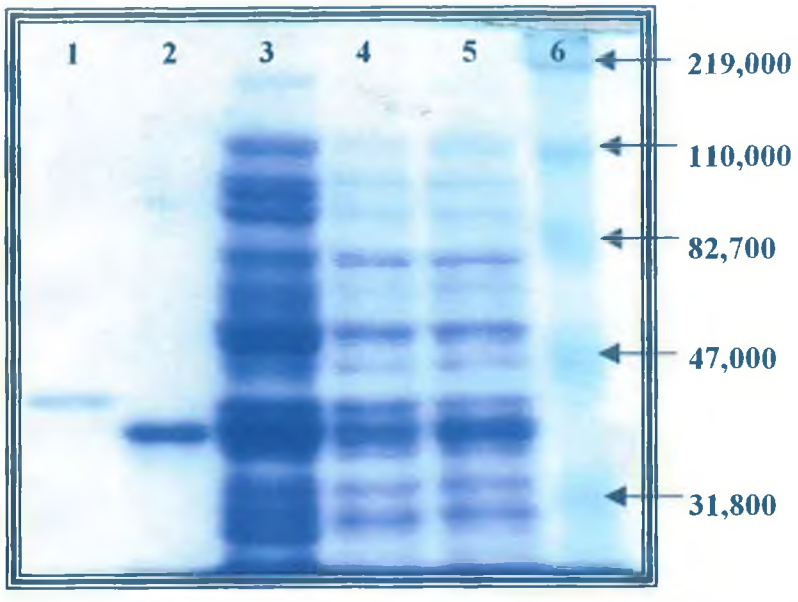


Figure 3.2.13: *SDS PAGE analysis of monomeric, dimeric and bifunctional scFvs expressed using the fed-batch method and purified by IMAC chromatography. Lane 1: Purified dimeric scFv; Lane 2: Purified monomeric scFv; Lane 3: Unpurified bifunctional scFv; Lane 4: Unpurified dimeric scFv; Lane 5: Unpurified monomeric scFv; Lane 6: Molecular weight markers*

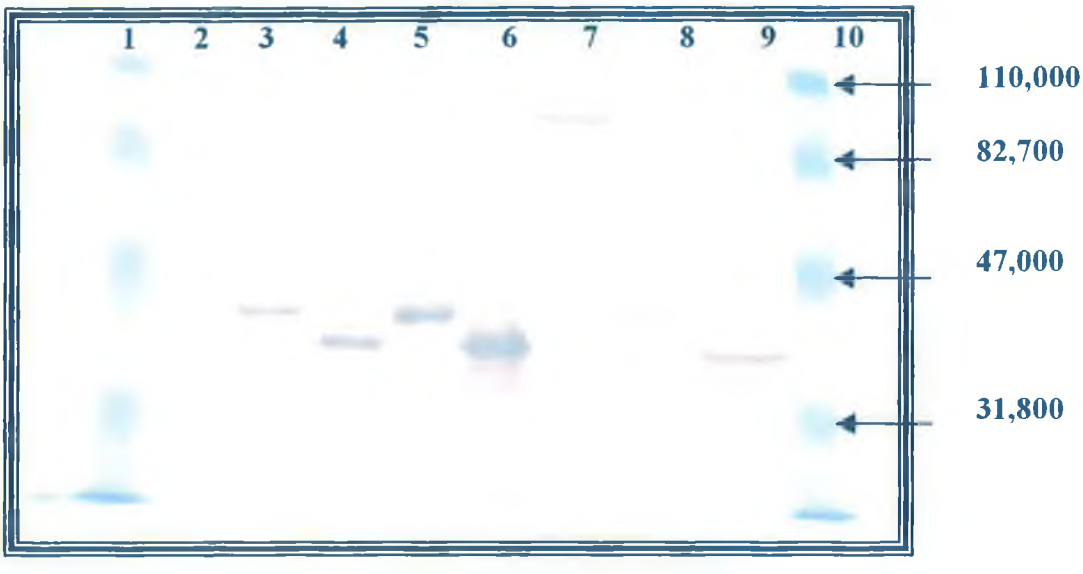


Figure 3.2.14: *Western Blot analysis of monomeric, dimeric and bifunctional scFvs expressed using the fed-batch method and purified by IMAC chromatography. Lane 1: Molecular weight markers; Lane 2: Negative control; Lane 3: Purified dimeric scFv (1 in 5 dilution); Lane 4: Purified monomeric scFv (1 in 5 dilution); Lane 5: Purified dimeric scFv (neat); Lane 6: Purified monomeric scFv (neat); Lane 7: Unpurified bifunctional scFv (neat); Lane 8: Unpurified dimeric scFv (neat); Lane 9: Unpurified monomeric scFv (neat); Lane 10: Molecular weight markers.*

Table 3.2.1: *Approximate expression levels achieved for monomeric and dimeric scFvs produced using the fed-batch method of expression and purified by IMAC chromatography. The concentration of scFvs was determined by BCA assay, using a quantified human scFv to as a standard for protein quantification.*

Antibody	Initial Culture Volume (mls)	Concentration (μg/ml)	Total Purified Volume (ml)	Total Expression (mg/L)
Monomeric scFv	200	309.17	6.25	9.28
Dimeric scFv	200	65.83	4	1.48

3.2.5 The development of a competitive immunoassay for the detection of M3G using genetically-derived scFv antibodies

Following the optimisation of expression and purification of genetically-derived scFvs, their use in a competitive immunoassay format for the detection of M3G was investigated. In order to maximise assay sensitivity, the optimal coating concentration of conjugate and working dilution of antibody had to be established, by means of a checkerboard ELISA.

3.2.5.1 Indirect checkerboard ELISA for determination of optimal assay parameters

The optimal concentration of coating conjugate and antibody working dilution were determined by indirect checkerboard ELISA. Varying coating concentrations of M3G-OVA coating conjugate and scFv working dilutions were optimised for each antibody. For maximum assay sensitivity to be attained, the minimum conjugate coating concentration, that exhibits a steep binding curve, containing an extended linear portion must be chosen. A dilution of antibody that yields 50 to 70% of maximal absorbance, located in the steepest part of the linear portion of the curve, should chosen for subsequent analysis. Checkerboard analysis of monomeric scFv, involved coating with M3G-OVA conjugate concentrations from, 100 µg/ml to 1.56 µg/ml. These were analysed against antibody dilutions, ranging from 1 in 5 to 1,280. Bound antibody was detected using anti-flag M1 monoclonal antibody, followed by peroxidase-labelled anti-mouse antibody, and visualised using *o*-PD substrate. A coating concentration of 3.125 µg/ml M3G-OVA and a 1 in 60 scFv dilution were chosen as optimal (Figure 3.2.15). For dimeric scFv, M3G-OVA coating concentrations from 50 to 0.79 µg/ml were analysed against antibody dilutions from 1 in 10 to 1 in 640, with a coating concentration of 6.25 µg/ml and an antibody dilution of 1 in 150, chosen as optimal (Figure 3.2.16). The indirect checkerboard of the bifunctional alkaline phosphatase-labelled scFv required no secondary antibody as bound antibody was detected directly using the enzyme tag, at the carboxy terminus

of the antibody. A coating concentration of 3.125 μg/ml, with an scFv dilution of 1 in 20 were chosen as optimal for use in competitive ELISA (Figure 3.2.17).

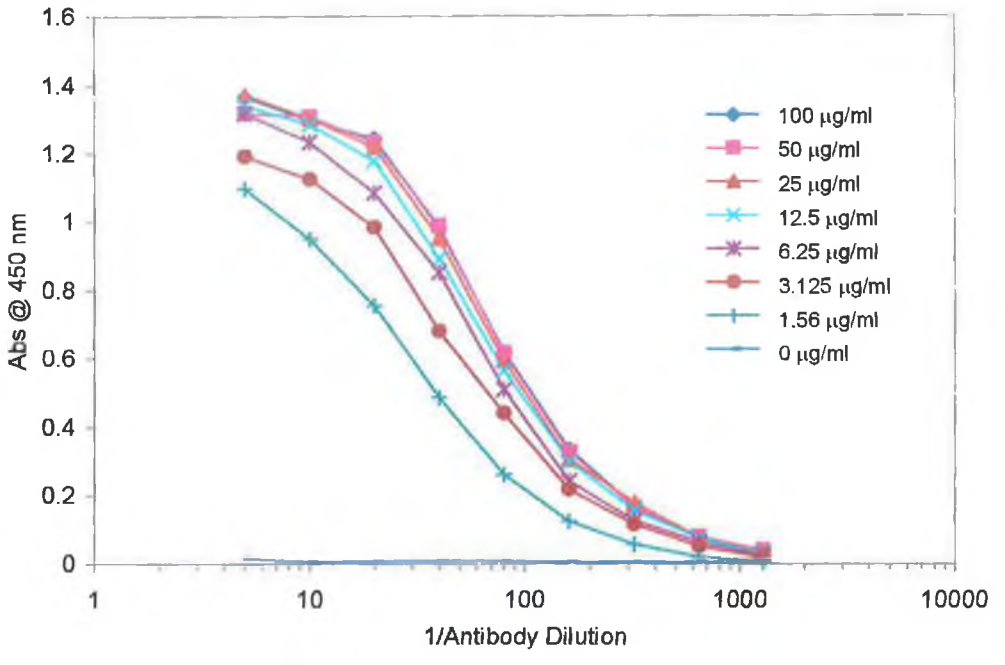


Figure 3.2.15: *Checkerboard ELISA of monomeric scFv expressed with the pAK 400 vector. An ELISA plate was coated with varying concentrations of M3G-OVA conjugate and varying dilutions of antibody were analysed. A conjugate coating concentration of 3.125 μg/ml was chosen as optimal, with an scFv dilution of 1/60. Bound scFv was detected using M1 anti-flag, followed by peroxidase-labelled anti-mouse antibody and colour visualised using the chromogenic substrate, o-PD.*

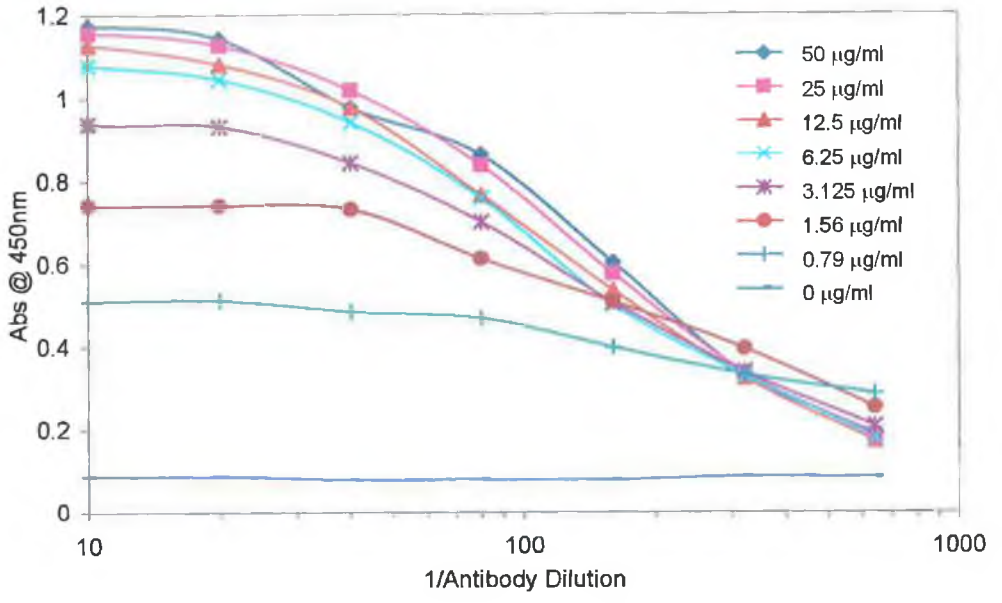


Figure 3.2.16: *Checkerboard of dimeric scFv expressed with the pAK 500 vector. A conjugate coating concentration of 6.25 µg/ml was chosen as optimal, with an scFv dilution of 1/150. Bound scFv was detected using M1 anti-flag, followed by peroxidase-labelled anti-mouse, and colour visualised using the chromogenic substrate o-PD.*

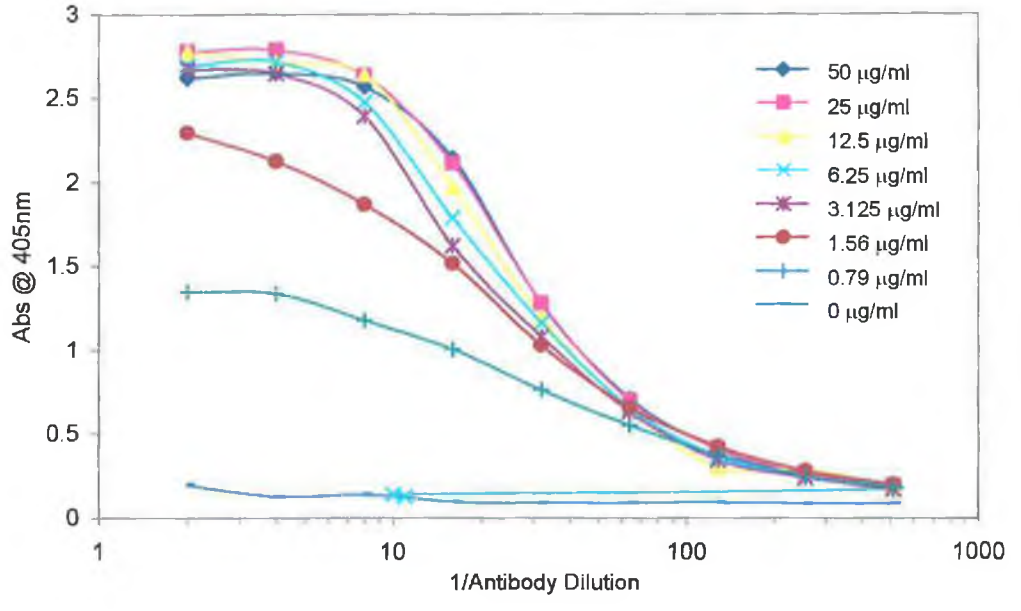


Figure 3.2.17: *Checkerboard of bifunctinal scFv expressed with the pAK 600 vector. A conjugate coating concentration of 3.125 µg/ml was chosen as optimal, with an scFv dilution of 1/20. Bound scFv was detected p-NPP chromogenic substrate.*

3.2.5.2 Development of a competitive ELISA assay for M3G using genetically-derived scFvs

Immunoassays exhibit a sigmoidal-shaped dose response curve, when response is plotted against the logarithm of analyte concentration. The curve consists of a relatively linear region around the point of 50% inhibition (IC_{50}); enclosed by two 'flat' asymptotic regions. The mean response is therefore a non-linear function of analyte concentration. Fitting a 'typical' linear standard curve to the data set will generate an overall bias in the dataset, particularly at the extremities. The 'reference standard' for fitting the mean concentration-response for immunoassay is a four-parameter logistic function (Findlay *et al.*, 2000). This model accurately reflects the relationship between measured response and logarithm of analyte concentration. Four parameter equations were fitted to immunoassay data sets using BIAevaluation 3.1 software, using the model described by the following equation:

$$R = R_{HI} - \frac{R_{HI} - R_{LO}}{1 + \left(\frac{Conc}{A_1}\right)^{A_2}}$$

Where:

R	=	*Expected Response*
R_{HI}	=	*Response at infinite analyte concentration*
R_{LO}	=	*Response at zero analyte concentration*
$Conc$	=	*Analyte concentration*
A_1	=	*Fitting Constant 1, concentration at IC_{50}*
A_2	=	*Fitting Constant 2, slope parameter that is typically equal to 1*

Calibration curves calculated using BIAevaluation software allow the degree of precision to be determined using the model generated to 'back-calculate' responses at each analyte concentration. A residual plot is also generated, which allows the user to analyse how close the model fits the dataset.

In order to determine intra-day assay variation, varying M3G concentrations were assayed five times on one day and the mean absorbance at each concentration was plotted against the log of analyte concentration. Intra-day studies for monomeric scFv yielded a calibration curve ranging from 763 to 195,000 pg/ml (Figure 3.2.18). The dimeric scFv and bifunctional scFvs yielded calibration curves ranging from to 763 781,250 pg/ml (Figures 3.2.19 and 3.2.20). The mean, standard deviations, coefficients of variation and percentage recoveries were calculated from the calibration curve for each antibody (Table 3.2.2 to 3.2.4).

The inter-day assay variation was calculated by performing five replicates of each sample over five separate days, giving an indication of the robustness of the assay. A calibration curve was constructed by dividing the mean absorbance response obtained at each M3G concentration (A) by the absorbance response in the presence of zero analyte (A0). Normalised absorbance values (A/A0) were plotted against log of M3G concentration to generate a calibration curve. Detection limits were set at the concentration of analyte that inhibited 10% of antibody binding (Hennion and Barcelo, 1998). Inter-day studies showed the monomeric scFv had detection limit of 2 ng/ml, with coefficients of variation (C.V.'s) obtained ranged from 3.23 to 9.8% obtained for the calibration curve (Figure 3.2.21 and Table 3.2.5). The dimeric scFv was less sensitive, with a detection limit of 9 ng/ml, with C.V.'s ranging from 2.67 to 9.6% for the dataset (Figure 3.2.22 and Table 3.2.6). The bifunctional scFv had a 5 ng/ml limit of detection (Figure 3.2.23). The mean, standard deviations, coefficients of variation and percentage recoveries were calculated from the calibration curves for each antibody (Table 3.2.5 to 3.2.7).

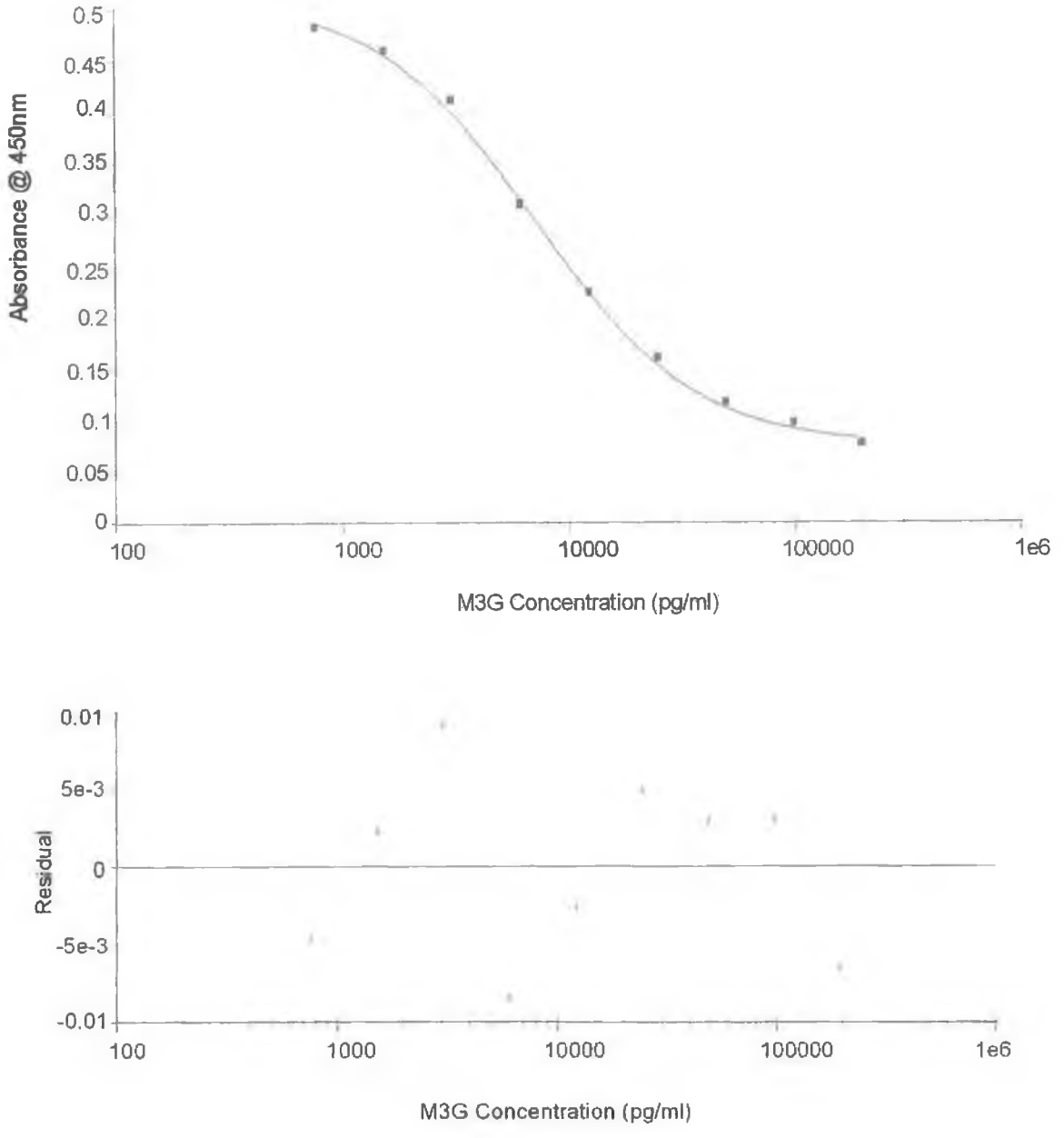


Figure 3.2.18: *Intra-day ELISA assay using pAK 400 monomeric scFv to detect free M3G in PBS. M3G-OVA was coated at 3.125 μg/ml and a 1 in 60 scFv dilution was used. Bound antibody was detected using M1 monoclonal anti-flag, followed by peroxidase-labelled anti-mouse antibody and visualized using o-PD. Absorbance was read at 450nm and related to M3G concentration using 4-parameter logistic model ranging from 763 pg/ml to 195,000 pg/ml.*

Table 3.2.2: *Intra-day assay coefficients of variation for the detection of free M3G using the monomeric scFv. Five sets of nine standards were assayed over one day and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard. Percentage recovery was calculated from the four-parameter model produced using BIAevaluation 3.1 software.*

Concentration (pg/ml)	Absorbance @ 450nm ± S.D.	% C.V.	Back calculated concentration (pg/ml)	% Recovery
195,312.50	0.076 ± 0.005	6.62%	192,927.00	98.78
97,656.25	0.095 ± 0.008	8.48%	86,358.68	88.43
48,828.13	0.115 ± 0.008	7.27%	46,464.42	95.16
24,414.06	0.159 ± 0.015	9.71%	23,730.72	97.20
12,207.03	0.223 ± 0.005	2.43%	12,659.68	103.71
6,103.52	0.308 ± 0.012	4.04%	66,43.33	108.84
3,051.76	0.409 ± 0.020	4.81%	2,897.35	94.94
1,525.88	0.458 ± 0.016	3.51%	1,576.75	103.30
762.94	0.481 ± 0.036	7.50%	1,025.86	134.50

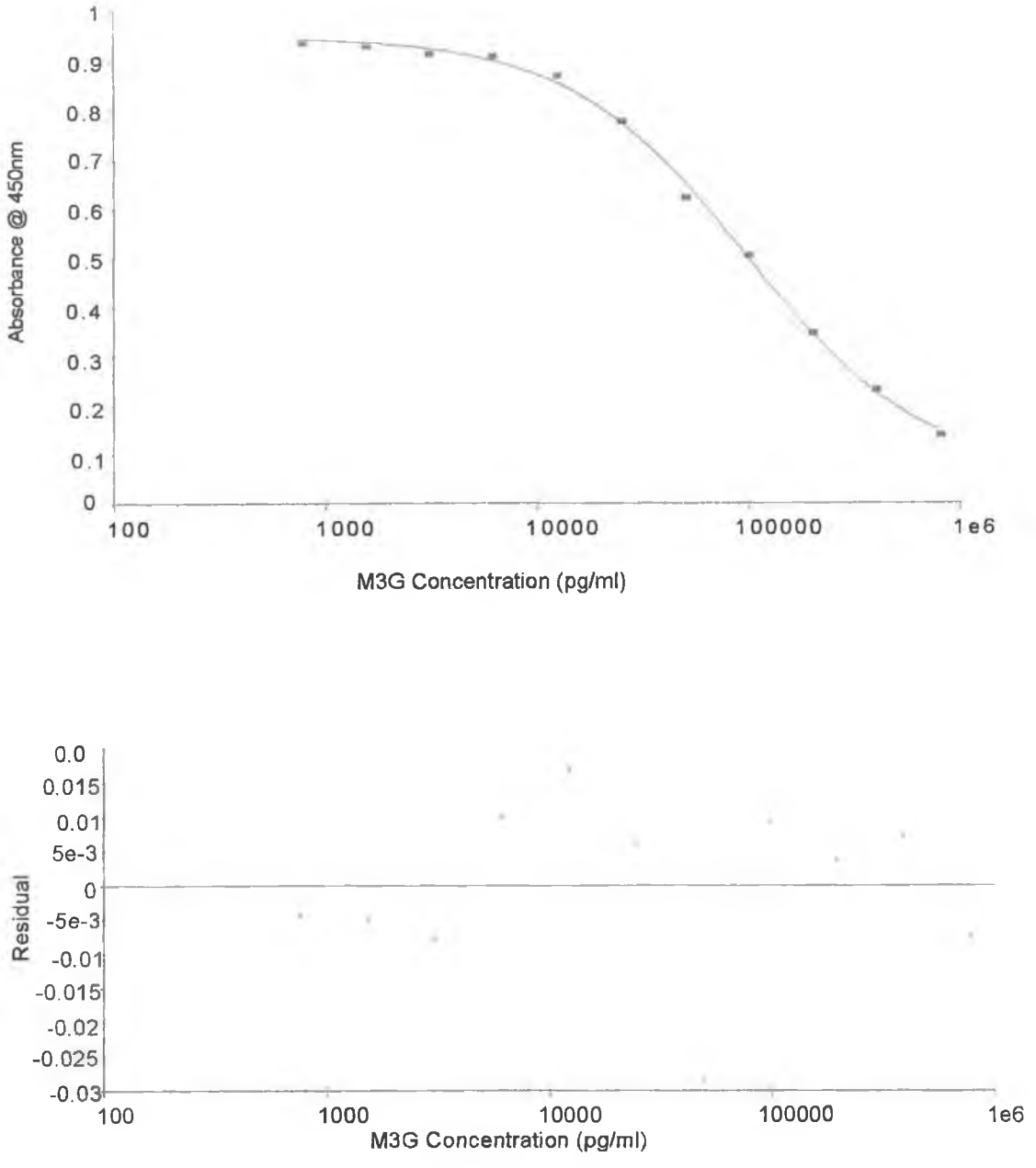


Figure 3.2.19: *Intra-day ELISA assay using pAK 500 dimeric scFv to detect free M3G in PBS. M3G-OVA was coated at 6.25 μg/ml and a 1 in 150 scFv dilution was used. Bound antibody was detected using M1 monoclonal anti-flag, followed by peroxidase-labelled anti-mouse antibody and visualized using o-PD. Absorbance was read at 450nm and related to M3G concentration using a 4-parameter logistic model that ranged from 763 pg/ml to 781,250 pg/ml.*

Table 3.2.3: *Intra-day assay coefficients of variation for the detection of free M3G using the dimeric scFv. Five sets of eleven standards were assayed over five different days and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard. Percentage recovery was calculated from the four-parameter model produced using BIAevaluation software.*

Concentration (pg/ml)	Absorbance @ 450nm ± S.D.	% C.V.	Back calculated concentration (pg/ml)	% Recovery
781,250	0.138 ± 0 .006	4.50%	856,468.9	109.63
390,625	0.230 ± 0.004	1.54%	371,514.3	95.11
195,312.50	0.345 ± 0.030	8.58%	191,879	98.24
97,656.25	0.503 ± 0.032	6.28%	93,806.06	96.06
48,828.13	0.620 ± 0.034	5.49%	55,876.56	114.44
24,414.06	0.777 ± 0.013	1.64%	23,416.27	95.91
12,207.03	0.868 ± 0.021	2.47%	9,933.61	81.38
6,103.52	0.908 ± 0.020	2.24%	4,858.06	79.59
3,051.76	0.915 ± 0.018	1.95%	4,001.51	131.12
1,525.88	0.930 ± 0.019	2.08%	2,120.70	138.98
762.94	0.938 ± 0.041	4.38%	1,284.12	168.31

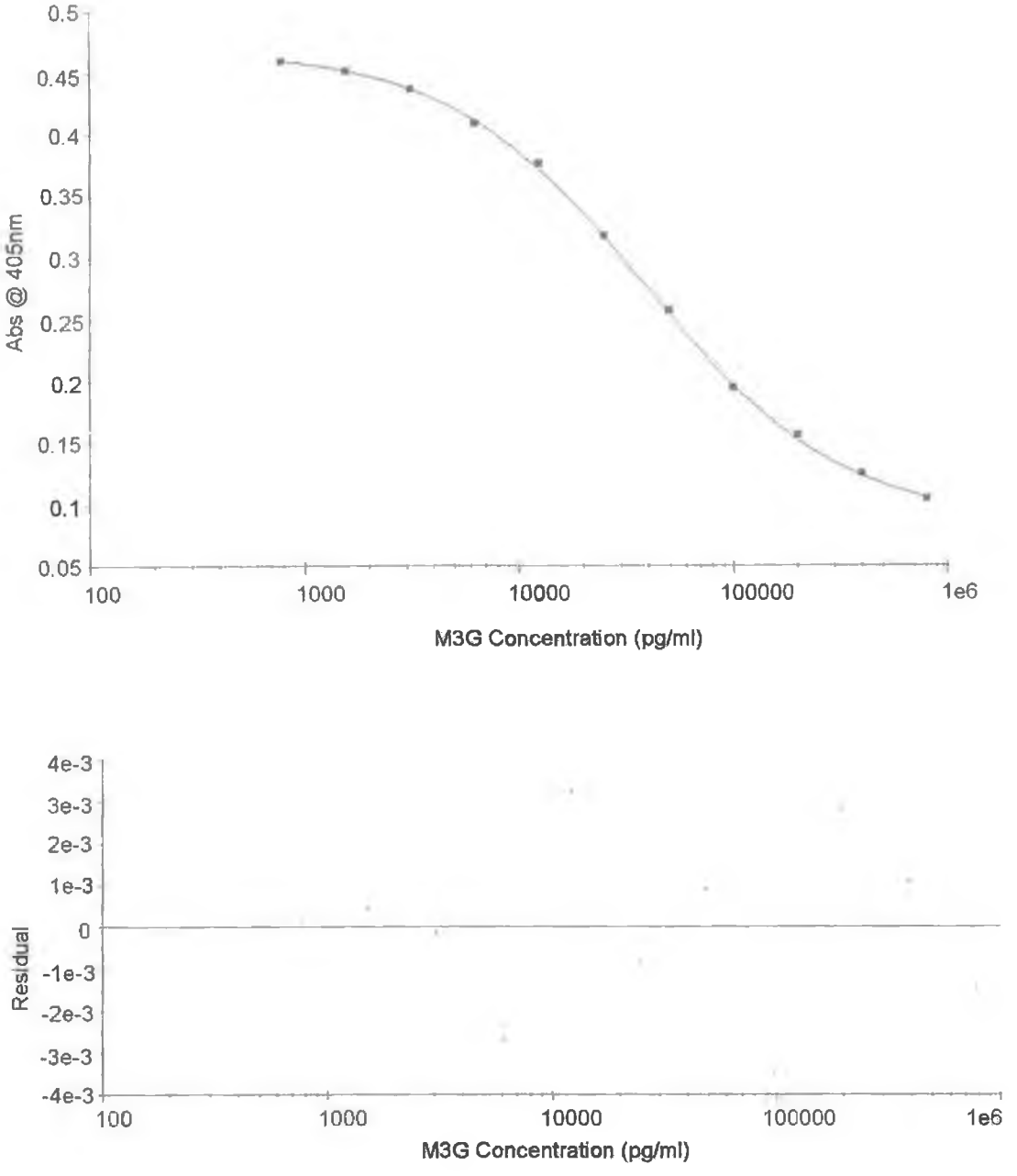


Figure 3.2.20: *Intra-day ELISA assay using pAK 600 enzyme-labelled dimeric scFv to detect free M3G in PBS. M3G-OVA was coated at 3.125 μg/ml and a 1 in 20 scFv dilution was used. Bound antibody was detected p-NPP substrate and absorbance read at 405nm. Mean response was related to M3G concentration using a 4-parameter logistic model that ranged from 763 pg/ml to 781,250 pg/ml.*

Table 3.2.4: *Intra-day assay coefficients of variation for the detection of free M3G using the bifunctional scFv, expressed with pAK 600 vector. Five sets of eleven standards were assayed over five different days and the C.V. 's were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard. Percentage recovery was calculated from the four-parameter model produced using BIAevaluation software.*

Concentration (pg/ml)	Absorbance @ 405nm ± S.D.	% C.V.	Back calculated concentration (pg/ml)	% Recovery
781,250	0.104 ± 0.005	4.81%	627,892	80.37
390,625	0.124 ± 0.003	2.44%	380,855	97.50
195,312.50	0.156 ± 0.008	5.17%	188,197	96.36
97,656.25	0.194 ± 0.003	1.38%	102,371	104.83
48,828.13	0.257 ±0.005	2.14%	484,34	99.19
24,414.06	0.317 ± 0.014	4.36%	24,863	101.84
12,207.03	0.376 ± 0.015	3.97%	11,758	96.32
6,103.52	0.409 ± 0.010	2.46%	6,502	106.53
3,051.76	0.437 ± 0.009	1.98%	3,094	101.38
1,525.88	0.452 ± 0.012	2.59%	1,537	100.72
762.94	0.460 ± 0.010	2.16%	838	109.82

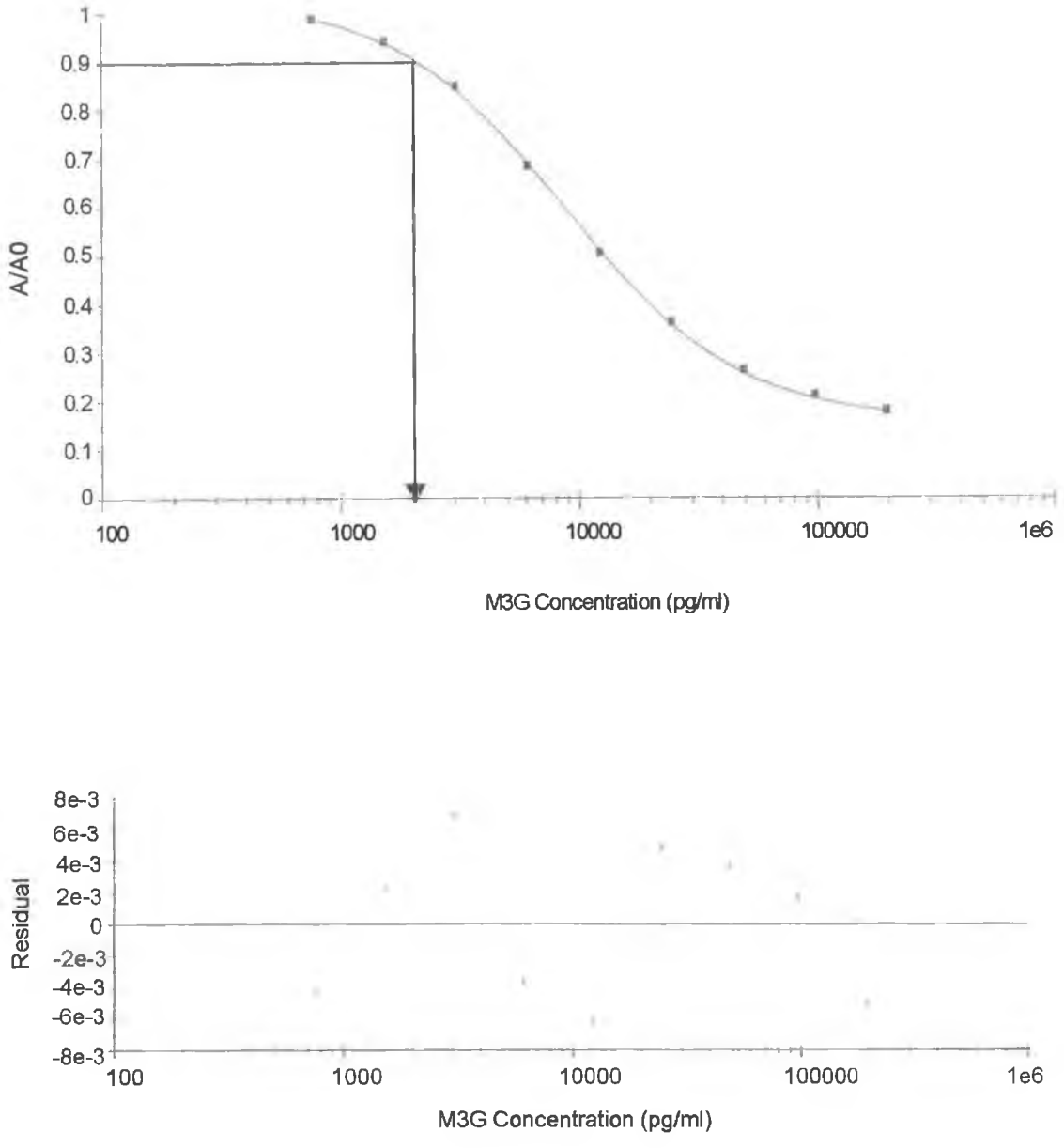


Figure 3.2.21: *Inter-day ELISA assay using pAK 400 monomeric scFv to detect free M3G in PBS. M3G-OVA was coated at 3.125 μg/ml and a 1 in 60 scFv dilution was used. Bound antibody was detected using M1 monoclonal anti-flag, followed by peroxidase-labelled anti-mouse antibody and visualized using o-PD. Absorbance was read at 450nm and related to M3G concentration using a 4-parameter logistic model that ranged from 763 pg/ml to 19,5000 pg/ml. The least detectable M3G concentration that led to 10% inhibition of antibody binding was shown to be 2 ng/ml.*

Table 3.2.5: *Inter-day assay coefficients of variation for the detection of free M3G using the monomeric scFv. Five sets of nine standards were assayed over five different days and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard. Percentage recovery was calculated from the four-parameter model produced using BIAevaluation software.*

Concentration (pg/ml)	A/A0 ± S.D.	% C.V.	Back calculated concentration (pg/ml)	% Recovery
195,312.50	0.173 ± 0.017	3.23%	166,075.65	85.03
97,656.25	0.205 ± 0.016	9.80%	102,085.29	104.54
48,828.13	0.259 ± 0.019	7.91%	48,388.43	99.10
24,414.06	0.357 ± 0.028	7.26%	24,165.61	98.98
12,207.03	0.498 ± 0.043	7.72%	12,709.79	104.12
6,103.52	0.680 ± 0.046	8.62%	6,256.66	102.51
3,051.76	0.843 ± 0.037	6.71%	2,953.38	96.77
1,525.88	0.937 ± 0.025	4.35%	1,494.34	97.94
762.94	0.982 ± 0.031	2.66%	811.57	106.37

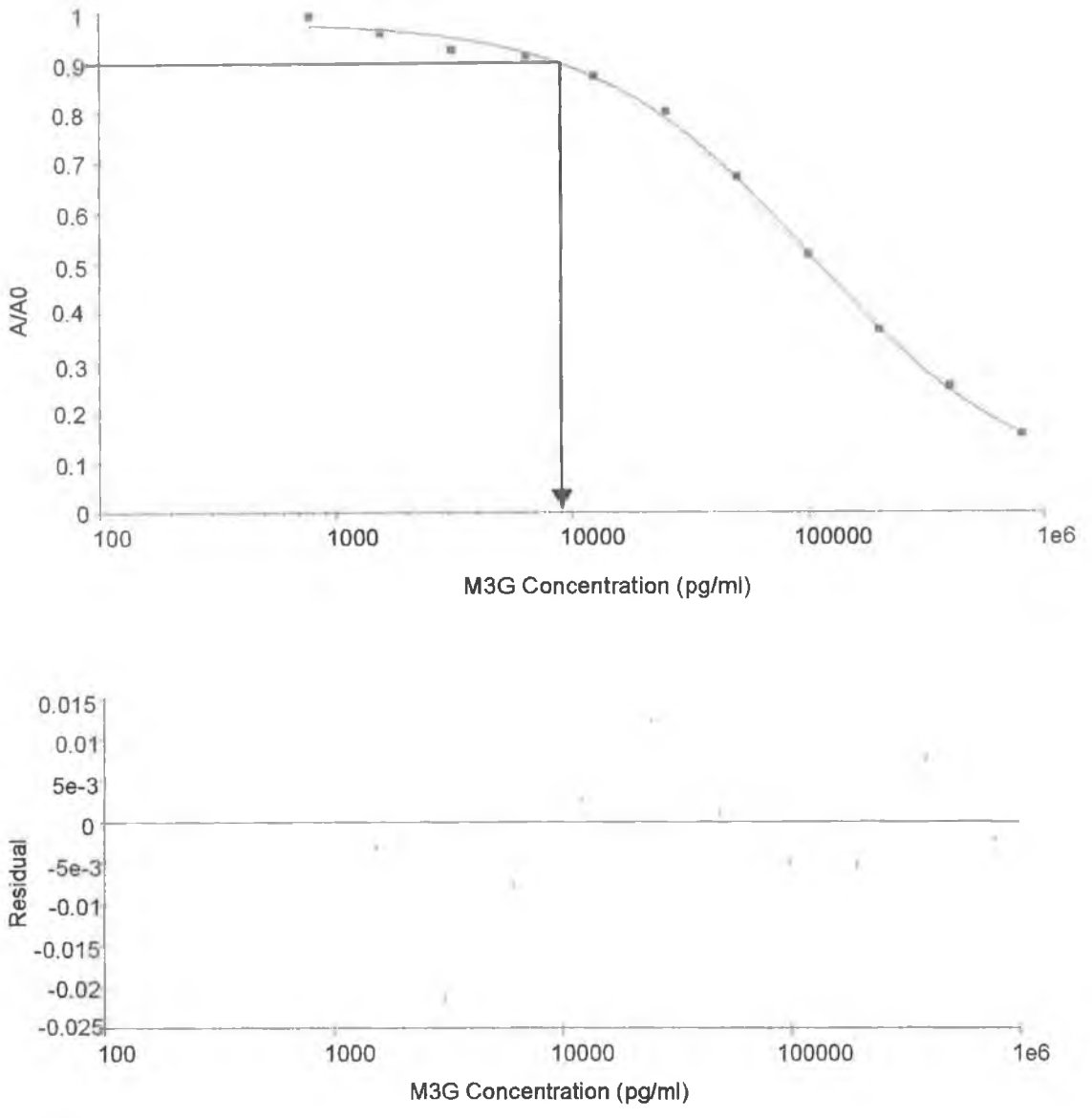


Figure 3.2.22: *Inter-day ELISA assay using pAK 500 dimeric scFv to detect free M3G in PBS. M3G-OVA was coated at 6.25 μg/ml and a 1 in 150 scFv dilution was used. Bound antibody was detected using M1 monoclonal anti-flag, followed by peroxidase-labelled anti-mouse antibody and visualized using o-PD. Absorbance was read at 450nm and related to M3G concentration using a 4-parameter logistic model that ranged from 763 pg/ml to 781,250 pg/ml. The least detectable M3G concentration that led to 10% inhibition of antibody binding was shown to be 9 ng/ml.*

Table 3.2.6: *Inter-day assay coefficients of variation for the detection of free M3G using the dimeric scFv. Five sets of eleven standards were assayed over five different days and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard. Percentage recovery was calculated from the four-parameter model produced using BIAevaluation software.*

Concentration (pg/ml)	A/A0 ± S.D.	% C.V.	Back calculated concentration (pg/ml)	% Recovery
781,250	0.158 ± 0.010	6.51%	799,747.22	102.37
390,625	0.255 ± 0.017	6.59%	372,184.29	95.28
195,312.50	0.367 ± 0.032	8.63%	200,524.64	102.67
97,656.25	0.518 ± 0.050	9.60%	99,812.59	102.21
48,828.13	0.672 ± 0.057	8.42%	48,590.63	99.51
24,414.06	0.803 ± 0.043	5.36%	22,398.04	91.74
12,207.03	0.874 ± 0.071	8.16%	11,880.70	97.33
6,103.52	0.914 ± 0.064	6.98%	6,999.53	114.68
3,051.76	0.928 ± 0.033	3.53%	5,354.82	175.47
1,525.88	0.962 ± 0.026	2.67%	1,835.36	120.28
762.94	0.995 ± 0.044	4.43%	<762.94	<100

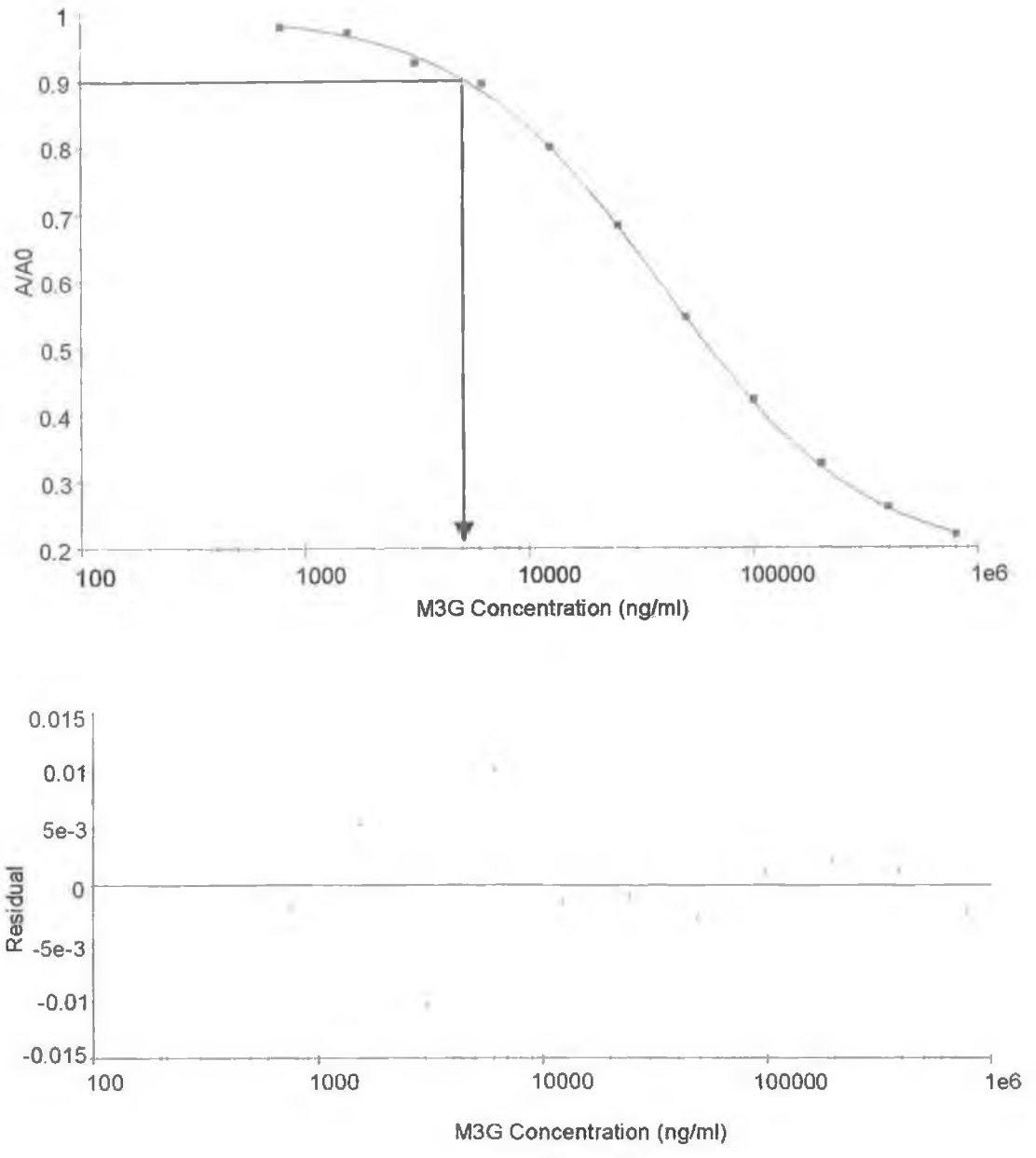


Figure 3.2.23: *Interday ELISA assay using pAK 600 enzyme-labelled dimeric scFv to detect free M3G in PBS. M3G-OVA was coated at 3.125 µg/ml and a 1 in 20 scFv dilution was used. Bound antibody was detected using p-NPP substrate and absorbance read at 405nm. The least detectable M3G concentration that led to 10% inhibition of antibody binding was shown to be 5 ng/ml.*

Table 3.2.7: *Inter-day assay coefficients of variation for the detection of free M3G using the dimeric enzyme-labelled scFv. Five sets of eleven standards were assayed over five different days and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard. Percentage recovery was calculated from the four-parameter model produced using BIAevaluation software.*

Concentration (pg/ml)	A/A0 ± S.D.	% C.V.	Back calculated concentration (pg/ml)	% Recovery
781,250	0.220 ± 0.010	4.77%	679,013	86.91
390,625	0.261 ± 0.004	1.38%	400,339	102.49
195,312.50	0.326 ± 0.005	1.49%	198,824	101.80
97,656.25	0.422 ± 0.018	4.15%	98,780	101.15
48,828.13	0.546 ± 0.031	5.67%	50,446	103.31
24,414.06	0.683 ± 0.015	2.13%	24,884	101.92
12,207.03	0.799 ± 0.021	2.68%	12,570	102.97
6,103.52	0.894 ± 0.039	4.31%	5,627	92.19
3,051.76	0.926 ± 0.015	1.66%	3,636	119.14
1,525.88	0.971 ± 0.012	1.20%	1,287	84.34
762.94	0.980 ± 0.011	1.09%	857	112.23

3.2.5.3 Cross reactivity studies

Cross reactivity measures the comparative reactivity of an antibody to its target analyte and structurally related compounds. One method to determine an antibody's cross reactivity is to measure the concentration of each compound required to displace the same amount of antibody. This is routinely performed at the point of 50% inhibition, i.e. the concentration of analyte required to give a normalised absorbance (A/A0) of 0.5. This is referred to as the IC_{50} value and a ratio of these values calculated for each compound is routinely used to calculate the percentage cross reactivity. This method is not always sufficient to estimate the specificity of an antibody because the displacement of curves of cross reactants are not always parallel to the standard curve over the entire working range. It is, therefore, recommended to measure cross reactivities at different concentrations over the entire working range (Hennion and Barcelo, 1998). The least detectable dose (LDD) and IC_{50} values were determined for each structurally-related compound as 90% A/A0 and 50% A/A0, respectively. The percentage cross reactivities were then estimated at the LDD (CR_{90}) and at the IC_{50} (CR_{50}) by expressing 100-fold the ratio of the M3G and of the cross-reactants.

Table 3.2.8 shows the characterised specificity of the monomeric scFv with the structurally related compounds. Cross reactivity is particularly visible for codeine, which exhibits a LDD value of 6 ng/ml, close to that of M3G, whereas the IC_{50} values for M3G and codeine are 13 and 55 ng/ml, respectively. As a general trend, cross reactivity is higher at low concentrations of analyte, than in the middle of the assay curve. The calculation of the percentage cross reactivity at different concentrations gives a more accurate estimation of possible interference in the assay. The dimeric scFv offered greater specificity to M3G, in comparison with the monomeric scFv, with the percentage cross reactivity against the various analytes remaining below 6% at the CR_{90} and 13% at the CR_{50} (Table 3.2.9). The dimerisation of the antibody, therefore, led to an increase in the specificity of the antibody. The bifunctional scFv displayed high levels of cross-reactivity towards other 'opiate-like' compounds. Heroin and codeine have lower LDD's than M3G, yielding over 154 and 333% cross

reactivity at CR_{90}. Cross reactivity is not as great in the IC_{50} area of the curve, with CR_{50}'s of 48 and 75% for heroin and codeine, respectively. Cross reactivity towards 6-MAM, however, is greater in this part of the curve, increasing from 15 to 75% between CR_{90} and CR_{50} values (Table 3.2.10). The decrease in specificity between dimeric antibody produced with pAK 500 and the enzyme-labelled dimeric antibody produced with pAK 600 has also been reported for an aflatoxin scFv (Dunne, 2004).

Table 3.2.8: *Specificity and cross reactivity of the monomeric scFv against various opiate and 'opiate-like' compounds. The cross reactivity is expressed as the least detectable dose (LDD), which was estimated at 90% A/A0, and at the dose required for 50% absorbance inhibition, IC_{50}. CR_{90} and CR_{50} were then expressed as 100-fold the ratio of the cross reactant to M3G.*

Compound	L.D.D. $_{90}$ (ng/ml)	IC_{50} (ng/ml)	CR_{90}	CR_{50}
M3G	2	13	100%	100%
Morphine	22	100	9%	13%
6-MAM	9	80	22%	16%
Heroin	8	80	25%	16%
Methadone	>10,000	>10,000	<0.1%	<0.1%
Codeine	6	55	33%	24%
Norcodeine	>10,000	>10,000	<0.1%	<0.1%
Dihydrocodeine	10	100	20%	13%

Table 3.2.9: *Specificity and cross reactivity of the dimeric scFv against various opiate and 'opiate-like' compounds. The cross reactivity is expressed as the least detectable dose (LDD), which was estimated at 90% A/A0, and at the dose required for 50% absorbance inhibition, IC_{50}. CR_{90} and CR_{50} were then expressed as 100-fold the ratio of the cross reactant to M3G.*

Compound	**L.D.D. $_{90}$ (ng/ml)**	**IC_{50} (ng/ml)**	**CR_{90}**	**CR_{50}**
M3G	8	120	100%	100%
Morphine	130	1700	6%	7%
6-MAM	180	1200	4%	11%
Heroin	275	1500	3%	9%
Methadone	>10000	>10000	<0.1%	<1%
Codeine	150	1000	5%	13%
Norcodeine	>10000	>10000	<0.1%	<1%
Dihydrocodeine	275	2250	3%	6%

Table 3.2.10: *Specificity and cross reactivity of the bifunctional scFv against various opiate and 'opiate-like' compounds. The cross reactivity is expressed as the least detectable dose (LDD), which was estimated at 90% A/A0, and at the dose required for 50% absorbance inhibition, IC_{50}. CR_{90} and CR_{50} were then expressed as 100-fold the ratio of the cross reactant to M3G.*

Compound	**L.D.D. $_{90}$ (ng/ml)**	**IC_{50} (ng/ml)**	**CR_{90}**	**CR_{50}**
M3G	2	60	100%	100%
Morphine	2.5	125	80%	48%
6-MAM	13	80	15.4%	75%
Heroin	1.3	125	154%	48%
Methadone	1,500	>10,000	<0.1%	<0.6%
Codeine	0.6	80	333%	75%
Norcodeine	1,500	>10,000	<0.1%	<0.6%
Dihydrocodeine	12.5	475	16%	12.6%

3.3 Discussion

This chapter describes the production of genetically-derived scFvs against morphine-3-glucuronide. Sequence analysis of the scFv allowed the amino acid sequence to be deduced. From this the complementary determining regions (CDRs) of the antibody were identified using the Kabat rules (Kabat *et al.*, 1991). The antibody 3-dimensional structure was modelled using Swiss model and DS Viewer Pro software (Figure 3.2.2 and 3.2.3) and was consistent with that expected of an anti-hapten antibody.

Soluble monomeric scFv was produced using the expression vector pAK 400, exploiting the strong Shine Dalgarno sequence for increased levels of protein expression. Fed-batch expression, followed by induction with 0.1 mM IPTG, proved the most efficient method, as levels as high as 9.3 mg/ml of pure scFv could be produced on a laboratory scale. The antibody could be purified by either of two affinity resins. The first involved using an affinity column, which employed M3G–BSA immobilised on a sepharose resin. This exploits the antibody's specificity for its target and yielded a very pure antibody upon elution with pH shock (Figure 3.2.11). The only drawback from this method was that the sample became diluted during the purification process, as the antibody did not dissociate rapidly following the pH decrease. Immobilised metal affinity chelate chromatography (IMAC) could also be used to purify the antibody via the hexahistidine tail located at the carboxy terminus of the antibody (Figure 3.2.13). Either method could be employed for simple, rapid and economical antibody purification. Western blot analysis of the antibody revealed a single band at approximately 32 kDa that corresponded to the molecular weight of a monomeric scFv (Figure 3.2.12 and 3.2.14).

Soluble dimeric scFv was produced using the pAK 500, expression vector. This vector encodes a double helix for dimerisation of the antibody fragment. The concentration of IPTG and induction lengths had to be increased to 0.5 mM and 8 hours to achieve optimal expression of antibody. The dimeric scFv displayed higher avidity for its target than the antibody in its monomeric state. For this reason purification via an antigen-affinity column was not possible, as conditions required to elute the antibody from the resin were too harsh to retain functionality of the antibody.

The antibody is expressed with a pentahistidine tag and this was utilised to purify the antibody by IMAC chromatography (Figure 3.2.13). Western blot analysis confirmed the dimeric nature of the scFv, with a band at 35 kDa, representing the reduced monomeric fusion protein comprising the scFv and the aliphatic helix. A faint band at 70 kDa, representing the unreduced dimeric scFv is also visible. This molecular weight is in agreement with the published literature. Rheinnecker *et al.* (1996) used the same helix to dimerise an scFv with a theoretical molecular weight of 31,400 Da and reported a molecular weight of 60,000 Da for the dimeric version. A similar helix employed by Kerschbaumer *et al.* (1997), generated a fusion protein with a molecular weight of 70 kDa. Pack *et al.* (1993) also reported a similar molecular weight for their scdHLX antibody, with only an insignificant amount of monomeric scFv being produced.

In an attempt to simplify and reduce assay time required for ELISA and western blot analysis, an alkaline phosphatase-labelled scFv was produced using the pAK 600, expression vector. In effect, this has the potential to eliminate the need for one or even two detection antibodies and the washing steps involved. This system has the ability to increase functional affinity of the antibody along with substantially decreasing assay time. This enzyme-labelled dimeric scFv required a 1 mM IPTG concentration and an induction time of 8 hours for optimum expression. The bifunctional nature of the antibody was confirmed by immunoblot analysis. This demonstrated that the antibody retained the ability of the parental antibody to bind M3G, while also possessing the enzymatic activity of alkaline phosphatase (Figure 3.2.10). Purification of this antibody could not be achieved by conventional antigen-affinity chromatography due to the extreme conditions required to elute the antibody, which may denature the enzyme label. The antibody did display a flag tag, which has been extensively characterised for protein purification (Einhauer[B] and Jungbauer, 2001). This purification strategy was investigated for the purification of the scFv–alkaline phosphatase fusion protein. However, sufficient binding to the anti–flag resin could not be achieved to separate the antibody from impurities in the lysate. Upon further investigation, it was observed that the column had less than 20% of the specified binding activity for pure flag protein. Einhauer[A] and Jungbauer (2001) have carried out kinetic analysis on the binding of the anti–flag antibody in the presence and absence of calcium and have revealed no difference on association and

dissociation rates was observed. As this technology relies on calcium dependence for efficient purification, this may explain the lack of affinity of the resin for the antibody, even with the presence of two flag tags per antibody. However, immunoblot analysis illustrated that the antibody could be specifically detected, even directly from crude lysate (Figure 3.2.10). This compares to similar findings by Lindner *et al.* (1997), who have reported that the *E. coli* lysate acts in conjunction with standard blocking agents to prevent non-specific binding. This eliminates the need for further purification of the protein. SDS-PAGE and western blot analysis of the scFv shows the molecular weight of the monomeric reduced form of the antibody was approximately 84 kDa. This corresponds to the reports in the literature of similar dimeric alkaline phosphatase-labelled antibodies with a molecular weight of 150 kDa, as determined by size exclusion chromatography (Suziki *et al.*, 1997).

There are a number of parameters that must first be optimised, for the successful development of competitive ELISAs. The concentration of coating conjugate must be low enough so as not to generate antibody bias towards solid phase but high enough so that there is a linear relationship between antibody concentration and absorbance. The dilution of antibody must also be optimised to maximise sensitivity. M3G-OVA coating concentrations of 3.125 μg/ml for monomeric and bifunctional antibodies and 6.25 μg/ml for the dimeric scFv were chosen as optimal. Monomeric antibody was diluted 1 in 60, dimeric scFv was diluted 1 in 150 and bifunctional scFv was diluted 1 in 20, as these concentrations corresponded to the point on the curve where the change in absorbance was greatest, with respect to change in antibody concentration.

Using these optimised parameters, competitive ELISAs were developed using each of the antibodies. Calibration curves were produced using a four-parameter model and from these curves least detectable doses of M3G were calculated as the concentration of M3G that exhibited a 10% inhibition of antibody binding or A/A0 of 0.9. Monomeric scFv displayed a calibration curve from 763 to 195,000 pg/ml with a Least Detectable Dose (LDD) of 2 ng/ml. Dimeric scFv had the ability to bind more M3G, with a calibration curve spanning from 763 to 781,250 pg/ml. The LDD was not as low, however, at 9 ng/ml. The assay developed with the bifunctional scFv had the advantage of needing considerably less assay time (< 2 hours) and reagents. The

antibody had a calibration curve from 763 to 781,250 pg/ml and a LDD of 5 ng/ml. This made the antibody particularly suited to rapid analysis. The robustness of each assay was analysed by calculating the variability of results on a number of separate days. Intra and Inter-assay coefficients of variation (C.V.'s) were below 10 % for each antibody, proving that each assay was not only sensitive but also reproducible. Percentage recoveries calculated from each model were all within 20% of actual concentration analysed for samples above the LDD level for each antibody. This demonstrates excellent sensitivity and reproducibility that can be achieved through the use of scFv antibodies in ELISA analysis.

An attempt to employ the dimeric scFv in a sandwich ELISA format as devised by Kerschbaumer *et al.* (1997), using immobilised dimeric antibody as a capture agent and an enzyme labelled scFv fusion protein for detection, was unsuccessful. However, this can be explained by the fact that as M3G is a small molecule and the epitope, once bound by one antibody, is inaccessible to the other antibody. Sensitive assays may be developed using this system for molecules that display multiple epitopes on their surface e.g. bacteria/viruses.

The extent to which each antibody cross reacted with structurally related compounds was also investigated. Cross reactivity was measured at different concentrations of analyte over the working range, as recommended by Hennion and Barcelo (1998). This reflects cross reactivity more accurately than a comparison between slopes of displacement curves as cross reactivity is generally greater in the area of around 10 % displacement. The least detectable doses (LDD) and IC_{50} values were determined for each structurally-related compound at 90% A/A0 and 50% A/A0, respectively. The percentage cross reactivity were then estimated at the LDD (CR_{90}) and at the IC_{50} (CR_{50}) by expressing 100-fold the ratio of the M3G and of the cross-reactants. The possibility of interferences can, therefore, be weighted with respect to the region of the curve where analysis is being carried out.

None of the three antibodies exhibited cross reactivity to norcodeine or methadone within the range tested (up to 10 μg/ml). This made the antibody suitable for detecting heroin residues in subjects who were receiving methadone replacement

therapy for heroin withdrawal. The highest degree of cross reactivity for the monomeric scFv was against codeine, followed by heroin, 6-MAM, dihydrocodeine and morphine. Although any cross reactivity with codeine is undesirable, due to its presence in over the counter medications, it was not found to significantly interfere in real sample analysis, as described in Chapter 4. Analysis of saliva samples following medication with the most common over the counter codeine remedies revealed an approximate 10 ng/ml over-estimation of morphine levels. This level of interference is not large enough to generate a false positive result in real sample analysis, as described in Chapter 4, as the cut-off level is set at 12 ng/ml. The dimeric scFv displayed increased specificity, with the highest interference observed for codeine at 50% inhibition (13%). The bifunctional scFv proved to be the least specific, preferentially recognising low concentrations of codeine over M3G. Morphine was also shown to cross react significantly. Structural differences between these cross reacting compounds occur only at the 3- and 6- positions (Figure 3.3.1). The antibody library was initially generated against a morphine-3-glucuronide conjugate, conjugated to the protein, BSA, through the glucuronide group at the 3-position. This part of the molecule was, therefore, 'hidden' from the immune response. The antibody recognises an epitope on the opposite side of the molecule, leading to the recognition of compounds, structurally identical to M3G in this region, by the antibody. Codeine differs from M3G only at the 3- position, the glucuronide moiety being substituted with a CH_3O group. Heroin, being the diacetyl derivative of morphine, possess two acetyl groups, at the 3- position, replacing the glucuronide group and at the 6- position, in place of an OH group. 6-MAM is the first metabolite observed following heroin intake and occurs through deacetylation of the molecule at the 3- position. Dihydrocodeine, which is found in combination analgesics, is also only distinguishable through the 3- position. Morphine, an opiate commonly administered for the control of pain, is also a major but short-lived metabolite of heroin. This is only distinguishable from M3G at the 3- position of the molecule, the glucuronide group being substituted by an -OH. For this reason the antibody would be expected to show a degree of cross reactivity.

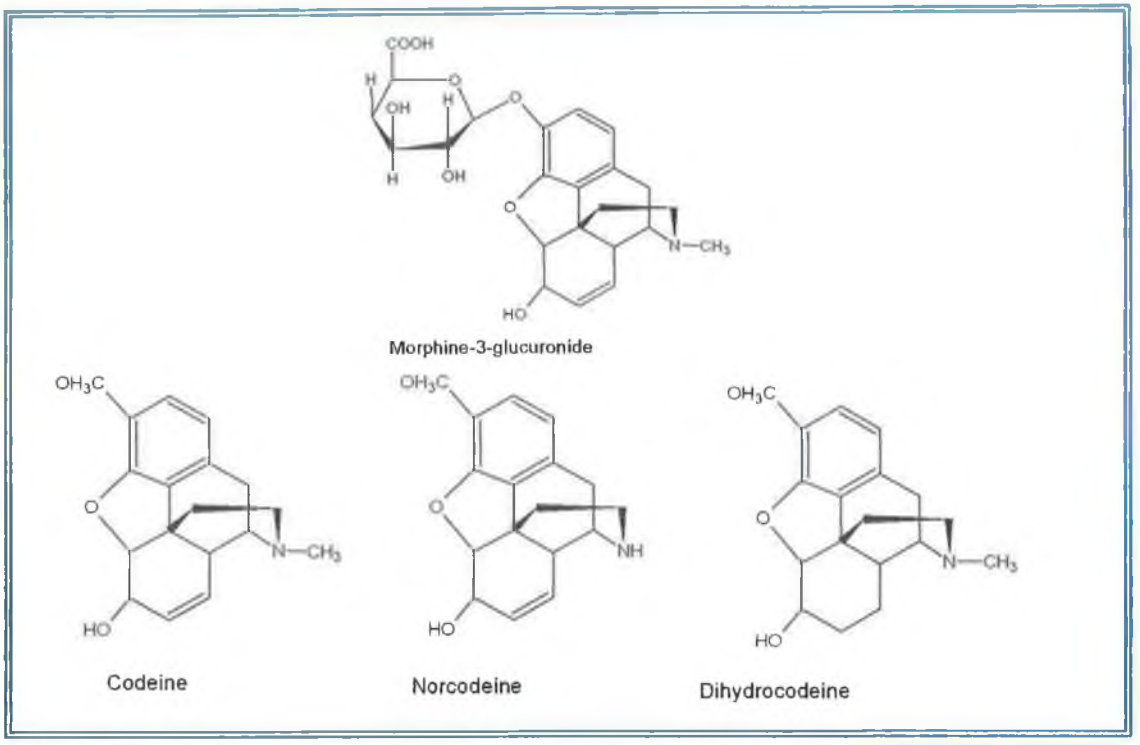


Figure 3.3.1: *Structure of codeines bearing a high degree of structural similarity to M3G*

The assays developed with all three scFvs achieved sensitivity comparable with other methods for the detection of M3G, reported in the literature. Elaborate analytical-based systems using HPLC, GC and MS can achieve high levels of sensitivity but such techniques require large capital investment and operation by skilled personal. Meng *et al.* (2000) developed a sensitive HPLC-based method for the detection of M3G in plasma. The assay showed excellent limits of quantification (0.45 ng/ml) with 82% recovery. The method involved a two-step solid phase extraction involving a hydrophobic column, followed by ionic exchange extraction. Samples were then analysed by reverse-phase HPLC, in combination with electrochemical and fluorometric detection systems. Schänzle *et al.* (1999) developed a method for M3G detection in body fluids using HPLC-MS. Limits of quantification for M3G were 5.4 pmol/ml (2.5 ng/ml) in serum and 50 pmol/ml (23 ng/ml) in urine, with an excellent percentage bias. Musshoff *et al.* (2004) reported a limit of quantification of 5 ng/ml M3G in urine with an LC-ESI-MS-MS based assay. The assay however, was subject to poor recoveries, with M3G recovery of only 45%, reported at 62.5 ng/ml. Whittington and Kharasch (2003) reported a HPLC-ESI-MS detection method for

M3G in plasma. The assay had a limit of quantification of 5 ng/ml, with a 90% recovery at 25 ng/ml. These methods all require a labour intensive sample extraction step. Beike *et al.* (1999) used polyclonal anti-M3G antibodies for this purpose and employed an immunoaffinity column for sample extraction of M3G from blood. Samples were analysed by HPLC and the assay had a limit of quantification of 10 ng/g of blood but suffered from a poor mean percentage recovery of 76%.

Immunoassay-based detection systems offer an appealing alternative to analytical systems, as they are rapid, economical and relatively simple to perform, without the requirement for elaborate, expensive equipment. Dillon[A] *et al.* (2003) developed an ELISA assay for M3G detection in urine. The assay achieved higher sensitivity than the ELISAs reported here, however the polyclonal antibodies employed, exhibited a lower degree of specificity for M3G. The antibodies were not capable of discriminating between codeine and M3G (92% and 97% for each antibody). This could possibly affect the integrity of the results, as codeine is found in many over the counter remedies and could therefore lead to a high percentage of 'false' positives. Dillon[B] *et al.* (2003) developed a biosensor-based assay to detect M3G. The assay had a detection limit of 3 ng/ml, with the scFv-based assay proving more specific for M3G than it's equivalent with polyclonal antibodies.

This chapter focused on the development of immunoassays using several forms of genetically-derived antibodies for the detection of M3G. Recombinant antibody technology offers an unlimited source of antibodies with defined affinities and specificities. Three forms of recombinant antibody were used in this chapter, each boasting its own advantages. Monomeric scFv was the simplest form of recombinant antibody expression. Through dimerisation of this antibody, specificity could be greatly increased. The production of a bifunctional scFv led to overall simplification of assay for M3G. Although this form of the antibody had exhibited higher cross reactivity with structurally related compounds, its advantage lay in the speed at which analysis could be performed. This antibody was particularly suited to 'on-site', screening analysis, as reagents needed were minimal and analysis time was rapid. Once a positive result was obtained, it could be confirmed using the more specific dimeric antibody or by a chosen analytical system. These antibodies offer a real alternative to large scale routine analysis by analytical means.

Chapter 4: Applications of Genetically-Derived scFv antibody Fragments

4.1 Introduction

4.1.1 Determination of affinity constant

There are various techniques used to determine the affinity constant of an antibody. These include equilibrium dialysis for haptens and dialysable antigen, immunoprecipitation, fluorescence-based measurements and ELISA-based methods. Two techniques for measuring affinities were used in this study. The first was an ELISA-based method described by Friguet *et al.* (1985) and the second involved a biosensor-based method, which measures solution phase affinity using 'real-time' biomolecular interaction BIAcore technology.

4.1.1.1 Antibody affinity

The affinity of an antibody gives a quantitative indication of the strength of the interaction between an antibody and its specific antigen (Djavadi-Ohaniance *et al.*, 1996). It is an important property that will determine the potential applicability of an antibody in various assay formats. The affinity of an antibody K_A is

$$[Ab] + [Ag] \underset{K_d}{\overset{K_a}{\longleftrightarrow}} [AbAg] \qquad \textit{Equation 4.1}$$

Where

[Ab]	=	Free Antibody Concentration
[Ag]	=	Free Antigen Concentration
[AbAg]	=	Antibody:Antigen Complex Concentration
K_a	=	Association Rate Constant
K_d	=	Dissociation Rate Constant

The equilibrium association constant K_A ($K_A=1/K_D$), of an antibody for its target antigen, is defined by the Law of Mass Action as:

$$\frac{K_a}{K_d} = K_A = \frac{[Ab]}{[AbAg]} = \frac{1}{K_D} \qquad \textit{Equation 4.2}$$

Where K_A and K_D are equilibrium association and dissociation constants respectively.

4.1.1.2 Affinity constant determination by ELISA

The method described by Friguet *et al.* (1985) involves measuring the amount of free antibody present following equilibration with various concentrations of free antigen. This involves mixing a fixed concentration of antibody with varying concentrations of antigen and allowing equilibrium to be attained while in solution. The amount of free antibody is measured using an indirect ELISA to capture any unbound, or unsaturated antibody.

$$\text{Antibody} + \text{Antigen} \longleftrightarrow \text{Complex}$$
$$[Ab] + [Ag] \longleftrightarrow [AbAg]$$

with the concentration of antibody sites, antigen sites and complex at equilibrium are given as [Ab], [Ag] and [AbAg], respectively. This is related to the total antibody and antigen sites, $[Ab]_{total}$ and $[Ag]_{total}$, by the following equation

$$[Ab] = [Ab]_{total} - [AbAg] \text{ and}$$
$$[Ag] = [Ag]_{total} - [AbAg] \qquad \textbf{\textit{Equation 4.3}}$$

K_D, the equilibrium dissociation constant is defined by:

$$K_D = [Ag][Ab]/[AbAg] \qquad \textbf{\textit{Equation 4.4}}$$

If $[Ag]_{total}$ is varied while $[Ab]_{total}$ is kept constant then:

$$K_D = \frac{[Ag]([Ab]_{total} - [AbAg])}{[AbAg]} \qquad \textbf{\textit{Equation 4.5}}$$

This can be rearranged to give:

$$\frac{[AbAg]}{[Ab]_{total}} = \frac{[Ag]}{([Ag] + K_D)} \qquad \textit{Equation 4.6}$$

Giving rise to the Klotz equation:

$$\frac{[Ab]_{total}}{[AbAg]} = \frac{K_D}{[Ag] + 1} \qquad \textit{Equation 4.7}$$

As $[Ag]_{total}$ is kept >> $[Ab]_{total}$, throughout the assay, [Ag] can be approximated to $[Ag]_{total}$.

The Klotz equation can therefore be rewritten as:

$$\frac{[Ab]_{total}}{[AbAg]} = \frac{K_D}{[Ag]_{total} + 1} \qquad \textit{Equation 4.8}$$

The fraction of bound antibody, $[AbAg]/ [Ab]_{total}$, is referred to as V, and this can be related to absorbance using the expression:

$$V = \frac{A_0 - A_1}{A_0} = \frac{[AbAg]}{[Ab]_{total}} \qquad \textit{Equation 4.9}$$

Where A_0 = Absorbance measured for total antibody concentration

A_1 = Absorbance measured in the presence of each antigen concentration

The Klotz equation becomes:

$$\frac{1}{V} = \frac{K_D}{[Ag]_{total} + 1} \qquad \textit{Equation 4.10}$$

A plot of 1/V versus 1/[Ag] will therefore yield a straight line graph, with a y-intercept of 1. The slope of the linear regression describes the overall equilibrium dissociation constant for the antibody:antigen interaction (K_D)

4.1.1.3 Prerequisites of the Friguet assay

For the correct determination of K_D, there are several criteria that must be fulfilled (Djavadi-Ohaniance *et al.*, 1996).

- Firstly as K_D is a temperature-dependant property, this factor must be kept constant throughout the analysis.

- Free antibody, that remains uncomplexed by antigen, is quantified by means of an indirect ELISA. A titration of antibody concentration versus absorbance will yield a linear curve that reaches a plateau at higher antibody concentrations. The concentration of antibody to be used in the analysis must be in the linear region of this curve, where concentration is directly proportional to absorbance.

- Within this linear region, the fraction of antibody retained by the solid phase must be less than 10% of the total antibody concentration. This is to ensure that there is minimal disruption of the equilibrium of the antibody:antigen mixtures in the solution phase. The disruption of solution phase equilibrium through coating with too much antigen, or prolonged incubation times, may lead to a gross underestimation of the antibody's true affinity (Holland and Steward, 1991)

4.1.2 Solution phase affinity determination by BIAcore

BIAcore technology was employed to determined equilibrium dissociation constants of monomeric and dimeric scFvs. This allowed a comparison to be made between ELISA and BIAcore methods of affinity determination. Solution phase affinity determination is based on the same principle as that described by Friguet *et al.* (1985), where the free concentration of one of the interactants is measured at equilibrium. The continual buffer flow and short contact time with the chip surface, in comparison to contact time in solid phase ELISA analysis, offers a decreased possibility of solution phase disruption, and, therefore, decreases the possibility of underestimating the affinity constant (Fitzpatrick, 2001). As BIAcore does not require any labelling or secondary reagents, the analysis is not restricted by any of the limiting factors imposed by these reagents.

Firstly, a standard curve of response observed for varying molar concentrations of antibody is constructed. A constant concentration of antibody, from within this range, is then mixed with varying concentrations of antigen and allowed to reach equilibrium. The mixtures are then passed over an antigen-coated sensor chip. The binding response observed is used to calculate the concentration of free antibody from the standard calibration curve. The concentration of free antibody is plotted against the concentration of antigen in each of the mixtures. A solution affinity fit, described below, is applied to the dataset using BIAevaluation software.

$$B_{free} = B - A - K_D + \sqrt{\frac{(A + B + K_D)^2}{4} - AB} \qquad \textit{Equation 4.11}$$

Where B_{free} is the concentration of free anti-M3G scFv

A is the total concentration of analyte

B is the total anti-M3G scFv concentration

K_D is the equilibrium dissociation constant

This describes a monovalent binding interaction between antibody and antigen. In theory, a bivalent molecule will deviate from this model, as the quantification assay will detect both free and monovalently bound antibody. Piehler *et al*, (1997), introduced a 'correction' factor into the model, that describes the distribution of antigen between the two binding sites of a bivalent antibody. The model assumes that free and monovalently bound antibody will give a similar response at the chip surface. The concentration of fully complexed antibody depends on the concentration of complex and the concentration of antibody binding sites i.e. two per antibody.

Therefore:

$$B_{free} = \frac{B}{2} - \frac{AB^2}{2B} \qquad \textit{Equation 4.12}$$

Giving rise to the expression:

$$B_{free} = \frac{B}{2} - \frac{\left[\frac{B + A + KD}{2} - \sqrt{\frac{(A + B + K_D)^2}{4} - A}\right]^2}{2B} \qquad \textit{Equation 4.13}$$

The symbols have the same meaning as in equation 4.11. This may be particularly important when analysing small haptens such as morphine and M3G. The model may sometimes be inappropriate for a bivalent antibody (Quinn and O'Kennedy, 2001). Whether or not the phenomenon of 'half-liganded' antibody interacting with the surface will depend on factors, such as flow rates, steric hindrance and the drug epitope density (Fitzpatrick, 2001). The uncontrolled manner of conjugation of the M3G molecule to OVA makes it impossible to predict the ratio of drug epitopes to protein. However flow rates can be increased to a maximal level that will still allow antibody interaction. This 'sweep' of buffer across the surface will decrease the probability of interactions between 'half liganded' antibody and sensor surface, while still facilitating monovalent antibody binding.

Equilibrium dissociation constants of monomeric, dimeric and bifunctional scFvs will be calculated using the Friguet method. This will be compared to the BIAcore solution phase method of equilibrium constant determination. The K_D values calculated will be used to determine the scFv that is best suited to analysis of morphine in saliva.

4.1.3 Studies on saliva as an alternative detection matrix for opiate use

Saliva is an ideal matrix to use for the detection of drug residues. Sample collection is non-invasive, can be easily supervised and is amenable to 'on-site' monitoring. Sample volume is adequate to yield a number of aliquots for multiple analyses. Salivary detection of drug residues is more indicative of recent drug use and/or impairment, as drug metabolites do not persist for long periods. For this reason, it was decided to employ scFv antibody fragments for the detection of morphine, the main metabolite of heroin found in saliva.

Analytical advances in the use of alternative biological matrices, such as saliva, have not been appropriately addressed in the laws of the majority of countries (Cone, 2001). Recent draft guidelines by the Substance Abuse and Mental Health Services Administration (SAMHSA), in the USA, include the requirement to test for heroin use in alternative matrices such as saliva and sweat (Presley *et al.*, 2003). The Criminal Justice Act (1990), here in Ireland allows Gardaí to take pubic hair, saliva, blood, urine and swabs in criminal cases. Many countries are investigating oral fluid analysis as an alternative to urine analysis to measure the level of impairment in the workplace and on the roadside.

A study by Tonnes *et al.*, (2004), conducted in Germany using the Dräger DrugTest® System, found that oral fluid correlated above 95% with serum levels for opiates. Persons found positive for opiate use in oral fluid were all deemed to be suffering from impairment of driving symptoms, as determined by a list of impairment symptoms given to Police officers. Bennet *et al.*, (2003) conducted a study of opiate use in a British Treatment Centre and found a correlation of 91% between oral fluid and urine analysis. Barrett *et al.*, (2001), found saliva analysis to correlate extremely well with urine analysis. Positive results showed 100% agreement, with negative results agreeing by 99%.

4.1.4 Cut-off concentrations for drugs of abuse in saliva

Currently, there are no cut-off levels established for levels of drug metabolites in saliva in Ireland. Most commercial-based tests work to the guidelines proposed by the Substance Abuse and Mental Health Service Administration (SAMSHA) for drug testing in oral fluid (Samyn *et al.*, 2002). These guidelines have recently been revised to include oral fluid as a detection matrix for federal workplace drug testing. The proposed revisions to mandatory guidelines for federal workplace drug testing programs (April 2004), list cut-off concentrations of drugs of abuse for screening and confirmatory tests as detailed in Table 4.1.1 and 4.1.2 (Federal Register, 2004).

For a laboratory to be certified to carry out analysis of oral fluid, the guidelines state that certain criteria must be met with regard to analysis of 3 performance test samples. The criteria for accreditation by Dept. of Health and Human Services (USA) are listed below:

(1) Have no false positive results

(2) Correctly identify and confirm at least 90% of the total drug challenges on the 3 sets of performance test samples

(3) Correctly determine the quantitative values for at least 80% of the total drug challenges to be within ± 20 percent or ± 2 standard deviations of the calculated reference group mean

(4) Have no quantitative value on a drug concentration that differs by more than 50% from the calculated reference group mean

(5) For an individual drug, correctly detect and quantify at least 50 % of the total drug challenges

(6) Must not obtain any quantitative value on a validity test sample that differs by more than ± 50% from the calculated reference group means

(7) For qualitative validity test samples, must correctly report at least 80% of the challenges for each qualitative validity test sample over the 3 sets of performance test samples

(8) Must not report any sample as adulterated with a compound that is not present in the sample

(Adapted from the Federal Register, 2004).

Table 4.1.1: *Initial Test Cut-off Concentrations of Drugs of Abuse in Saliva*

Drug	**(ng/ml of Saliva)**
THC and metabolites	4
Opiate metabolites other than 6-MAM	40
Opiate metabolite 6-MAM	4
Amphetamines	51
Cocaine metabolites	20
MDMA	50

Table 4.1.2: *Confirmatory Test Cut-off Concentrations of Drugs of Abuse in Saliva*

Drug	**(ng/ml of Saliva)**
THC Parent drug	2
Cocaine (or benzoylecgonine)	8
Opiates:	
Morphine	40
Codeine	40
6-Acetylmorphine	4
Phencyclidine	10
Amphetamines:	
Amphetamine	50
Methamphetamines (must also contain amphetamine):	50
MDMA	50
MDA	50
MDEA	50

4.1.5 Composition and biochemistry of saliva as a matrix for analytical detection of illicit drug residues

Saliva is a dilute aqueous fluid (99%), containing electrolytes and small amounts of protein. The protein content of saliva is less than 1% that of plasma (Samyn *et al.*, 1999). There is also a certain amount of cell debris from epithelial cells of the mouth and food residues present in saliva (Hold *et al.*, 1995). Salivary glands are fed by blood flow therefore any drug circulating in plasma must pass through the capillary wall, the basal membrane and the membrane of epithelial cells before entering saliva (Samyn *et al.*, 1999). This will determine the rate at which any drug enters saliva. Drugs may be transported into saliva by passive diffusion, active processes across a concentration gradient or by filtration through pores in the membrane. Low molecular weight substances such as electrolytes, some IgA and some drugs such as Lithium are actively excreted into saliva. Low molecular weight, polar molecules, such as ethanol and glycerol, enter the plasma by filtration (Kidwell *et al.*, 1998). Most drugs appear to enter saliva by passive diffusion across a concentration gradient. The concentration of free drug present in saliva is, therefore, dependant on the different chemical and physiological properties of the drug and saliva. Saliva has little protein binding capacity and for this reason, parental drugs are predominantly present as they are more lipophilic than their hydrophilic metabolites (Samyn *et al.*, 1999). Once transported to saliva they must have some degree of water solubility, however, to prevent them from diffusing back into the plasma. This generally occurs through an equilibrium ionisation process, determined by drug concentrations and salivary pH. The ratio of saliva to plasma concentrations of a drug is derived from the Henderson-Hasselbalch equation and the equation for mass-balance (Kidwell *et al.*, 1998):

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \qquad \textit{Equation 4.14}$$

$$[A] = [A^-] + [HA] \qquad \textit{Equation 4.15}$$

Where [HA] is the concentration of non-ionised drug, $[A^-]$ is the concentration of ionised drug and [A] is the total concentration of ionised and non-ionised drug.

The total amount of drug in either form present in a biological matrix is therefore described by:

$$\frac{[A]}{[HA]} = 1 + 10^{(pH - pKa)} \qquad \textit{Equation 4.16}$$

The ratio of drug in saliva:plasma (S/P) can then be described by:

$$\frac{S}{P} = \frac{[A_{saliva}][HA_{plasma}]}{[A_{plasma}][HA_{saliva}]} = \frac{1 + 10^{(pH_{saliva} - pKa)}}{1 + 10^{(pH_{plasma} - pKa)}} \qquad \textit{Equation 4.17}$$

As only unbound drug can cross through cellular membranes, drugs bound to plasma and saliva proteins must be taken into account:

For an Acidic Drug:

$$\frac{S}{P} = \frac{1 + 10^{(pH_{saliva} - pKa)}}{1 + 10^{(pH_{plasma} - pKa)}} \times \frac{Free_{plasma}}{Free_{saliva}} \qquad \textit{Equation 4.18}$$

For a Basic Drug:

$$\frac{S}{P} = \frac{1 + 10^{(pka - pH_{saliva})}}{1 + 10^{(pH_{plasma} - pKa)}} \times \frac{Free_{plasma}}{Free_{saliva}} \qquad \textit{Equation 4.19}$$

The fraction of free drug in saliva is generally accepted to be 1, because the amount of protein in saliva is negligible, with respect to that of plasma (Kidwell *et al.*, 1998).

4.1.6 Salivary pH and opiate detection

The normal pH of blood is 7.4, while that of saliva is generally between 5.6 and 7, which can increase to pH 8, with stimulation (Kidwell *et al.*, 1998). This means that the pH of saliva will affect the degree of ionisation and, therefore, the S/P ratio, especially for drugs with a pKa close to that of salivary pH. Opiates are weakly basic

drugs and will have a S/P ratio of greater than 1. Small changes in the pH of saliva will affect the S/P ratio, the ratio decreasing, with increasing pH. The pH of saliva is determined by secretions of the major salivary glands (parotid, submandibular and sublingual) and to a lesser extent by labial, buccal and palatal glands (Hold *et al.*, 1995). This makes the pH of saliva difficult to adulterate for test purposes, offering a major advantage over urinary analysis. Widely available products like 'urine luck', which contains pyridinium chlorochromate (PCC), may be used to adversely affect standard opiate recoveries from urine (Wu *et al.*, 1999). The collection procedure of saliva samples can also be closely monitored, without the need to invade personal privacy, making external tampering more difficult. This makes saliva an ideal detection matrix.

4.2 Results

4.2.1 Determination of the affinity constant of monomeric, dimeric and bifunctional scFvs by ELISA

The conditions under which there was minimal disruption of the solution-phase equilibrium of each antibody were first investigated. An indirect ELISA was carried out at various concentrations of antigen coatings, from 5 to 0.25 μg/ml M3G-OVA. Serial dilutions of each antibody, that exhibited a concentration-response relationship in the linear portion of the curve, were applied to ELISA plates coated with various concentrations of antigen and incubated for 10, 15 and 30 mins, respectively, at room temperature. After incubation, the liquid from the wells was transferred to a second set of wells coated with an identical concentration of antigen. Both sets of wells were developed simultaneously. The constant concentration of antibody used for affinity analysis was chosen from the linear portion of this plot. Within the linear region of each curve, the fraction of antibody retained by the coated antigen was calculated from the ratio of the slopes for each set of wells, $(S_1\text{-}S_2/S_1)$, where S_1 is the slope obtained for the first set of coated wells and S_2 is the slope obtained for the second set of coated wells. If the amount of antibody retained on the first set of wells was greater than 10%, the assay was repeated using either a decreased antigen coating or decreased incubation time, until the ratio of slopes differed by less than 10%.

An indirect ELISA was carried out to construct a standard curve that could then be used to quantify the nominal concentration of free antibody that remains unsaturated at each antigen concentration. The standard curve of nominal concentration of monomeric scFv versus absorbance is shown in Figure 4.2.1. This involved coating the plate with 0.25 μg/ml M3G-OVA. Nominal concentrations of antibody were then incubated for 10 minutes at room temperature before bound antibody was detected with secondary antibody. Figure 4.2.2 shows that under these conditions the displacement of monomeric scFv from solution phase equilibrium was 7.8%. The nominal concentration of antibody that remained bound in the antibody:antigen complex (V) at each concentration of antigen was then calculated from the standard curve (Figure 4.2.1). The calculated nominal concentrations of free and bound

monomeric scFv at each concentration of M3G and morphine are shown in Tables 4.2.1 and 4.2.2. A Klotz plot of 1/V versus 1/[Ag] was then prepared (Figure 4.2.3 and 4.2.4). This yielded a linear curve with a y-intercept of 1. The slope of this graph defines the equilibrium dissociation constant (K_D) of the interaction between monomeric scFv and each target antigen. The equilibrium dissociation constant describing the interaction between monomeric scFv and M3G was determined to be 3.125×10^{-9} M (Figure 4.2.3). The interaction between monomeric scFv and morphine was determined to have an equilibrium dissociation constant of 1.73×10^{-8} M (Figure 4.2.4).

Affintiy studies were also carried out on the dimeric scFv. Plates were coated with 0.5 μg/ml M3G-OVA and the antibody was incubated on the plate for 10 minutes at room temperature. Absorbance values at each nominal concentration of antibody were used to construct a standard curve (Figure 4.2.5). Under these conditions there was negligible disruption of the solution phase equilibrium (Figure 4.2.6). The calculated nominal concentrations of free and bound antibody at each concentration of M3G and morphine are shown in Table 4.2.3 and 4.2.5. From these values Klotz plots were constructed to describe each interaction. Dimeric scFv was determined to have a K_D of 2.4×10^{-8} M for M3G and 2.08×10^{-8} M for morphine (Figure 4.2.7 and Figure 4.2.9). Completely unsaturated or monovalently bound antibody will be detected in the final indirect ELISA, as this antibody is bivalent in nature. This is not factored into the Klotz plot, leading to a possible overestimation of actual K_D (Fitzpatrick, 2001). Stevens, (1987), introduced a correction factor based on the binomial distribution theory to provide a 'corrected' concentration of complexed antibody for K_D calculation. To take account of the bivalency of the scFv, $(1/V)^{1/2}$ is plotted against 1/[Ag], to determine the 'corrected' equilibrium dissociation constant. 'Corrected' concentrations of free dimeric antibody are shown in Tables 4.2.4 and 4.2.6. This correction factor yielded a K_D of 5.62×10^{-9} M for M3G, representing a four-fold increase in affinity and a K_D corrected to 7.5×10^{-9} M, for morphine (Figures 4.2.8 and 4.2.10).

Friguet analysis on the bifunctional scFv was carried out using the standard curve in Figure 4.2.11 to calculate the nominal concentration of free antibody (Friguet *et al.*,

1985). As the antibody possessed an enzyme label, this binding interaction was monitored directly, without the use of a secondary antibody. Figure 4.2.12 proves that less than 5% of this antibody was displaced from solution phase equilibrium when the optimised coating concentration and antibody incubation period were employed (1 μg/ml M3G-OVA and a 15 minute incubation at room temperature). Nominal concentrations of free antibody at each antigen concentration were calculated from the standard curve. These values are shown in Tables 4.2.7 and 4.2.9. Klotz plots of bound antibody (1/V) versus concentration of antigen revealed an equilibrium dissociation constant of 3.63×10^{-8} M for M3G (Figure 4.2.13) and 3.61×10^{-8} M for morphine (Figure 4.2.15). When the bivalency of the antibody is taken into account and the 'corrected' concentration of free antibody calculated (Tables 4.2.8 and 4.2.10), the K_D for these interactions were 'corrected' to 1.09×10^{-8} M (Figure 4.2.14) for M3G and 1.24×10^{-8} M for morphine (Figure 4.2.16). A summary of the equilibrium dissociation constants determined for each antibody using ELISA analysis is shown in Table 4.2.12.

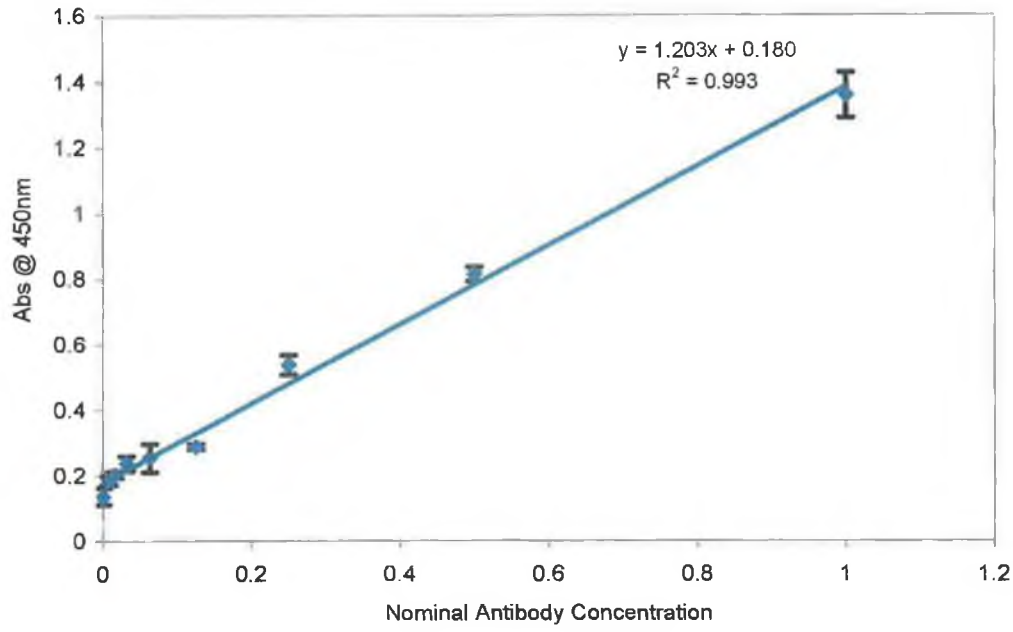


Figure 4.2.1: *Standard curve of nominal antibody concentration of monomeric scFv versus absorbance @ 450nm. Results shown are the average of triplicate measurements ± standard deviation. The highest concentration of antibody that lay within the linear region of the curve was assigned a nominal antibody concentration of '1'. Serial dilutions up to a 1 in 128 dilution were prepared, corresponding to 1, 0.5, 0.25, 0.125, 0.0615, 0.0313, 0.0156, 0.0078 and 0, respectively. The antibody dilutions were added to a plate coated with 0.25 μg/ml M3G-OVA for 10 mins at room temperature. The incubation period was predetermined to ensure that minimal disruption of the solution phase equilibrium, between antibody:antigen interaction, took place. Bound antibody was detected with a peroxidase-labelled anti-histidine antibody, followed by o-PD substrate. The linear plot was used to determine the bound fraction of antibody present at equilibrium in the antigen mixtures.*

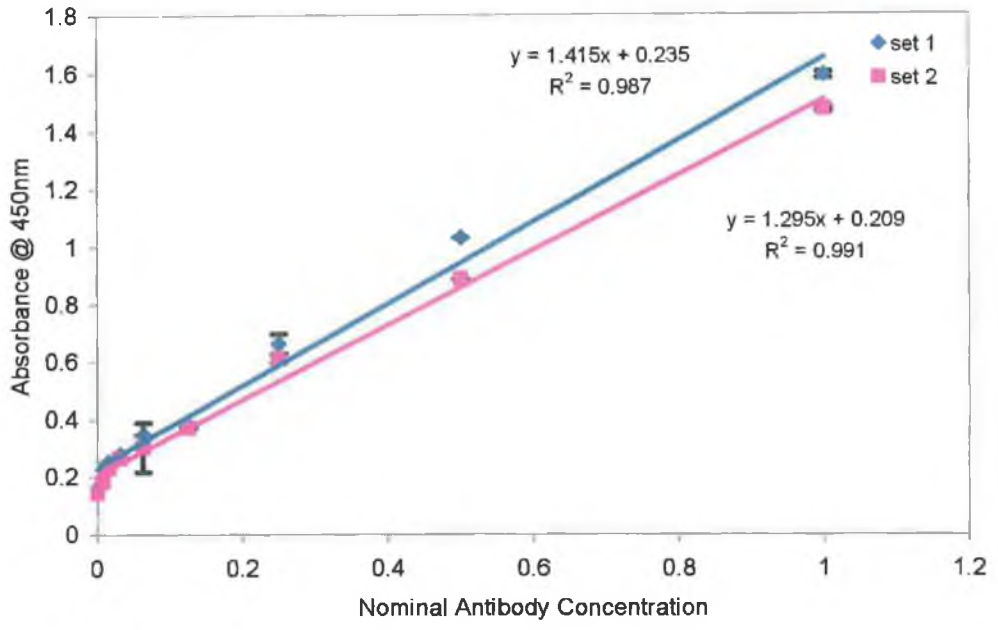


Figure 4.2.2: *Determination of the percentage displacement of monomeric scFv from solution phase equilibrium. Nominal antibody concentrations from the linear range were applied to an ELISA plate coated with 0.25 μg/ml M3G-OVA for 10 mins at room temperature. After this time the liquid in the wells was transferred to a second set of identical coated wells, for the same incubation period. The absorbance for each set of wells was monitored at 450nm. The fraction of antibody retained on the coated-antigen in the first plate is deduced from the ratio (Slope1- Slope2 / Slope 1). In this case the amount of antibody retained on the coated suface represents 7.8% of the total amount of antibody incubated in the plate.*

Table 4.2.1: *Calculation of free antibody concentration. The nominal concentrations of antibody were calculated with reference to the standard curve. Bound antibody was calculated to yield the values for 1/V. A plot of 1/[Ag] versus 1/V was linear. The equilibrium dissociation constant (K_D) is defined by the slope of this graph.*

[M3G] mol/L	1/[M3G] L/mol	A/A0	Nominal Antibody Concentration	1/V
0	∞	1	0.845	∞
6.25E-07	1.60E+06	0.090	0.082	0.998
3.13E-07	3.20E+06	0.097	0.088	1.005
1.56E-07	6.40E+06	0.119	0.108	1.028
7.81E-08	1.28E+07	0.131	0.119	1.041
3.90E-08	2.56E+07	0.130	0.102	1.150
1.95E-08	5.13E+07	0.166	0.129	1.199
9.75E-09	1.03E+08	0.236	0.184	1.310
4.88E-09	2.05E+08	0.381	0.297	1.614
2.44E-09	4.10E+08	0.566	0.442	2.306

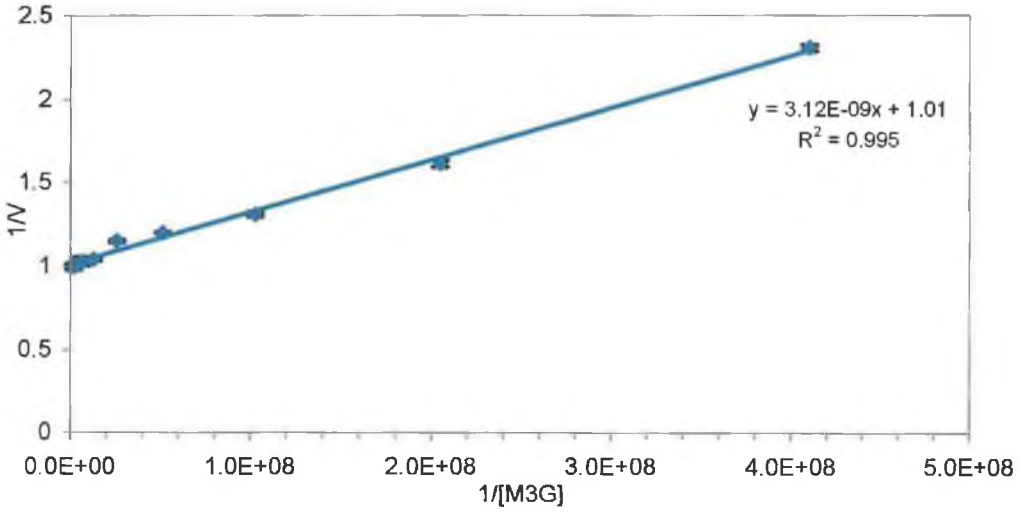


Figure 4.2.3: *Determination of the equilibrium dissociation constant for the interaction between monomeric scFv and M3G by the method described by Friguet et al. (1985). A constant concentration of antibody, within the linear range, was incubated with various concentrations of M3G and incubated overnight to reach equilibrium. The mixtures were added to an antigen-coated plate for 10 mins and the concentration of unbound antibody was determined using the standard curve constructed in Figure 4.2.1. The equilibrium dissociation constant for the interaction between monomeric scFv and M3G was determined to be 3.12 x 10^{-9} M.*

Table 4.2.2: *Calculation of free antibody concentration. The nominal concentrations of antibody were calculated with reference to the standard curve. Bound antibody was calculated to yield the values for 1/V. A plot of 1/[Ag] versus 1/V was linear. The equilibrium dissociation constant (K_D) is defined by the slope of this graph.*

[Morphine] mol/L	1/[Morphine] L/mol	A/A0	Nominal Antibody Concentration	1/V
0	∞	1	0.755	∞
1.00E-05	1.00E+05	0.087	0.069	1.095
5.00E-06	2.00E+05	0.094	0.075	1.104
2.50E-06	4.00E+05	0.104	0.083	1.116
1.25E-06	8.00E+05	0.120	0.095	1.136
6.25E-07	1.60E+06	0.147	0.117	1.172
3.13E-07	3.20E+06	0.140	0.100	1.163
1.56E-07	6.40E+06	0.183	0.131	1.225
7.81E-08	1.28E+07	0.267	0.191	1.365
3.91E-08	2.56E+07	0.377	0.270	1.606
1.95E-08	5.12E+07	0.495	0.354	1.979

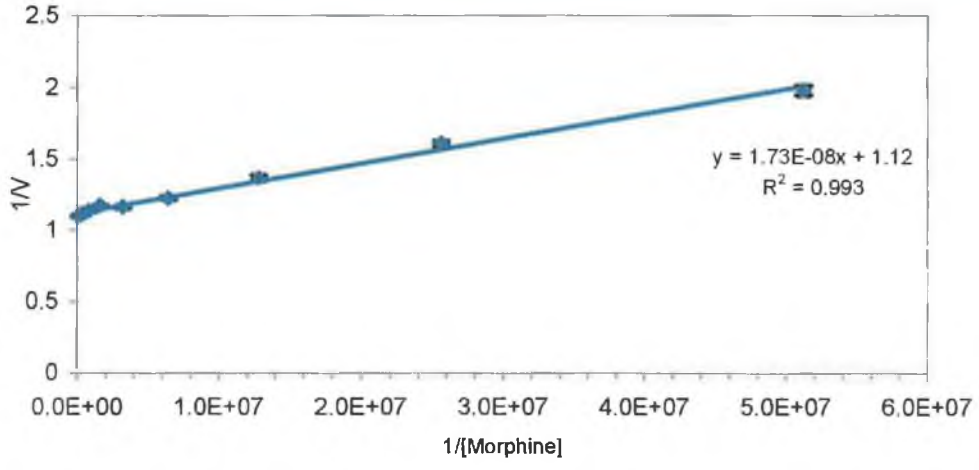


Figure 4.2.4: *Determination of equilibrium dissociation constant for the interaction between monomeric scFv and morphine by the method described by Friguet et al. (1985). A constant concentration of antibody, within the linear range, was incubated with various concentrations morphine and allowed to reach equilibrium overnight. The mixtures were added to an antigen-coated plate for 10 mins and the concentration of unbound antibody was determined using the standard curve constructed in Figure 4.2.1. The equilibrium dissociation constant for the interaction between monomeric scFv and morphine was determined to be 1.73 x 10^{-8} M.*

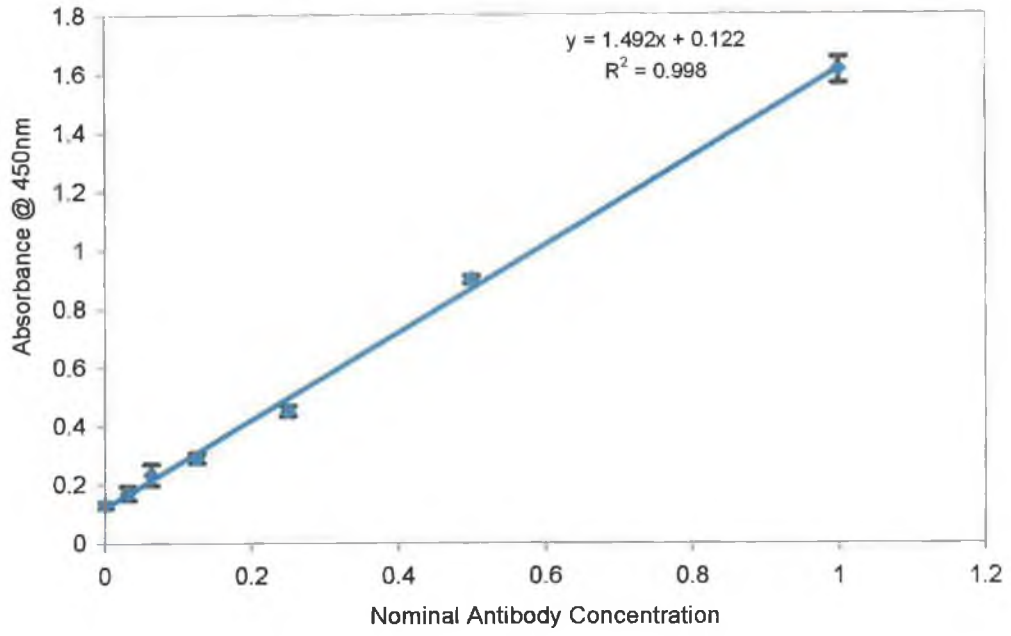


Figure 4.2.5: *Standard curve of nominal antibody concentration of dimeric scFv versus absorbance @ 450nm. Results shown are the average of triplicate measurements ± standard deviation. The highest concentration of antibody that lay within the linear region of the curve was assigned a nominal antibody concentration of '1'. Serial dilutions up to 1 in 32 were prepared, corresponding to 1, 0.5, 0.25, 0.125, 0.0615 0.0313, and 0, respectively. Each antibody dilution was added to an antigen-coated plate (0.5 µg/ml M3G-OVA) for10 mins at room temperature. The incubation period was predetermined to ensure that minimal disruption of the solution phase equilibrium of the antibody:antigen interaction took place. Bound antibody was detected with a peroxidase-labelled anti-histidine antibody, followed by o-PD substrate. The linear plot was used to determine the bound fraction of antibody present at equilibrium in the antigen mixtures.*

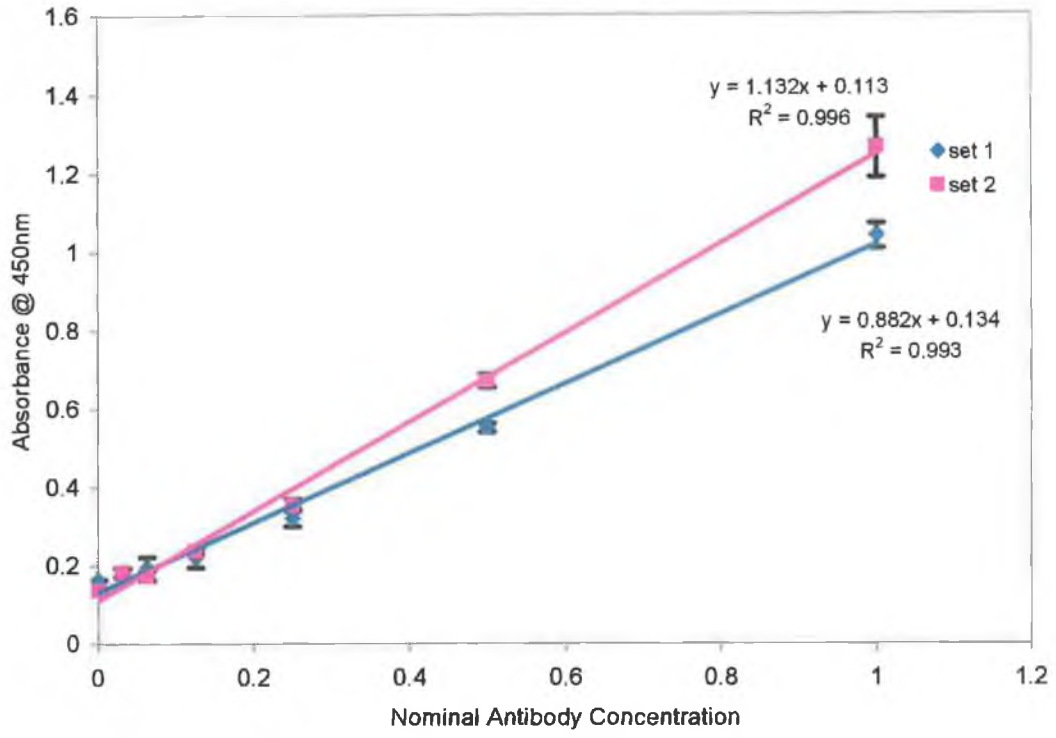


Figure 4.2.6: *Determination of the percentage displacement of dimeric scFv from solution phase equilibrium. Nominal antibody concentrations from the linear range were applied to an ELISA plate coated with 0.5 μg/ml M3G-OVA for 10 mins at room temperature. After this time the liquid in the wells was transferred to a second set of identical coated wells for the same incubation period. The absorbance for each set of wells was monitored at 450nm. In this case the amount of antibody retained on the coated antigen is negligible as the slope of the second dataset is higher than that observed for the first.*

Table 4.2.3: *Calculation of nominal free antibody concentration.*

[M3G] mol/L	1/[M3G] L/mol	A/A0	Nominal Antibody Concentration	1/V
0	∞	1	0.657	∞
1.25E-06	8.00E+05	0.108	0.088	0.907
6.25E-07	1.60E+06	0.122	0.099	0.918
3.13E-07	3.20E+06	0.138	0.112	0.932
1.56E-07	6.40E+06	0.123	0.062	1.599
7.81E-08	1.28E+07	0.147	0.074	1.662
3.90E-08	2.56E+07	0.230	0.116	1.930
1.95E-08	5.13E+07	0.476	0.239	3.668
9.75E-09	1.03E+08	0.771	0.387	4.368
4.88E-09	2.05E+08	0.838	0.420	6.168
2.44E-09	4.10E+08	0.909	0.456	10.95

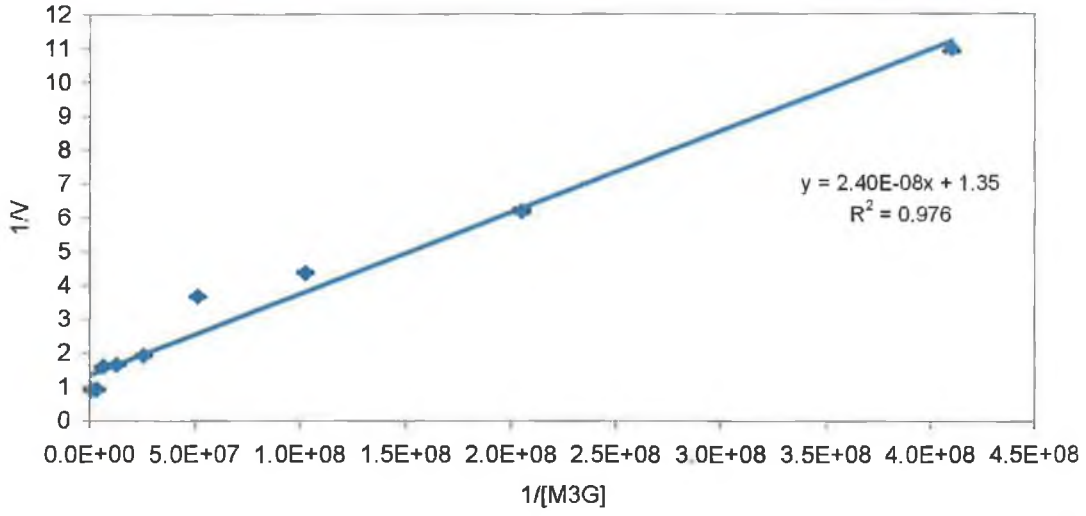


Figure 4.2.7: *Determination of the equilibrium dissociation constant for the interaction between dimeric scFv and M3G by the method described by Friguet et al. (1985). A constant concentration of antibody, within the linear range, was incubated with various concentrations of antigen until equilibrium was reached. The mixtures were added to an antigen-coated plate for 10 mins and the concentration of unbound antibody was determined using the standard curve constructed in Figure 4.2.5. The equilibrium dissociation constant for the interaction between dimeric scFv and M3G was determined to be 2.4 x 10^{-8} M.*

Table 4.2.4: *'Corrected' concentrations of free antibody according to Stevens (1987).*

[M3G] mol/L	1/[M3G] L/mol	Nominal Antibody Concentration	1/V	$1/V^{1/2}$
0	∞	0.657	∞	∞
1.25E-06	8.00E+05	0.088	0.907	0.952
6.25E-07	1.60E+06	0.099	0.918	0.958
3.13E-07	3.20E+06	0.112	0.932	0.965
1.56E-07	6.40E+06	0.062	1.599	1.265
7.81E-08	1.28E+07	0.074	1.662	1.289
3.90E-08	2.56E+07	0.116	1.930	1.389
1.95E-08	5.13E+07	0.239	3.668	1.915
9.75E-09	1.03E+08	0.387	4.368	2.090
4.88E-09	2.05E+08	0.420	6.168	2.483
2.44E-09	4.10E+08	0.4556	10.95	3.309

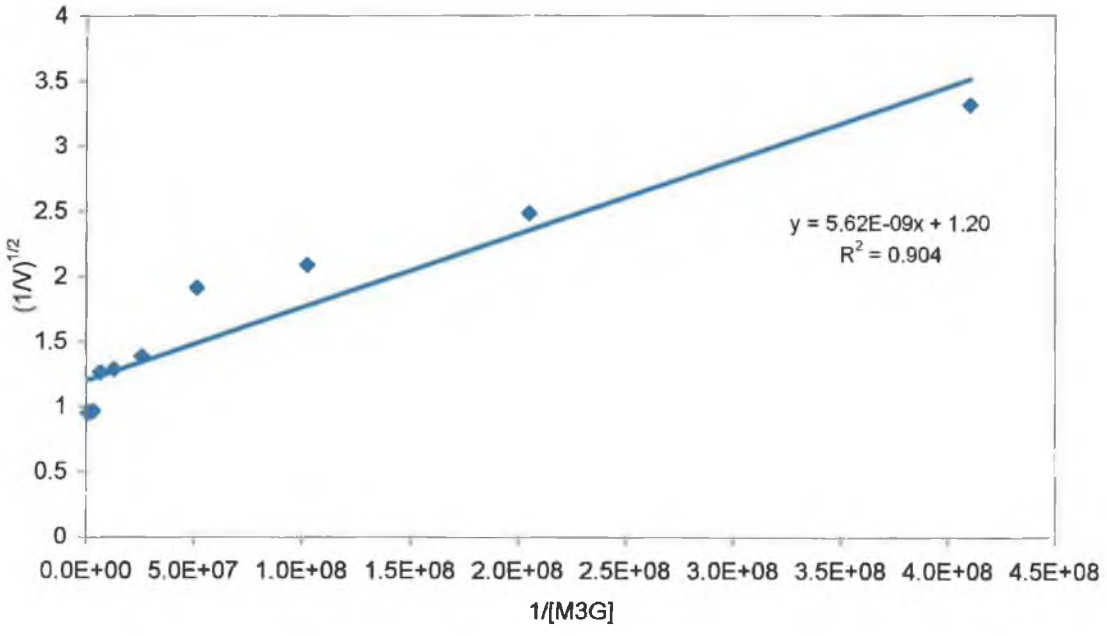


Figure 4.2.8: *Friguet assay to determine the equilibrium dissociation constant of dimeric scFv for M3G using 'corrected' antibody concentrations described by Stevens (1987) to account for the bivalent nature of the molecule. The corrected value of K_D was calculated to be 5.62 x 10^{-9} M, which represents over a four-fold increase in affinity*

Table 4.2.5: *Calculation of nominal free antibody concentration.*

[Morphine] mol/L	1/[Morphine] L/mol	A/A0	Nominal Antibody Concentration	1/V
0	∞	1	0.821	∞
1.00E-05	1.00E+05	0.081	0.078	1.088
5.00E-06	2.00E+05	0.107	0.104	1.120
2.50E-06	4.00E+05	0.116	0.113	1.131
1.25E-06	8.00E+05	0.117	0.114	1.133
6.25E-07	1.60E+06	0.112	0.075	1.126
3.13E-07	3.20E+06	0.132	0.088	1.151
1.56E-07	6.40E+06	0.151	0.101	1.178
7.81E-08	1.28E+07	0.199	0.133	1.249
3.91E-08	2.56E+07	0.354	0.237	1.549
1.95E-08	5.12E+07	0.609	0.408	2.556
9.77E-09	1.02E+08	0.675	0.452	3.074

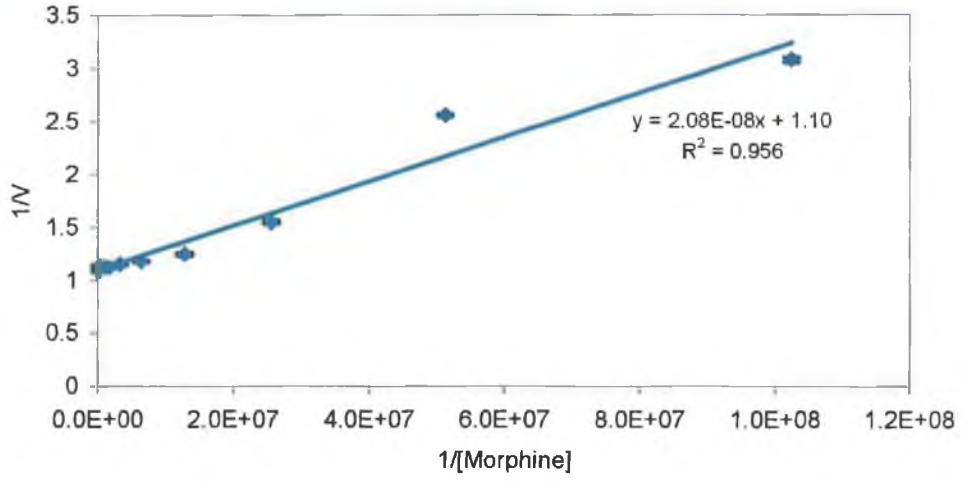


Figure 4.2.9: *Determination of the equilibrium dissociation constant for the interaction between dimeric scFv and morphine by the method described by Friguet et al. (1985). A constant concentration of antibody within the linear range was incubated with various concentrations of morphine and incubated until equilibrium was reached. The mixtures were added to an antigen-coated plate for 10 mins and the concentration of unbound antibody was determined using the standard curve constructed in Figure 4.2.5. The equilibrium dissociation constant for the interaction between dimeric scFv and morphine was determined to be 2.08 x 10^{-8} M.*

Table 4.2.6: *'Corrected' concentration of free antibody according to Stevens (1987)*

[Morphine] mol/L	1/[Morphine] L/mol	Nominal Antibody Concentration	1/V	$1/V^{1/2}$
0	∞	0.821	∞	∞
1.00E-05	1.00E+05	0.078	1.088	1.042
5.00E-06	2.00E+05	0.104	1.120	1.058
2.50E-06	4.00E+05	0.113	1.131	1.063
1.25E-06	8.00E+05	0.114	1.133	1.064
6.25E-07	1.60E+06	0.075	1.126	1.061
3.13E-07	3.20E+06	0.088	1.151	1.073
1.56E-07	6.40E+06	0.101	1.178	1.085
7.81E-08	1.28E+07	0.133	1.249	1.117
3.91E-08	2.56E+07	0.237	1.549	1.245
1.95E-08	5.12E+07	0.408	2.556	1.599
9.77E-09	1.02E+08	0.452	3.074	1.753

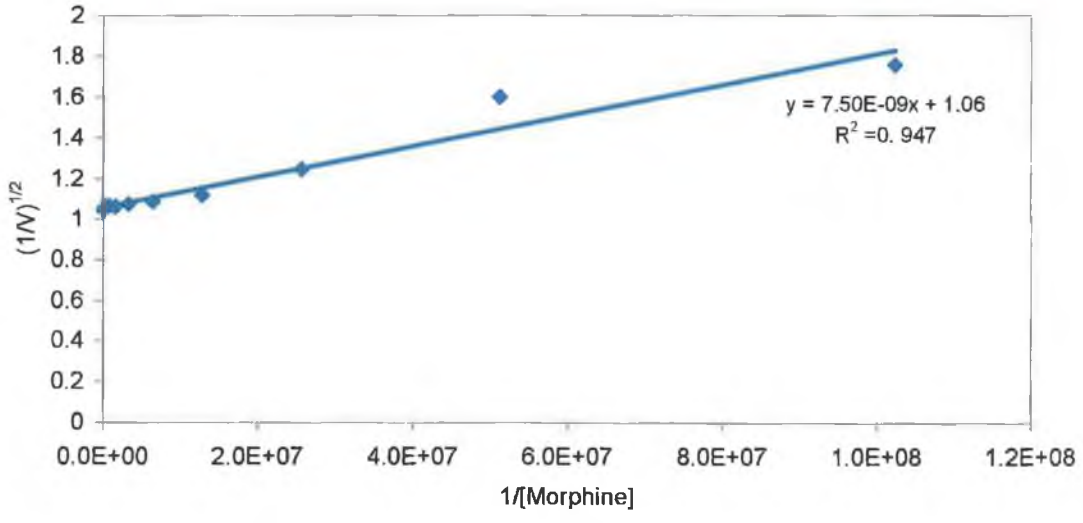


Figure 4.2.10: *Friguet assay to determine to determine the equilibrium dissociation constant of dimeric scFv for morphine using 'corrected' antibody concentrations described by Stevens (1987) to account for the bivalent nature of the molecule. The corrected value of K_D was calculated to be 7.5 x 10^{-9} M, which represents over a two-fold increase in affinity.*

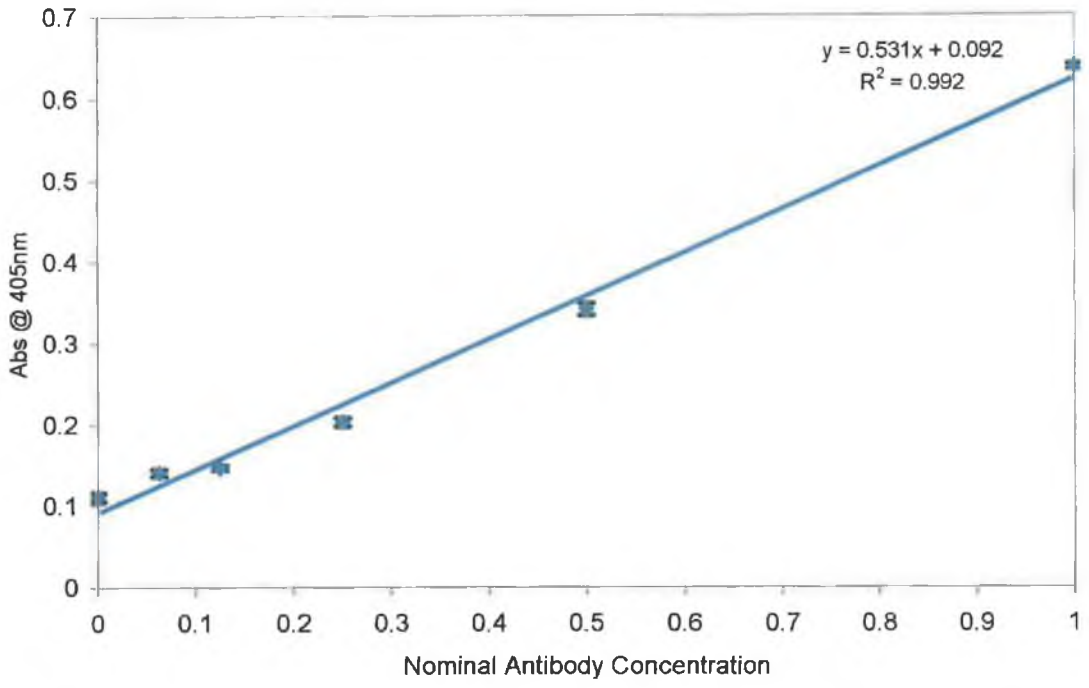


Figure 4.2.11: *Standard curve of nominal antibody concentration of dimeric enzyme-labelled scFv versus absorbance @ 405nm. Results shown are the average of triplicate measurements ± standard deviation. The highest concentration of antibody that lay within the linear region of the curve was assigned a nominal antibody concentration of '1'. Serial dilutions up to 1 in 16 were prepared, corresponding to 1, 0.5, 0.25, 0.125, 0.0615, and 0, respectively. Each antibody dilution was added to an antigen-coated plate (1 μg/ml M3G-OVA) for15 mins at room temperature. The incubation period was predetermined to ensure that minimal disruption of the solution phase equilibrium took place. The linear plot was used to determine the bound fraction of antibody present at equilibrium in the antigen mixtures.*

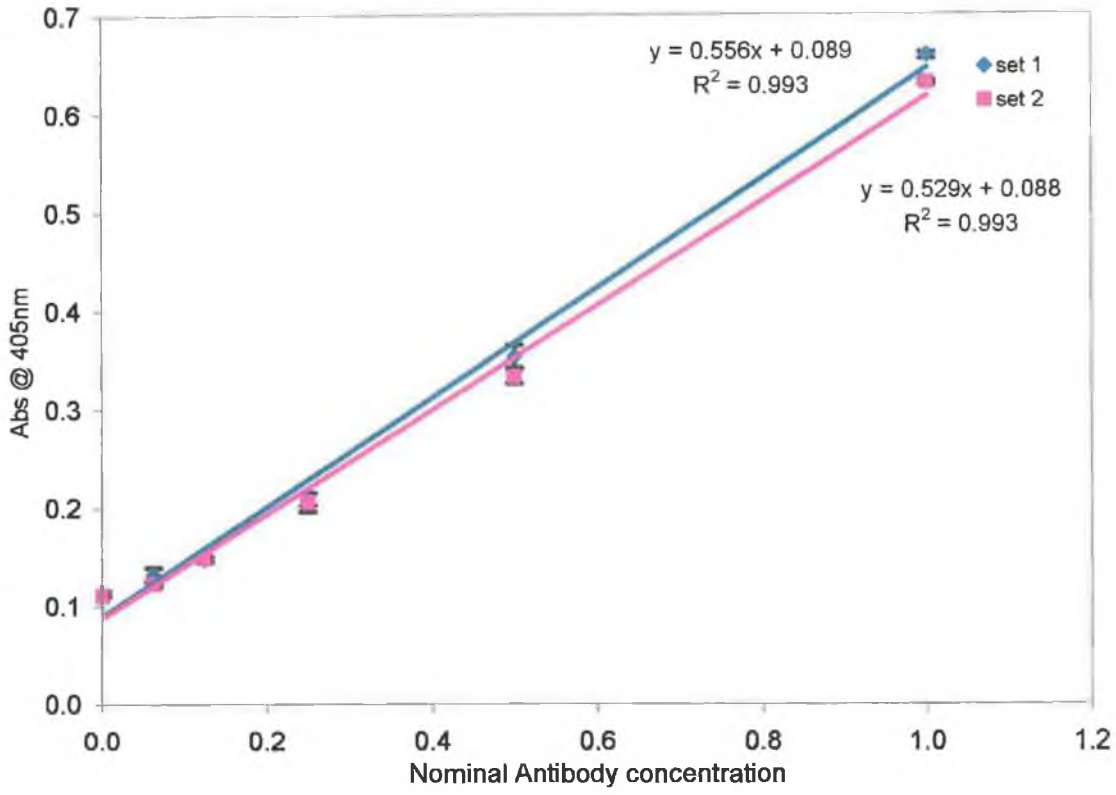


Figure 4.2.12: *Determination of the percentage displacement of the bifunctional scFv from solution phase equilibrium. Nominal antibody concentrations from the linear range were applied to an ELISA plate coated with 1 μg/ml M3G-OVA for 15 mins at room temperature. After this time the liquid in the wells was transferred to a second set of identical coated wells for the same incubation time. The absorbance for each set of wells was monitored at 405nm. The fraction of antibody retained on the coated antigen in the first plate is deduced from the ratio (Slope1 - Slope2 / Slope 1). In this case the amount of antibody retained on the coated antigen represents 4.8% of the total amount of antibody incubated in the plate.*

Table 4.2.7: *Determination of nominal free antibody concentration*

[M3G] mol/L	1/[M3G] L/mol	A/A0	Nominal Antibody Concentration	1/V
0	∞	1	1.183	∞
1.00E-06	1.00E+06	0.138	0.085	1.077
5.00E-07	2.00E+06	0.148	0.097	1.089
2.50E-07	4.00E+06	0.157	0.109	1.101
1.25E-07	8.00E+06	0.184	0.143	1.138
6.25E-08	1.60E+07	0.283	0.269	1.295
3.13E-08	3.20E+07	0.522	0.574	1.941
1.56E-08	6.40E+07	0.749	0.863	3.699
7.81E-09	1.28E+08	0.829	0.966	5.441

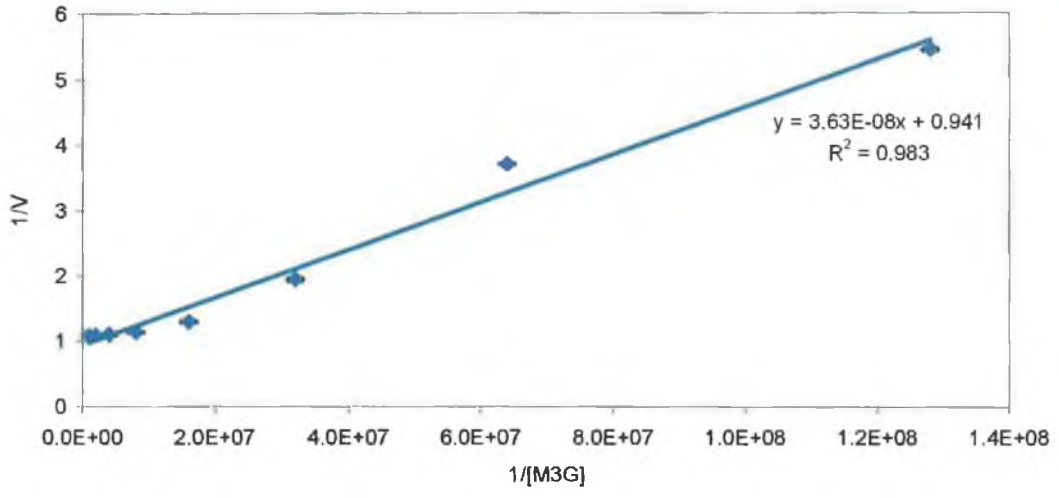


Figure 4.2.13: *Determination of the equilibrium dissociation constant for the interaction between bifunctional scFv and M3G by the method described by Friguet et al. (1985). A constant concentration of antibody, within the linear range, was incubated with various concentrations of M3G and incubated until equilibrium was reached. The mixtures were added to an antigen-coated plate for 15 mins and the concentration of unbound antibody was determined using the standard curve constructed in Figure 4.2.11. The equilibrium dissociation constant for the interaction between bifunctional scFv and M3G was determined to be 3.63 x 10^{-8} M.*

Table 4.2.8: *'Corrected' antibody concentration according to Stevens (1987).*

[M3G] mol/L	1/[M3G] L/mol	Nominal Antibody Concentration	1/V	$(1/V)^{1/2}$
0	∞	1.183	∞	∞
1.00E-06	1.00E+06	0.085	1.077	1.038
5.00E-07	2.00E+06	0.097	1.089	1.044
2.50E-07	4.00E+06	0.109	1.101	1.049
1.25E-07	8.00E+06	0.143	1.138	1.067
6.25E-08	1.60E+07	0.269	1.295	1.138
3.13E-08	3.20E+07	0.574	1.941	1.393
1.56E-08	6.40E+07	0.863	3.699	1.923
7.81E-09	1.28E+08	0.966	5.441	2.333

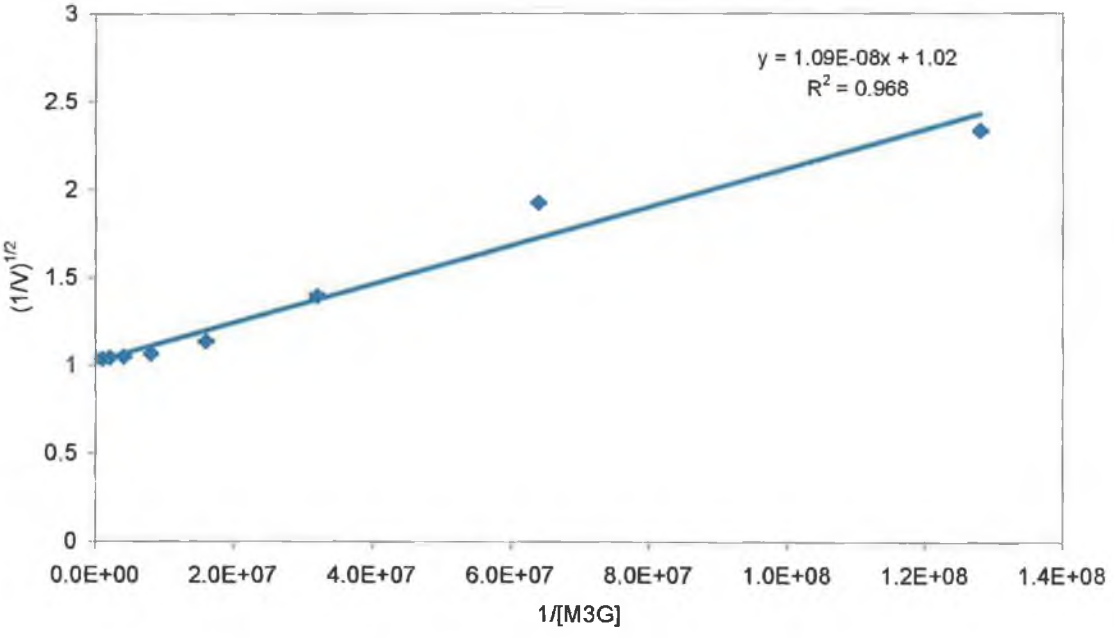


Figure 4.2.14: *Friguet assay to determine to determine the equilibrium dissociation constant of bifunctional scFv for M3G using 'corrected' antibody concentrations described by Stevens (1987) to account for the bivalent nature of the molecule. The corrected value of K_D was calculated to be 1.09 x 10^{-8} M, which represents over a three-fold increase in affinity.*

Table 4.2.9: *Determination of free antibody Concentration.*

[Morphine] mol/L	1/[Morphine] L/mol	A/A0	Nominal Antibody Concentration	1/V
0	∞	1	1.245	∞
1.00E-06	1.00E+06	0.201693	0.178	1.167
5.00E-07	2.00E+06	0.230842	0.216	1.211
2.50E-07	4.00E+06	0.261401	0.257	1.261
1.25E-07	8.00E+06	0.328632	0.347	1.387
6.25E-08	1.60E+07	0.433004	0.487	1.642
3.13E-08	3.20E+07	0.603197	0.714	2.347
1.56E-08	6.40E+07	0.726845	0.880	3.409

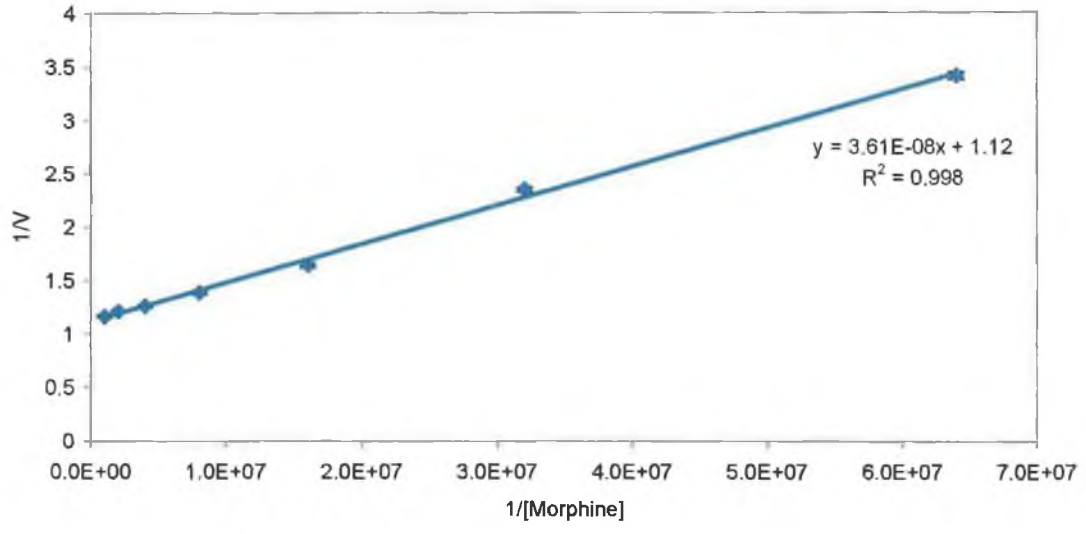


Figure 4.2.15: *Determination of the equilibrium dissociation constant for the interaction between bifunctional scFv and morphine by the method described by Friguet et al. (1985). A constant concentration of antibody within the linear range was incubated with various concentrations of morphine and incubated until equilibrium was reached. The mixtures were added to an antigen-coated plate for 15 mins and the concentration of unbound antibody was determined using the standard curve constructed in Figure 4.2.11. The equilibrium dissociation constant for the interaction between bifunctional scFv and morphine was determined to be 3.61 x 10^{-8} M.*

Table 4.2.10: *'Corrected' concentrations of free antibody according to Stevens (1987)*

[Morphine] mol/L	1/[Morphine] L/mol	Nominal Antibody Concentration	1/V	$(1/V)^{1/2}$
0	∞	1.245	∞	∞
1.00E-06	1.00E+06	0.178	1.167	1.080
5.00E-07	2.00E+06	0.216	1.211	1.100
2.50E-07	4.00E+06	0.257	1.261	1.123
1.25E-07	8.00E+06	0.347	1.387	1.178
6.25E-08	1.60E+07	0.487	1.642	1.282
3.13E-08	3.20E+07	0.714	2.347	1.532
1.56E-08	6.40E+07	0.880	3.409	1.846

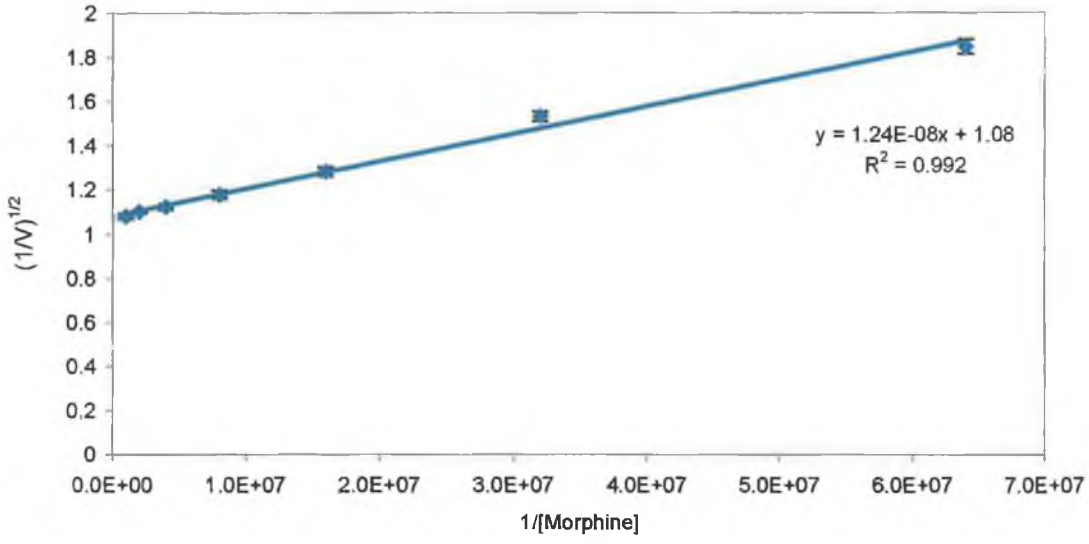


Figure 4.2.16: *Friguet assay to determine to determine the equilibrium dissociation constant of bifunctional scFv for morphine using 'corrected' antibody concentrations described by Stevens (1987) to account for the bivalent nature of the molecule. The corrected value of K_D was calculated to be 1.24 x 10^{-8} M, which represents a three-fold increase in affinity.*

4.2.2 Solution phase affinity determination by BIAcore analysis

The first requirement for measuring affinity constants using BIAcore technology is that the antibody is specific for the M3G moiety of the coated sensor chip surface. Purified scFv was injected over a 'capped' dextran surface, an OVA-coated surface and a M3G-OVA-coated surface. The antibody was shown to be specific for the M3G moiety of the sensor chip surface (Figure 4.2.17).

Immobilisation of the M3G-OVA chip was carried out as described in Section 2.7.2. A dextran-coated sensor chip was activated with EDC/NHS and approximately 8,000 response units of M3G-OVA was immobilised on the chip surface. Known molar concentrations of anti-M3G scFvs were serially diluted and passed over an M3G-OVA-coated sensor chip at a flow rate of 5 µl/min to construct a standard curve of antibody concentration versus response units observed. The surface was regenerated between injections with a 1-minute pulse of 10 mM NaOH. A standard curve of antibody concentration versus response units observed was prepared to quantify free, uncomplexed antibody. A known concentration of antibody was then mixed with varying concentrations of antigen and allowed to reach equilibrium at 37°C for 2 hours. Each mixture was analysed for uncomplexed antibody by passing 10 µl of the equilibrated sample over an M3G-OVA coated surface. The concentration of free antibody was quantified from the standard calibration curve. The concentration of free antibody was plotted against the concentration of antigen. A solution affinity fit, described in equation 4.11, was applied to the dataset using BIAevaluation software.

Response units observed at each concentration of monomeric and dimeric scFv were used to construct two standard curves (Figures 4.2.18 and 4.2.21). Free concentrations of each antibody were calculated from the respective graphs. The solution affinity fit for monomeric scFv yielded a K_D value of 1.79×10^{-8} M ± 1.85×10^{-9} M for its interaction with M3G (Figure 4.2.19), and 1.01×10^{-7} M ± 1.07×10^{-8} M for its interaction with morphine (Figure 4.2.20).

Dimeric scFv, although bivalent in nature, was also fitted with a monovalent model, as the dataset could not be defined using the 'corrected' bivalent model described by

Piehler *et al.*, (1997). It has previously been observed that bivalent molecules do not necessarily behave in a bivalent manner, under the conditions used for analysis (Quinn and O'Kennedy, 2001). It can only be assumed that the binding of the dimeric antibody lies somewhere between a monovalent and bivalent interaction. This has an effect on the fitting of the solution affinity model. A comparison of Figures 4.2.22 and 4.2.23 show that the effect of this monovalent model is more pronounced for the interaction with M3G, as it yields a poor global fit. Dimeric scFv was shown to have an equilibrium dissociation constant of 1.09×10^{-8} M ± 2.97×10^{-9} M for M3G (Figure 4.2.22) and 1.12×10^{-7} M ± 2.98×10^{-8} M for morphine (Figure 4.2.23). A summary of the equilibrium dissociation constants determined using solution phase BIAcore analysis is shown in Table 4.2.12.

As discussed in Chapter 3, the bifunctional scFv could not be successfully purified. This meant that the actual concentration of scFv could not be determined. An accurate determination of antibody concentration is a pre-requisite for solution phase analysis using BIAcore. For this reason no analysis was carried out on the bifunctional scFv.

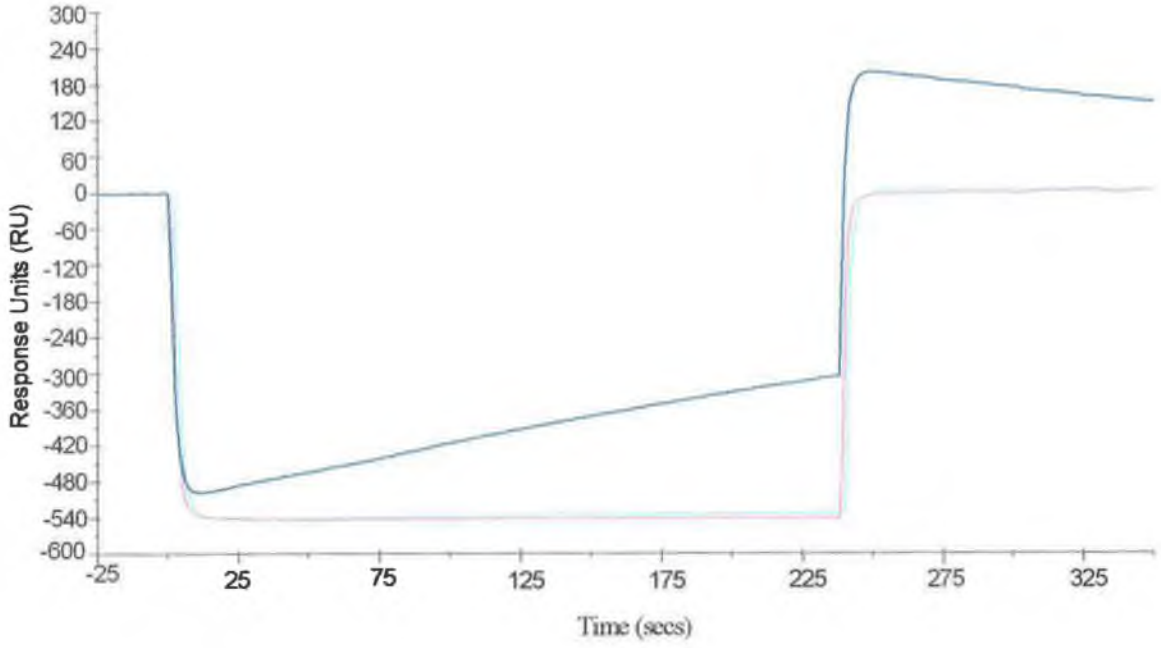


Figure 4.2.17: *Overlay plot demonstrating binding of IMAC-purified monomeric scFv to immobilised M3G - OVA, OVA and a 'capped' dextran surface. This resulted in negligible binding to the control OVA (—) and dextran surfaces (—). However, 200 response units were observed to bind to the immobilised M3G-OVA (—) surface. This indicates that the scFv is specific to the M3G portion of the conjugate.*

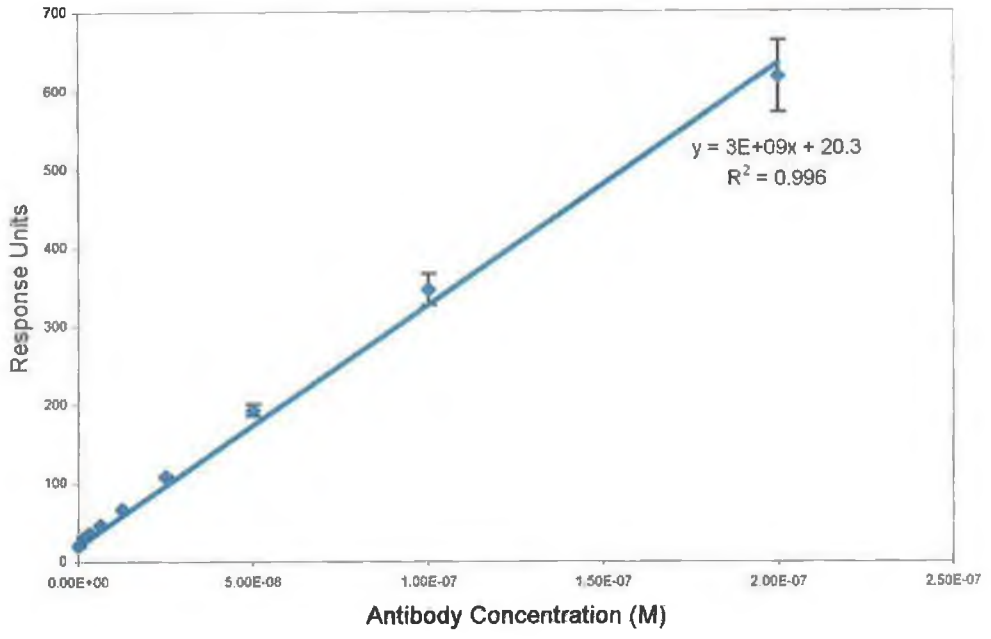


Figure 4.2.18: *Standard Curve showing response of monomeric scFv versus concentration, performed on BIAcore. Serial dilutions of known concentrations of antibody were passed over an M3G-OVA-coated surface for 2 mins at a flow rate of 5 µl per minute. A standard curve of response units observed versus antibody concentration was constructed to calculate the concentration of free antibody present in each of the equilibrium mixtures. Results shown are the average of triplicate measurements ± standard deviation.*

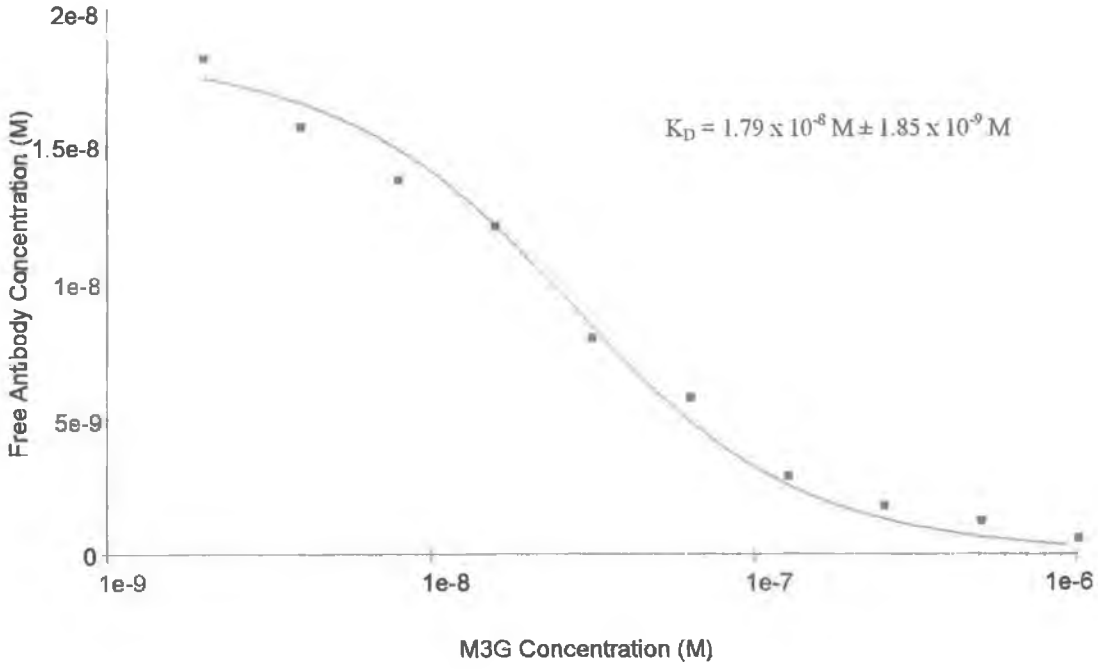


Figure 4.2.19: *Determination of overall solution equilibrium affinity constant between monomeric scFv and M3G on an M3G-OVA-coated surface. The results show M3G concentration (M) plotted against the calculated values of free antibody concentration (M). A 1:1 interaction model was used to describe the interaction and fitted to the data set using BIAevalution software. The equilibrium dissociation constant ± standard error was derived to be 1.79 x 10^{-8} M ± 1.85 x 10^{-9} M.*

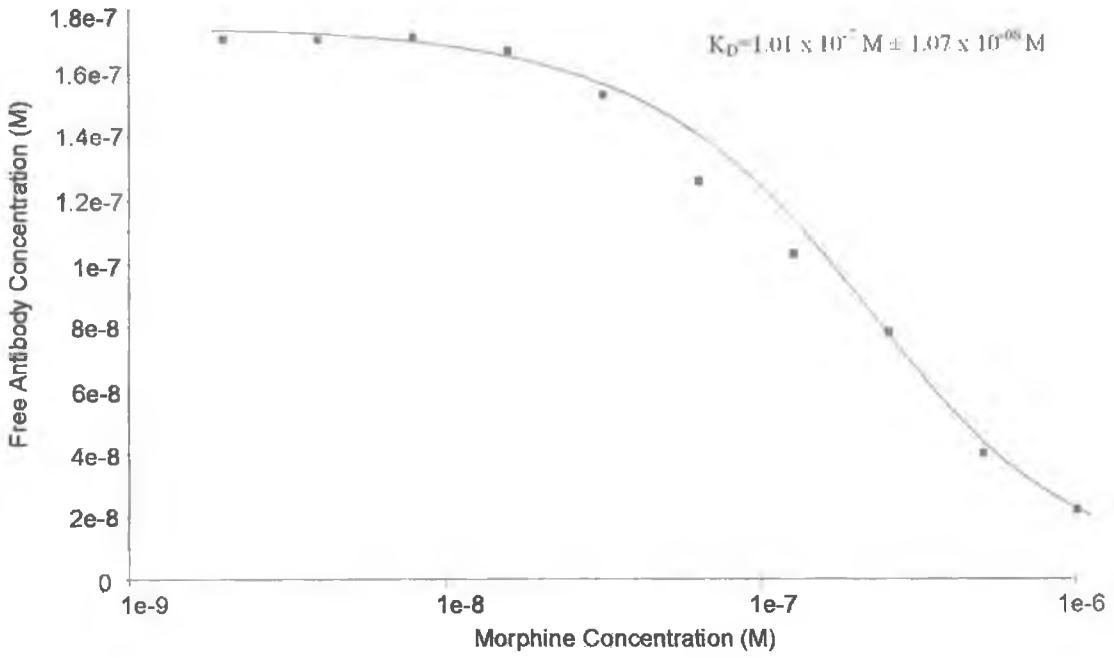


Figure 4.2.20: *Determination of overall solution equilibrium affinity constant between monomeric scFv and morphine on an M3G-OVA-coated surface. The results show morphine concentration (M) plotted against the calculated values of free antibody concentration (M). A 1:1 interaction model was used to describe the interaction and fitted to the data set using BIAevalution software. The equilibrium dissociation constant ± standard error was derived to be 1.01 x 10^{-7} M ± 1.07 x 10^{-8} M.*

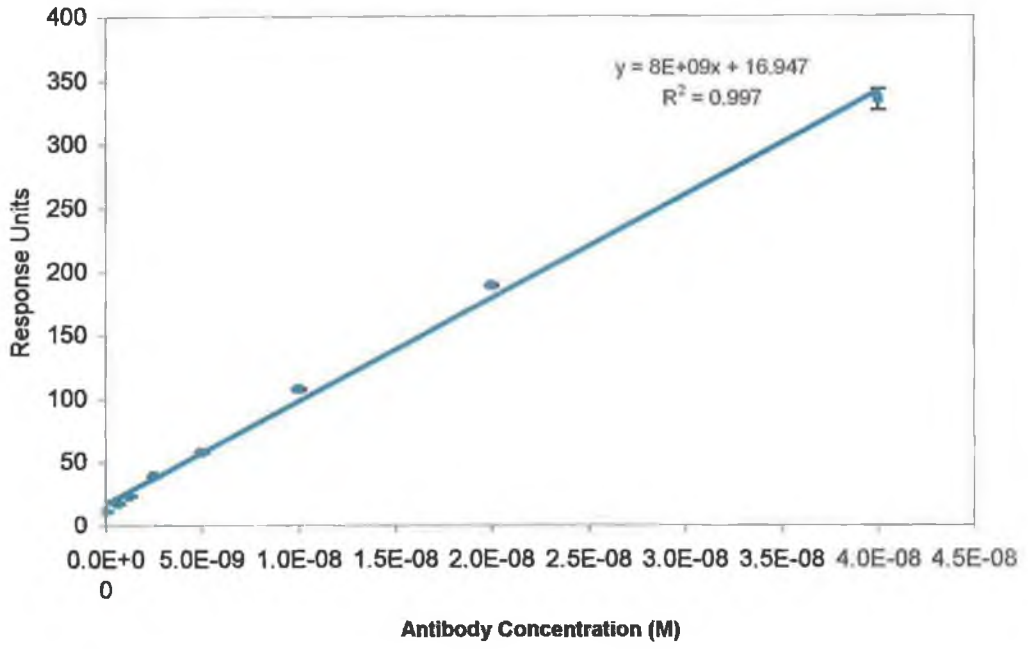


Figure 4.2.21: *Standard Curve illustrating response observed at various concentrations of dimeric scFv, performed on BIAcore. Serial dilutions of known concentrations of antibody were passed over an M3G-OVA-coated surface for 2 mins at a flow rate of 5 µl per minute. A standard curve of response units observed versus antibody concentration was constructed to calculate the concentration of free antibody present at equilibrium. Results shown are the average of triplicate measurements ± standard deviation.*

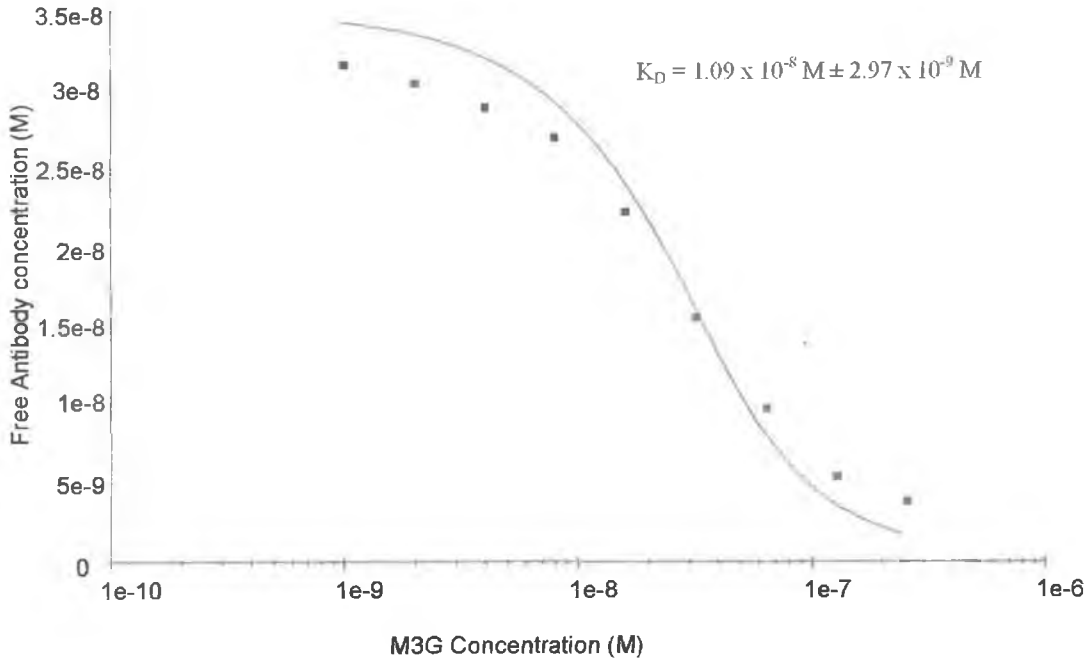


Figure 4.2.22: *Determination of overall solution equilibrium affinity constant between dimeric scFv and M3G on an M3G-OVA coated surface. The results show M3G concentration (M) plotted against the calculated values of free antibody concentration (M). A 1:1 interaction model was used to describe the interaction and fitted to the data set using BIAevalution software. The equilibrium dissociation constant ± standard error was derived to be 1.09 x 10^{-8} M ± 2.97 x 10^{-9} M. The global fit provided by the model yielded a poor fit. This is due to the bivalency of the antibody. It can only be assumed that the binding interaction lies somewhere between that of a monovalent and bivalent interaction.*

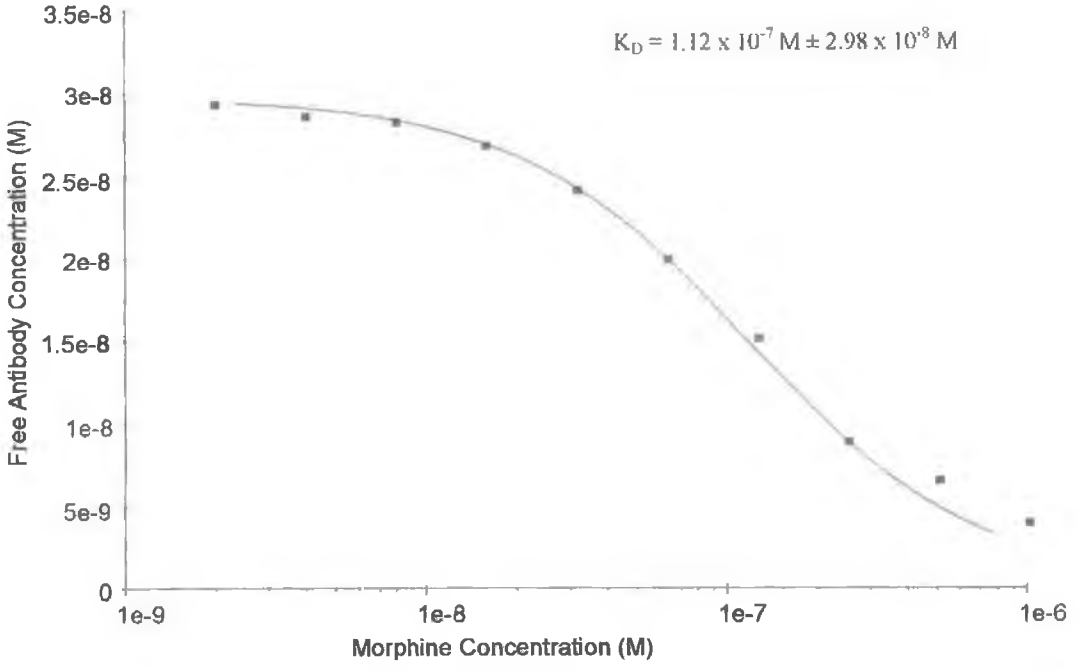


Figure 4.2.23: *Determination of overall solution equilibrium affinity constant between dimeric scFv and morphine on an M3G-OVA-coated surface. The results show morphine concentration (M) plotted against the calculated values of free antibody concentration (M). A 1:1 interaction model was used to describe the interaction and fitted to the data set using BIAevalution software. The equilibrium dissociation constant ± standard error was derived to be 1.12 x 10^{-7} M ± 2.98 x 10^{-8} M.*

Table 4.2.11: *Equilibrium dissociation constants, K_D, as determined by the method of Friguet et al., (1985) for the interaction between monomeric, dimeric and bifunctional scFv, and target antigens, M3G and morphine.*

	K_D for M3G	Corrected K_D for M3G	K_D for Morphine	Corrected K_D for Morphine
Monomeric scFv	3.12×10^{-9} M	Not Applicable	1.73×10^{-8} M	Not Applicable
Dimeric scFv	2.40×10^{-8} M	5.62×10^{-9} M	2.08×10^{-8} M	$7.50\ 10^{-9}$ M
Bifuctional scFv	3.63×10^{-8} M	1.09×10^{-9} M	3.61×10^{-8} M	1.24×10^{-8} M

Table 4.2.12: *Equilibrium dissociation constants, K_D, as determined by solution phase analysis on BIAcore, describing the interaction between monomeric and dimeric, and target antigens, M3G and morphine.*

	K_D for M3G	K_D for Morphine
Monomeric scFv	1.79×10^{-8} M $\pm$ 1.85×10^{-9} M	1.01×10^{-7} M $\pm$ 1.07×10^{-8} M
Dimeric scFv	1.09×10^{-8} M $\pm$ 2.97×10^{-9} M	1.12×10^{-7} M $\pm$ 2.98×10^{-8} M

4.2.3 Development of an ELISA-based assay for morphine in saliva

The cross reactivity potential and high affinity of monomeric scFv for morphine was exploited in a case study carried out to determine the feasibility of using morphine levels present in saliva to indicate recent heroin use. As the level of saliva production is depleted in drug users, it was decided to minimise the sample volume required for analysis (Fanning, 2002). This was chosen as an alternative to sample dilution as this introduces further errors into the analysis. Concerns existed that salivary morphine concentrations could be extremely low (Cone *et al.*, 1993). Therefore, sample dilution would decrease the probability of successful identification of drug use.

In order to assess any possible matrix effects of saliva, a competitive ELISA assay was first carried out in PBS. The assay was carried out in exactly the same manner as described in Chapter 3, using morphine as the standard free analyte. The minimum sample volume required for analysis was determined to be 25 µl, as saliva was to be the final detection matrix. The assay was repeated over 5 separate days and the relationship between normalised absorbance at 450nm (A/A0) and free morphine concentration is plotted in Figure 4.2.24. The mean absorbance values, standard deviations, coefficients of variation and percentage recoveries for the assay are shown in Table 4.2.13. This proves that a robust competitive ELISA for free morphine could be developed in an end sample volume of as little as 25 µl.

An indirect checkerboard was performed to determine the optimal conjugate coating and antibody dilution for use in saliva. Optimised parameters included coating with 3.125 µg/ml of M3G-OVA conjugate and a 1 in 60 dilution of scFv. A competitive ELISA was carried out, as previously described in Chapter 3, with the exception of using 25 µl of 'spiked saliva sample', assayed with 25 µl of scFv. A calibration curve was constructed by dividing the mean absorbance response obtained at each morphine concentration (A) by the absorbance response in the presence of zero analyte (A0). Normalised absorbance values (A/A0) were plotted against log of morphine concentration to generate a calibration curve. The relationship between normalised absorbance at 450nm (A/A0) and concentration of free morphine in saliva samples is shown in Figure 4.2.25. The values shown represent an inter-day assay,

where five replicates of each sample were performed on five separate days. The mean absorbance values, standard deviations, coefficients of variation and percentage recoveries for the assay are shown in Table 4.2.14. This gives an indication of the robustness of the assay, as saliva samples from different donors were used on each occasion. The range of detection for the assay was from 6 to 1,563 ng/ml in saliva, with a least detectable dose or LDD of 12 ng/ml. The percentage recovery within this range was between – 8% and + 13%. The least detectable dose or LDD was determined to be the concentration of morphine that inhibited 10% of antibody binding (Hennion and Barcelo, 1998). This was chosen over the limit of 75% to 125% percentage recovery, set by Findlay *et al.* (2000), as it was thought that upper and lower limits of quantification would be more accurate if they did not fall within the asymptotic regions of the curve.

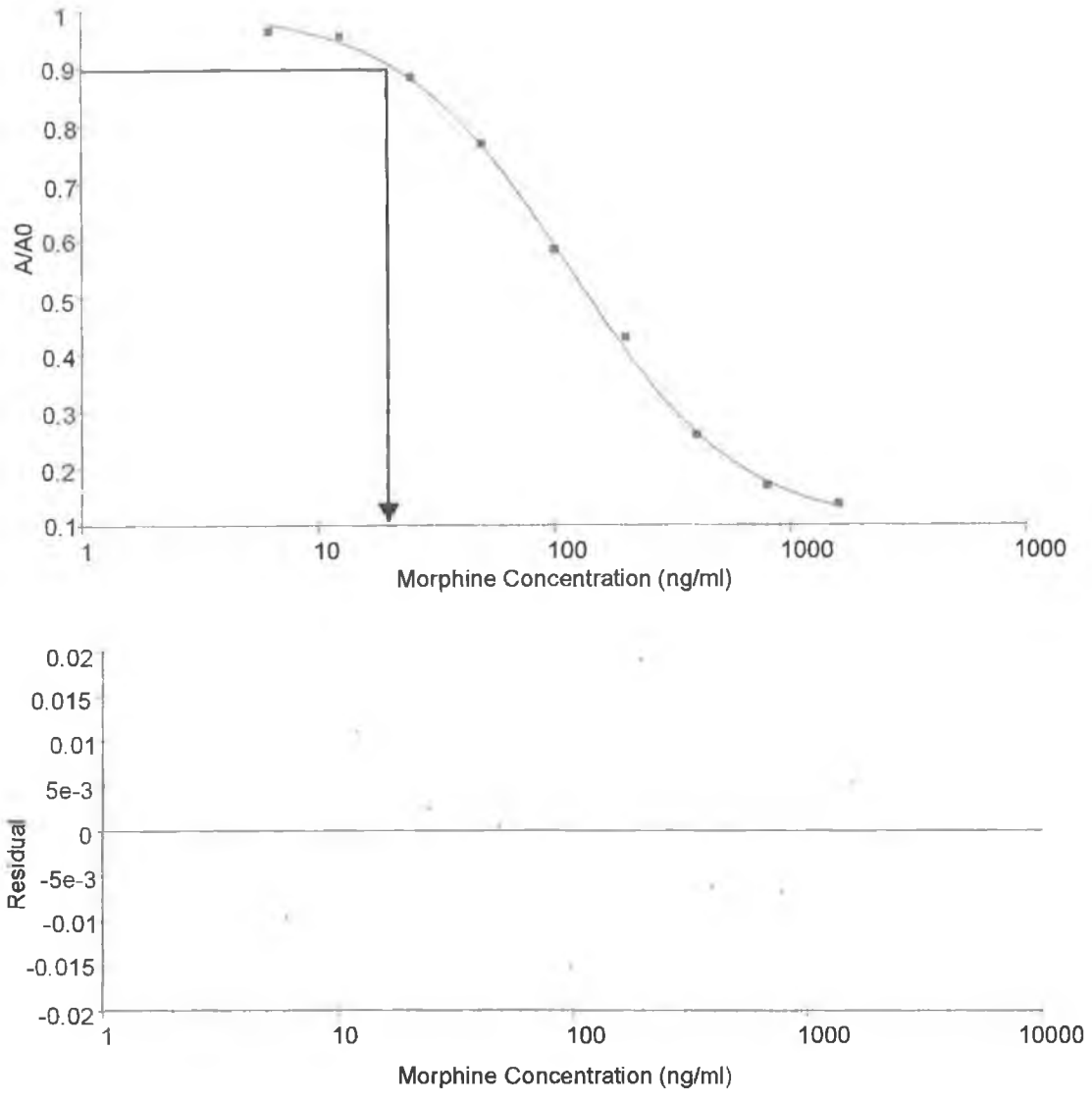


Figure 4.2.24: *Inter-day ELISA assay using pAK 400 monomeric scFv to detect free morphine in PBS. M3G-OVA was coated at 3.125 μg/ml and a 25 μl of a 1 in 60 dilution of scFv was used to assay 25 μl of morphine standards in PBS. Bound antibody was detected using M1 monoclonal anti-flag antibody, followed by a peroxidase-labelled anti-mouse antibody and visualized using o-PD. Absorbance was read at 450nm and related to morphine concentration using a 4-parameter logistic model that ranged from 6.1 ng/ml to 1,562.5 ng/ml. The least detectable morphine concentration that led to 10% inhibition of antibody binding was shown to be 20 ng/ml.*

Table 4.2.13: *Inter-day assay coefficients of variation for the detection of free morphine using the monomeric scFv. Five sets of nine standards were assayed over five different days and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard. Percentage recovery was calculated from the four-parameter model produced using BIAevaluation software.*

Concentration (ng/ml)	A/A0 ± SD	% CV	Back calculated concentration (ng/ml)	% Recovery
1,562.50	0.138 ± 0.007	5.08%	1,428.78	91.44%
781.25	0.171 ± 0.012	5.70%	859.76	110.05%
390.63	0.259 ± 0.025	5.52%	413.59	105.88%
195.31	0.431 ± 0.041	9.61%	183.48	93.94%
97.66	0.583 ± 0.053	9.17%	104.82	107.34%
48.83	0.769 ± 0.074	9.53%	49.74	101.88%
24.41	0.886 ± 0.049	9.45%	24.07	101.17%
12.21	0.958 ± 0.055	7.04%	11.37	93.14%
6.10	0.967 ± 0.049	5.07%	8.46	138.61%

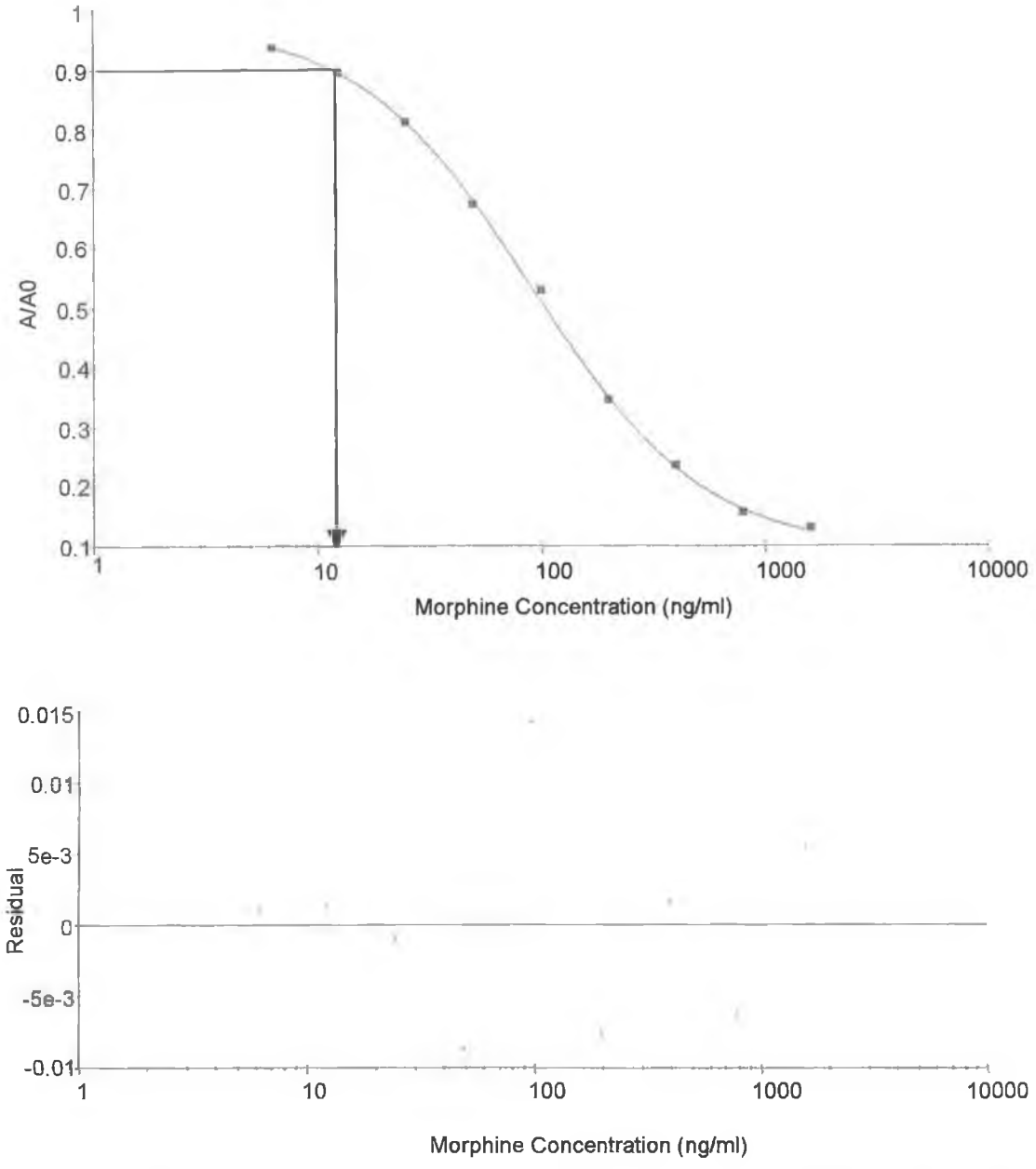


Figure 4.2.25: *Inter-day ELISA assay using pAK 400 monomeric scFv to detect free morphine in saliva. M3G-OVA was coated at 3.125 μg/ml and a 25 μl of a 1 in 60 dilution of scFv was used to assay 25 μl of saliva 'spiked' with morphine. Bound antibody was detected using M1 monoclonal anti-flag antibody, followed by a peroxidase-labelled anti-mouse antibody and visualized using o-PD. Absorbance was read at 450nm and related to morphine concentration using a 4-parameter logistic model that ranged from 6.1 ng/ml to 1,562.5 ng/ml. The least detectable morphine concentration that led to 10% inhibition of antibody binding was shown to be 12 ng/ml.*

Table 4.2.14: *Inter-day assay coefficients of variation for the detection of free morphine in saliva using the monomeric scFv. Five sets of nine standards were assayed over five different days and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard. Percentage recovery was calculated from the four-parameter model produced using BIAevaluation software.*

Morphine Concentration (ng/ml)	A/A0 ± S.D.	% CV	Back calculated concentration (ng/ml)	% Recovery
1,562.50	0.130 ± 0.012	8.93%	1,443.94	92.43%
781.25	0.156 ± 0.014	9.19%	882.42	112.95%
390.63	0.235 ± 0.021	9.00%	392.36	100.44%
195.31	0.345 ± 0.018	5.08%	203.37	104.13%
97.66	0.529 ± 0.030	5.71%	92.84	95.07%
48.83	0.674 ± 0.027	4.05%	50.95	104.34%
24.41	0.812 ± 0.011	1.39%	24.60	100.76%
12.21	0.896 ± 0.037	4.18%	12.08	98.96%
6.10	0.939 ± 0.016	1.72%	5.96	97.65%

4.2.4 Analysis of saliva samples from drug users

A pilot study was carried out to determine the feasibility of using morphine levels in present in saliva to indicate recent heroin use. The study involved testing a prototype saliva collection device obtained from Trinity Biotech to collect saliva samples from patients attending the Trinity Court Drug Treatment Centre, Pearse St, Dublin 2. The device consisted of a 'cotton wool-like' material that was used to absorb the sample and a plastic plunger that was used to extract the liquid. Clients that participated in the study were all thought to have a high probability of recent drug use. This was identified by staff at the Centre through past experience of the patients' behaviour and routine urine analysis. Patients were invited to talk about their experiences with screening for drug use, methods of sample collection and biological matrices used for analysis. All patients expressed a strong dislike of giving urine samples. Many experienced difficulty with being asked to urinate on demand and felt that sample collection posed an invasion of privacy. All patients interviewed expressed a more favourable attitude to giving a saliva sample for monitoring.

Participation in the study was voluntary for all patients and written consent was obtained prior to any sample collection (Appendix 1A). All patients, with the exception of patient 6, were on methadone replacement therapy, consisting of differing methadone dosages. Patients were asked for a brief history of their recent drug use throughout the last week. This was carried out in the absence of Clinic personnel to encourage patients to fully disclose any recent drug use. This confidentiality was found to be important. Patients were unwilling to divulge this type of information in the presence of a nurse as it could affect their dosage of methadone given in the rehabilitation program, if they were known to have relapsed. Patients with a higher risk of relapse were put on a low dose program, while patients who had been 'clean' from drugs for a longer period were on a higher dose of methadone. Patients 'self-confessed' recent drug use is outlined in Table 4.2.15.

All analysis was covered by a confidentiality agreement, where samples were referred to using a system of a number and initial for sample labelling. Patients were asked to chew on the 'cotton-wool-like material' for a couple of minutes. Once the material

was saturated, it was inserted into the plunger device and extracted. All patients, with the exception of patient 8, were willing to use the prototype collection device. Patient 8 was put off by the texture of the 'cotton wool-like material' and instead opted to provide a spit sample. This however, proved unsuitable for analysis. Volumes of saliva provided by patients varied from 1.8 mls for patient 1 to 25 µl for patient 6. It was clearly evident that the ability to provide larger sample volumes was diminished through increased drug abuse. Laboratory analysis was carried out in DCU, as described in Section 2.5.12.4, with each sample being tested in triplicate. Results shown in Table 4.2.16 illustrate that appreciable concentrations of morphine were present in the saliva of all patients tested.

Table 4.2.15: *Summary of interviews with patients tested regarding their recent drug use history*

Patient Number	Program	Recent Drug Use	Medications
1 (A-D)	Rehab-High dose methadone	Heroin: within 5 days Benzodiazepines Cannabis: smoked with 24 hours	Methadone
2 (S-C)	Low dose methadone	Heroin: within 15-20 mins Diazepam: (10mgs x 20) prior to visit Dalmane: (30 mgs x 10) prior to visit	Methadone Zimovane (Benzodiazepine)
3 (F-W)	Low dose methadone	Heroin: within 24 hours Benzodiazepines: within 24 hours	Methadone
4 (JMK)	Low dose methadone	Heroin: within 24-48 hours Cannabis: within 24 hours	Methadone Valium
5 (JOD)	Low dose methadone	Heroin: within 24 hours Cannabis: within 24 hours	Methadone Valium
6 (R-H)	New client; no program	Heroin: within hours DF118's (dihydrocodeine): within minutes	
7 (AOR)	Low dose methadone	Heroin: within 48hours	Methadone
8 (A-D)	Low dose methadone	Heroin: within 24 hours Cannabis:smoked with 24 hours	Methadone Stillnox Valium Benzodiazepines

Table 4.2.16: *Levels of morphine detected in saliva samples obtained from client attending a drug rehabilitation programme in Trinity Court Drug Treatment Centre.*

Sample	Assayed	A/AO	Back Calculated Morphine Concentration (ng/ml)	% C.V.
Patient 1	Neat	0.732	38.89	20.18%
Patient 2	Neat	0.253	340.70	0.70%
Patient 3	1 in 20	0.201	10,102.88	3.10%
Patient 4	Neat	0.826	22.72	43.56%
Patient 5	1 in 10	0.693	466.29	2.06%
Patient 6	1 in 50	0.167	36,982.25	8.64%
Patient 7	1 in 10	0.647	571.36	2.88%

4.2.5 Development of a BIAcore-based assay using monomeric scFv for Morphine in saliva

4.2.5.1 Preconcentration studies

In order to optimise immobilisation of hapten-carrier to the sensor chip surface, a pre-concentration step is necessary to identify the optimal pH conditions for immobilisation. At pH values above 3, the carboxymethylated surface of the CM5 sensor chip is negatively charged. For efficient immobilisation, the pH of the ligand should possess a net positive charge in order to maximise electrostatic interactions. This is achieved by using a buffer with a pH below the pI of the ligand to be immobilised.

Preconcentration studies involved preparing solutions of 100 μg/ml hapten-protein conjugate in 10 mM sodium acetate solution at a range of varying pHs. Sodium acetate buffer was chosen due to its low ionic strength so as to maximise ionic interactions. These solutions were then passed over an underivatised chip surface and the amount of electrostatic interaction monitored. The pH, which gave the highest degree of electrostatic interaction, i.e. highest binding, was chosen to perform immobilisations. The optimal pH for immobilisation was determined to be pH 4.9, as shown in Figure 4.2.26.

4.2.5.2 Immobilisation of M3G-OVA

The immobilisation of M3G-OVA was carried out as described in Section 2.7.2. A solution of EDC/NHS was passed over the sensor chip surface to activate the carboxyl groups of the dextran layer. M3G-OVA, at a concentration of 100 μg/ml in 10 mM sodium acetate, pH 4.9 was then passed over the activated surface in random order for 40 mins, at a flow rate of 2 μl per minute. The activated NHS esters on the chip surface react with the amine groups on the M3G conjugate, producing an immobilisation profile as shown in Figure 4.2.27. NHS esters were then deactivated

using a solution of 1 M ethanolamine, pH 8.5, to block any unreacted sites. This process is known as 'capping'.

4.2.5.3 Regeneration conditions

Regeneration of the sensor chip surface is essential if it is to be used repeatedly in an assay format. All bound analyte must be removed without damaging the coating on the sensor chip surface. Regeneration conditions must be optimised to achieve the maximum number of regeneration cycles from one surface. This is done to calculate the reproducibility of the surface but also to minimise the number of chips used for an assay and therefore the expense involved. For this reason, the least stringent regeneration solution that is sufficiently strong enough to remove all bound analyte is chosen to maximise the life of the sensor chip, while ensuring reproducibility.

20 µl of a 1 in 400 dilution of monomeric scFv, diluted in HBS, was injected over the chip surface; this was followed by a 1-minute pulse with 10 mM NaOH. This regeneration solution proved adequate to remove all bound scFv from the surface. The surface could be regenerated up to 50 times without any significant loss in antibody binding activity (Figure 4.2.28).

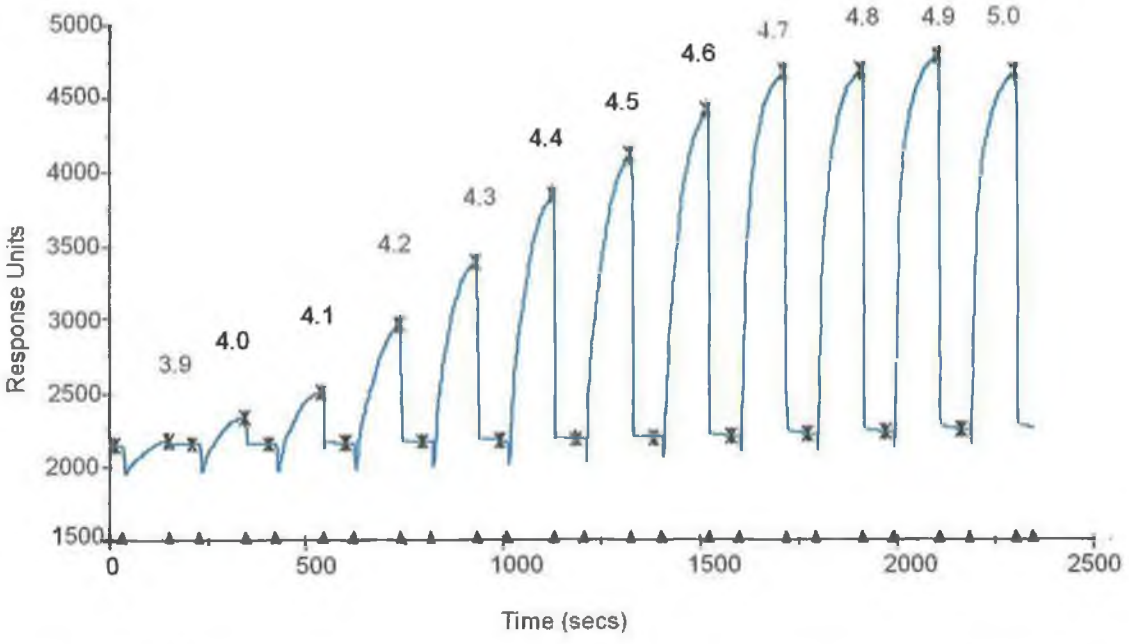


Figure 4.2.26: *Preconcentration of M3G-OVA conjugate onto the surface of a CM-5 dextran chip. Solutions of 100 μg/ml of M3G-OVA in 10 mM acetate buffer at a range of pH from 3.9 to 5.0 were passed over the same unactivated chip surface at 2 ml/min for 2 min. The optimal pH for immobilisation was determined to be pH 4.9.*

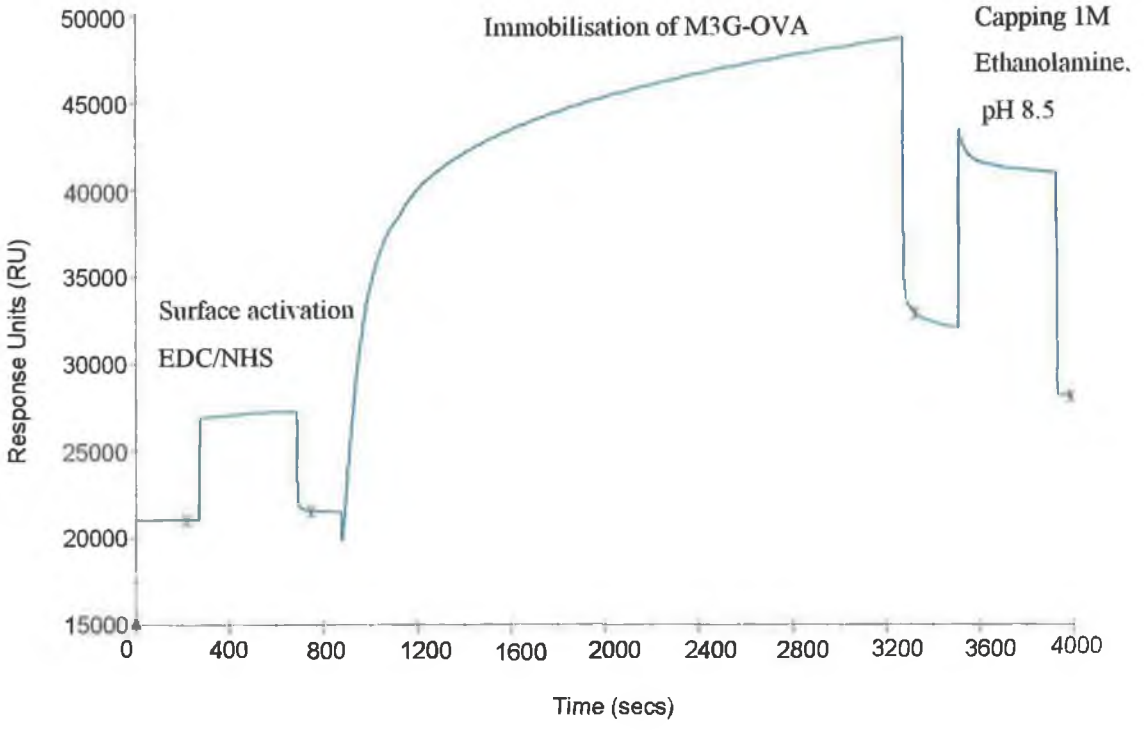


Figure 4.2.27: *Immobilisation of 100 μg/ml M3G-OVA in 10 mM sodium acetate buffer, pH 4.9. The dextran surface is activated with EDC/NHS. 100 μg/ml of M3G-OVA in 10 mM sodium acetate, pH 4.9, is passed over the activated surface, for 40 mins, at a flow rate of 2 μl per minute. The surface is then capped using 1 M ethanolamine, pH 8.5, to block any unreacted sites. Approximately 6,500 response units of conjugate binding were recorded.*

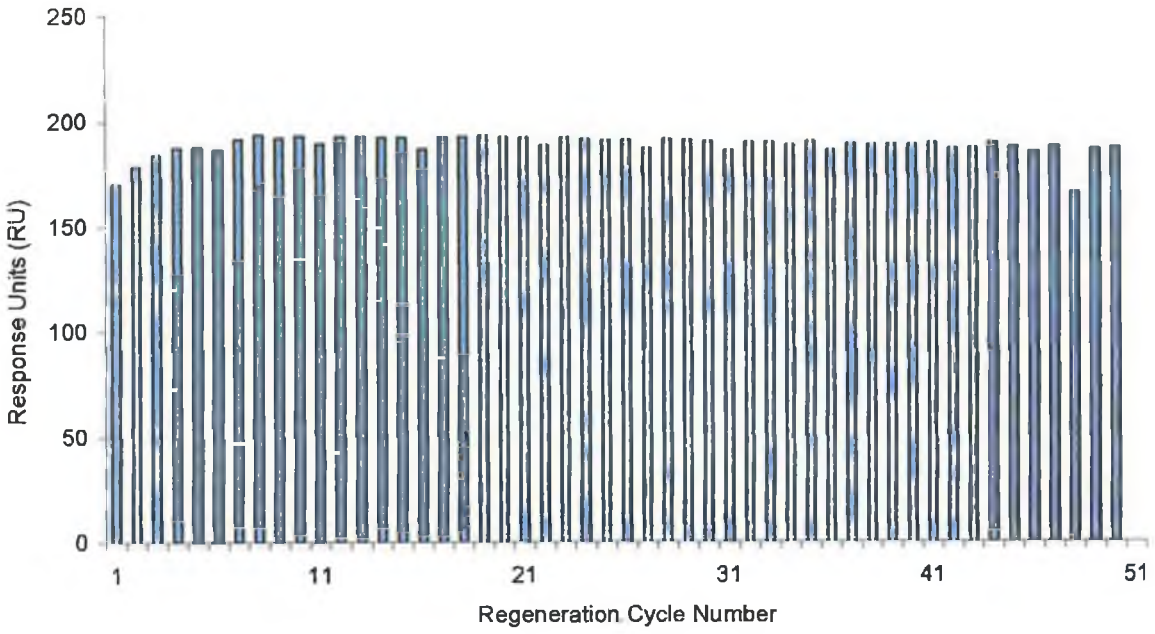


Figure 4.2.28: *Typical regeneration profile for approximately 50 cycles of a 4-minute binding pulse of purified monomeric scFv to the surface of a chip immobilised with M3G–OVA. A 1 in 400 dilution of antibody was used and the surface regenerated with a 1-minute pulse of 10 mM NaOH. The ligand binding capacity was found to be variable for the first five regenerations, for this reason, the surface was regenerated five times before use in an assay.*

4.2.5.4 Determination of the range of detection of morphine in HBS buffer using a BIAcore inhibition assay format

In order to investigate any possible matrix effects of saliva on an optical biosensor, such as BIAcore, a preliminary assay was carried out to determine the detection range of the monomeric scFv for morphine in HBS buffer. Varying concentrations of morphine ranging from 1562,500 pg/ml to 763 pg/ml were prepared in HBS. Each concentration of morphine was incubated with an equal volume of the monomeric scFv diluted to 1/200 (to ensure a final dilution of 1/400) and allowed to equilibrate for 30 min at 37°C. 20 µl of equilibrated samples was injected over the sensor chip surface, at a flow rate of 5 µl per minute, in random order. Each sample was analysed in triplicate, with a 1-minute injection pulse of 10 mM NaOH to regenerate the surface between each injection.

The inhibition assay format employed proved to be more sensitive than ELISA for the detection of morphine with an assay range of 382 to 781,250 pg/ml achieved in HBS. This represents a 20-fold increase in assay sensitivity. The assay was repeated using 3 replicates of each standard over one day for intra–day variability studies and using three replicates of each standard over three days for inter–day variability studies (Figure 4.2.29). CV's for both assays were below the required margins of 10 %. Average response units ± standard deviation, % C.V.'s and % recoveries for the inter-day assay, as shown in Table 4.2.17.

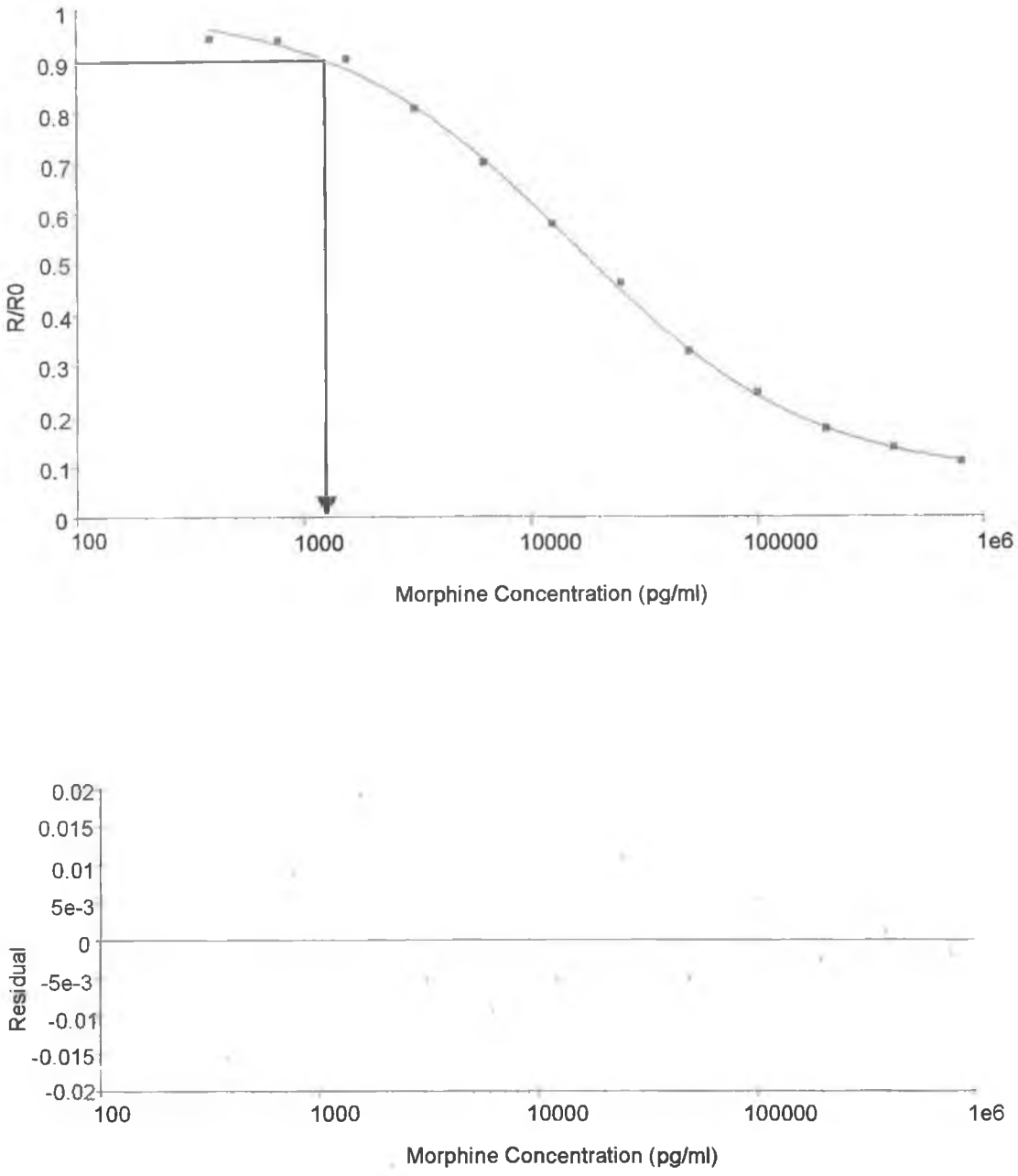


Figure 4.2.29: *Inter-day assay curve for inhibition assay for free morphine in PBS solution using monomeric scFv antibody. Three replicates of each standard were analysed on three separate days. CV's were all below 10%. M3G–OVA was immobilised on the sensor chip surface. The range of detection was found to be from 381.5 to 781,250 pg/ml. The binding response at each free morphine concentration (R) was divided by the antibody response in the absence of free morphine (R_0) to give a normalised binding response. The least detectable morphine concentration that led to 10% inhibition of antibody binding was shown to be 1.2 ng/ml.*

Table 4.2.17: *Inter-day assay coefficients of variation for the detection of free morphine in HBS using the monomeric scFv in an inhibition assay format on BIAcore. Three sets of twelve standards were assayed over three different days and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard. Percentage recovery was calculated from the four-parameter model produced using BIAevaluation software.*

Concentration Morphine (pg/ml)	R/R0 ± S.D.	% C.V.	Back Calculated morphine Concentration (pg/ml)	% Recovery
781,250.0	0.109 ± 0.004	3.82%	852,023.3	109.1%
390,625.0	0.136 ± 0.013	9.37%	401,021.3	102.7%
195,312.5	0.173 ± 0.008	4.50%	203,827.4	104.4%
97,656.3	0.245 ± 0.017	6.84%	94,194.3	96.5%
48,828.1	0.327 ± 0.026	8.09%	51,143.3	104.7%
24,414.1	0.462 ± 0.022	4.86%	23,169.1	96.0%
12,207.0	0.578 ± 0.023	4.04%	12,612.1	103.3%
6,103.5	0.699 ± 0.055	7.93%	6,618.2	108.4%
3,051.8	0.806 ± 0.037	4.62%	3,225.0	105.7%
1,525.9	0.904 ± 0.009	0.96%	1,202.8	80.0%
762.9	0.942 ± 0.028	2.99%	662.2	86.8%
381.5	0.947 ± 0.031	3.24%	601.2	157.6%

4.2.5.5 Development of an inhibition assay for morphine in saliva using BIAcore analysis

Saliva, as a biological sample can be heterogeneous in nature. Its viscosity, colour and texture will vary between different individuals. These parameters will also vary for the saliva of any one individual, depending on recent intake of food and fluids. For this reason, it is not an ideal matrix for use in an assay that is based on optical recognition by refractive index change.

When a drug-negative saliva sample, containing no antibody, was injected over the sensor chip surface, a positive binding response was observed. It had already been proven that the antibody was specific for the drug moiety of the chip surface (Figure 4.2.17) and, therefore, it was determined that the saliva sample matrix was responsible for the background binding. Initially it was tried to counteract this background by passing the saliva sample simultaneously over OVA and M3G-OVA coated surfaces. The background interaction of the saliva sample with the OVA surface could then be reference subtracted from the specific antibody interaction with the M3G-OVA-coated surface. This however proved unsuccessful as the saliva matrix behaved differently in the presence of an antibody binding interaction. Binding reponses to the OVA surface were larger than that at the M3G surface. Such problems with using saliva on BIAcore have previously been described by Fanning (2002). Dilution of the sample in buffer was proven insufficient to eliminate the problem due to the variation in the composition of saliva samples.

In order to circumvent some of the 'stickiness' associated with the matrix, sample volume was decreased from that used in the model buffer system. 12 µl of sample was passed over the surface, which meant that surface contact time for saliva samples was decreased from 4 mins in the buffer system to 2.4 mins. This decreased the level of background binding response to approximately 50 units for a negative sample.

Varying concentrations of morphine were prepared in saliva from 312,5000 pg/ml to 1,524 pg/ml. Each concentration of morphine was incubated with an equal volume of

the monomeric scFv diluted to 1/200 (to ensure a final dilution of 1/400) and allowed to equilibrate for 30 min at 37°C. 12 µl of equilibrated samples was injected over the sensor chip surface, at a flow rate of 5 µl per minute, in random order. Each sample was analysed in triplicate, with a 1-minute injection pulse of 10 mM NaOH, proving sufficient to regenerate the surface between each injection.

The detection range of the assay (1562,500 pg/ml to 762 pg/ml) was slightly less sensitive than that in a model buffer system. However, the assay was still more sensitive than ELISA-based assay. The assay was repeated using 3 replicates of each standard over one day for intra–day variability studies and using three replicates of each standard over three days for inter–day variability studies (Figure 4.2.30). Coefficients of variation were below 14% for the assay, with all but the lowest standard yielding a % recovery within ± 25% of the actual concentration. Table 4.2.18 illustrates average response units ± standard deviation, % C.V.'s and % recoveries for the inter-day assay.

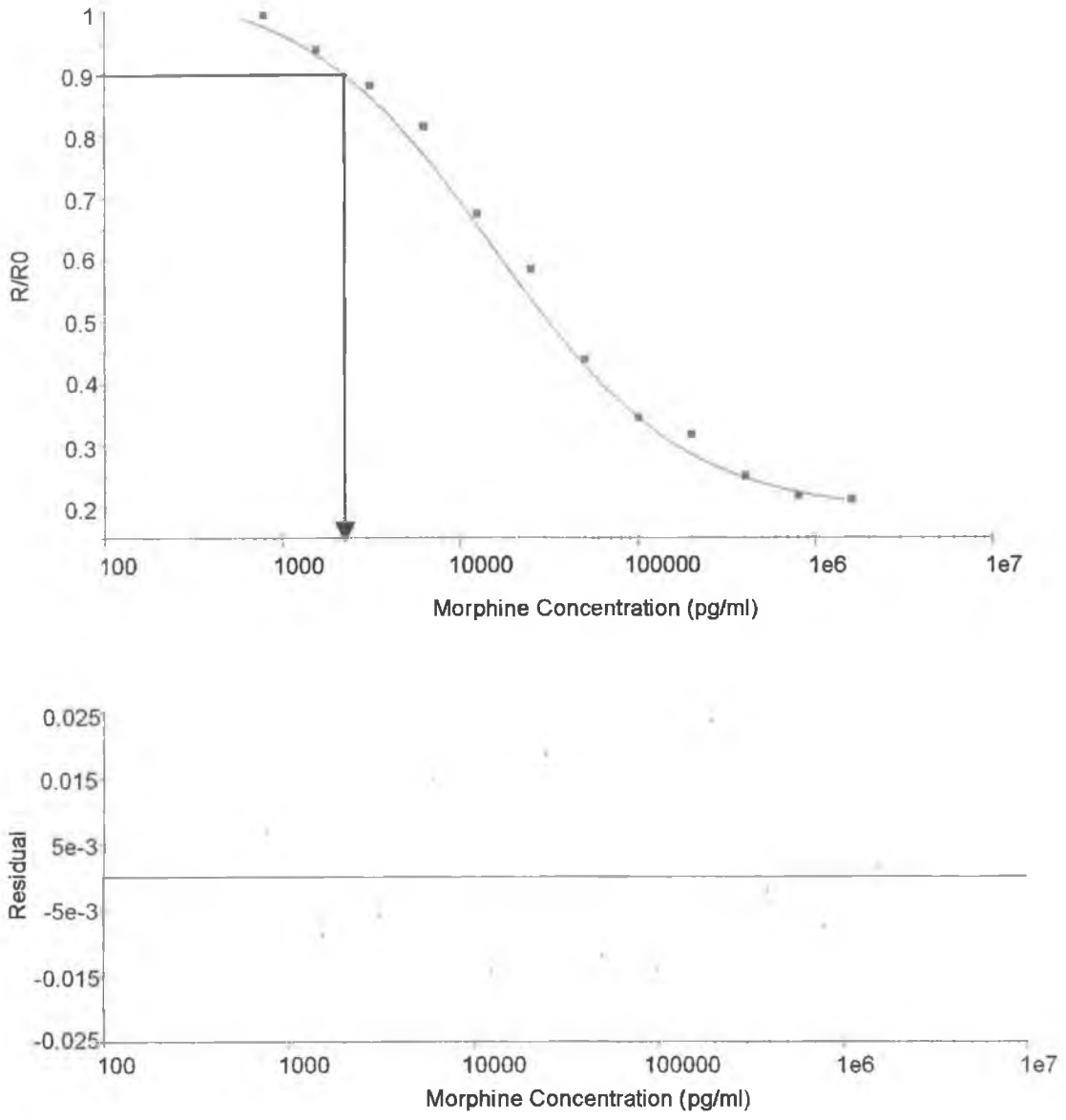


Figure 4.2.30: *Inter-day assay curve for an inhibition assay for free morphine in saliva solution using monomeric scFv antibody. Three replicates of each standard were analysed on three separate days. CV's were all below 14%. M3G–OVA was immobilised on the sensor chip surface. The range of detection was found to be from 783 pg/ml to 1,563 ng/ml. The binding response at each free morphine concentration (R) was divided by the antibody response in the absence of free morphine (R_0) to give a normalised binding response. The least detectable morphine concentration that led to 10% inhibition of antibody binding was shown to be 2.5 ng/ml.*

Table 4.2.18: *Inter-day assay coefficients of variation for the detection of free morphine in saliva using the monomeric scFv in an inhibition assay format on BIAcore. Three sets of twelve standards were assayed over three different days and the C.V.'s were calculated as the standard deviation (S.D.) expressed as a percentage of the mean values for each standard. Percentage recovery was calculated from the four-parameter model produced using BIAevaluation software.*

Concentration Morphine (pg/ml)	R/R0 ± S.D.	% C.V.	Back Calculated morphine Concentration (pg/ml)	% Recovery
1562,500.00	0.212 ± 0.011	5.15%	1261,703.0	80.8%
781,250.00	0.218 ± 0.025	11.33%	683,834.1	87.5%
390,625.00	0.249 ± 0.012	4.83%	423,512.4	108.4%
195,312.50	0.317 ± 0.036	11.42%	160,930.5	82.4%
97,656.25	0.345 ± 0.044	12.72%	122,427.5	125.4%
48,828.13	0.439 ± 0.043	9.79%	55,308.3	113.3%
24,414.06	0.585 ± 0.028	4.83%	22,177.5	90.8%
12,207.03	0.675 ± 0.092	13.65%	14,453.3	118.4%
6,103.52	0.815 ± 0.107	13.07%	6,241.3	97.8%
3,051.76	0.882 ± 0.082	9.27%	3,528.6	115.6%
1,525.88	0.940 ± 0.012	1.33%	1,724.6	113.0%
762.94	0.995 ± 0.094	9.41%	1,118.6	146.6%

4.2.6 Development of a Lateral Flow Immunoassay (LFIA) for M3G using monomeric and dimeric scFv

This section describes the development of an indirect competitive lateral flow immunoassay (LFIA) for the detection of M3G. Initial attempts to develop a direct assay were found to suffer from poor sensitivity. Daly[B] *et al.*, (2001), reported that an indirect assay format proved more sensitive in an LFIA for the detection of aflatoxin. It was therefore decided to employ an indirect assay format using monomeric and dimeric scFvs to detect morphine. The assay format is outlined in Figure 4.2.31. A colloidal carbon M3G-OVA conjugate was prepared by physically absorping the colloidal carbon to the protein moiety of the conjugate, as described in Section 2.8.1.

A monoclonal M1 anti-flag antibody was sprayed onto a nitrocellulose membrane as the test line, 3mm below the control line, which consisted of an anti-horse antibody sprayed onto the nitrocellulose. A droplet of running buffer containing free M3G, M3G-OVA-carbon-conjugate, the anti-M3G scFv and a carbon-conjugated horse antibody was mixed and allowed to flow along the nitrocellulose. The free M3G and the M3G-OVA-carbon conjugate compete for binding to the anti-M3G scFv, as the sample droplet travels along the membrane. The intensity of the generated signal increases, as the concentration of free M3G in the sample droplet deceases, because the numbers of antibodies available for binding the M3G-OVA-carbon conjugate increases.

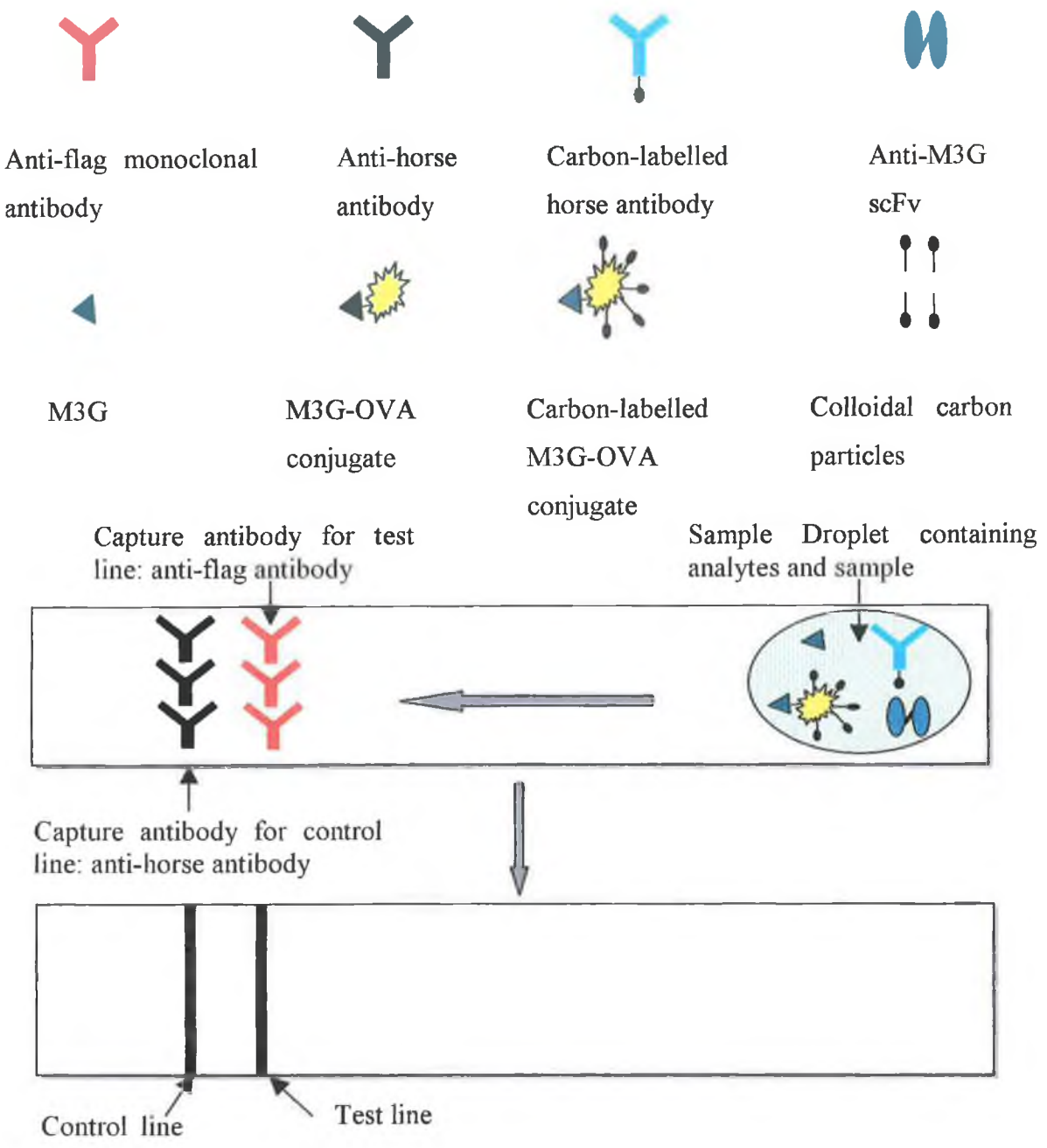


Figure 4.2.31: *Schematic representation of an indirect competitive LFIA for the detection of M3G. Capture antibodies (anti-horse antibody, control line and anti-flag antibody, test line) were sprayed onto the nitrocellulose strip, 3 mm apart. A sample droplet consisting of free M3G, M3G-OVA-carbon conjugate, the anti-M3G scFv and carbon-conjugated horse antibody in running buffer was prepared. Free M3G and carbon-conjugated M3G-OVA compete for binding to the anti-M3G scFv as the sample runs along the strip. The signal is generated due to binding of the dark carbon colloid. The signal generated will decrease as the concentration of free M3G in the sample increases.*

4.2.6.1 Optimisation of flow rate for spraying nitrocellulose strips

The rate at which the antibody was sprayed onto the nitrocellulose membrane was initially optimised. Various flow rates, ranging from 100 – 500 nl/sec, were tested, by spraying 10 µl of a 500 µg/ml solution of anti-horse antibody onto the nitrocellulose membrane, using the Linomat 5 sample application device. Signals were developed using 1 µl of carbon-conjugated horse antibody in 100 µl of running buffer, which was allowed run up the nitrocellulose strip. A flow-rate of 400 nl/sec was chosen as optimal for use in subsequent assays, as it produced a definite line, with minimal dispersion, in as short a time as possible (Figure 4.2.32).

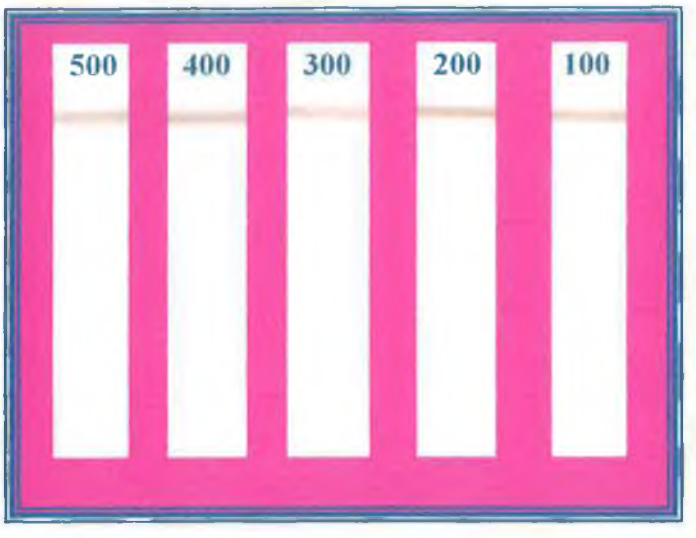


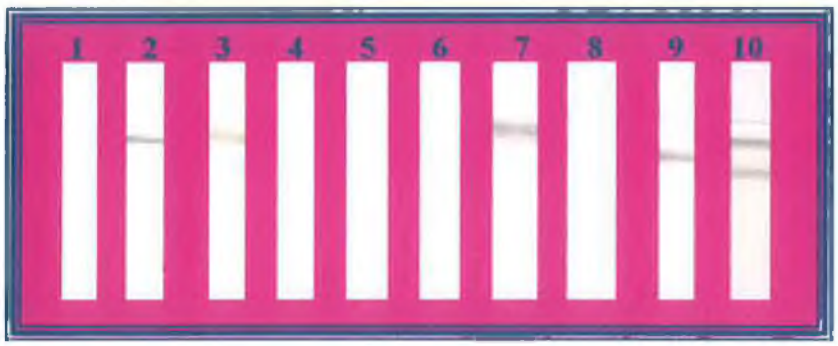
Figure 4.2.32: *Optimisation of flow rate for spraying capture antibodies onto nitrocellulose strips. Flow rates of 100, 200, 300, 400 and 500 nl/sec, were investigated for spraying of capture antibody. 400 nl/sec was chosen as optimal for assay generation as it gave a clear and definite signal, in as short a time as possible.*

4.2.6.2 Choice of antibody for the development of control line

The inclusion of a control line is necessary in any valid lateral flow immunoassay to confirm that the sample has travelled past the test line capture antibody, validating any positive or negative signal generated. The choice of antibody for control line generation is critical as any interference with, or cross-reactivity to, any components in the sample will lead to false positive or negative results.

A preferred control line would detect that all analytes had been included in the sample. The use of an anti-OVA or anti-M3G antibody would validate the presence of the labelled M3G-OVA tracer and the fact that the sample had indeed passed above the test line capture zone. As neither of these antibodies was available, a compromise was made and a number of other antibody pairs were investigated for the generation of the control line signal. One such example was a carbon-labelled rabbit antibody paired with an anti-rabbit capture antibody. The carbon-labelled rabbit antibody interacted non-specifically with the BSA solution used in the preservation of the monoclonal anti-flag antibody. This meant that the carbon-labelled control antibodies bound to the test line, generating a false negative signal.

To investigate any possible non-specific interactions of the horse/anti-horse antibody pairing, various combinations of analytes were sprayed onto the nitrocellulose. Non-specific interactions were visualised using combinations of carbon-labelled M3G-OVA, horse and anti-horse antibodies. The pairing of a carbon-labelled horse antibody and goat anti-horse capture antibody was found not to cause interference with any of the other components of the assay system (Figure 4.2.33). Lane 1 demonstrates that the anti-horse antibody does not bind either M3G-OVA or scFv. Lanes 2 and 3 prove that it binds to the carbon-labelled horse antibody in the presence of both monomeric and dimeric scFvs. Lane 4 demonstrates that the horse antibody does not bind monomeric scFv. Lane 5 indicates that the horse antibody does not interfere with test capture line. Lane 6 illustrates that the horse antibody does not bind to the M3G-OVA conjugate. Lane 7 shows that the carbon-labelled horse antibody specifically binds to the anti-horse antibody in the presenece of anti-flag antibody. Lane 8 demonstrates that carbon-labelled M3G-OVA does not bind to either anti-horse or anti-flag antibodies non-specifically. Lane 9 illustrates that carbon-labelled M3G-OVA does not bind to anti-horse, horse or anti-flag antibodies, non-specifically. Lane 10 proves that carbon-labelled M3G-OVA binds specifically to monomeric scFv, which in turn is captured by the anti-flag antibody. Therefore, this pair was chosen for further assay development.



1	Sprayed	250 µg/ml anti-horse antibody
	Added	1 µl carbon-labelled M3G-OVA + monomeric scFv
2	Sprayed	250 µg/ml Anti-horse antibody
	Added	1 µl carbon-labelled horse antibody+ monomeric scFv
3	Sprayed	250 µg/ml anti-horse antibody
	Added	1 µl carbon-labelled horse antibody + dimeric scFv
4	Sprayed	500 µg/ml horse antibody
	Added	1 µl carbon-labelled monomeric scFv
5	Sprayed	750 µg/ml anti-flag
	Added	1µl carbon-labelled horse antibody
6	Sprayed	500 µg/ml M3G-OVA
	Added	1 µl carbon-labelled horse antibody
7	Sprayed	500 µg/ml anti-horse antibody 750 µg/ml anti-flag antibody
	Added	1 µl carbon-labelled horse antibody
8	Sprayed	500 µg/ml anti-horse antibody 750 µg/ml anti-flag antibody
	Added	1 µl carbon-labelled M3G-OVA
9	Sprayed	500 µg/ml anti-horse antibody 750 µg/ml anti-flag antibody
	Added	1 µl carbon-labelled horse antibody + 1 µl carbon-labelled M3G-OVA
10	Sprayed	500 µg/ml anti-horse antibody 750 µg/ml anti-flag antibody
	Added	1 µl carbon-labelled horse antibody + 10 µl monomeric scFv + 1 µl carbon-labelled M3G-OVA

Figure 4.2.33: *Investigation of possible interferences of control line antibodies with other assay components.*

4.2.6.3 Optimisation of capture antibody concentration for generation of control line

The concentration of the goat anti-horse antibody capture antibody for generation of the control line was optimised. Various concentrations of the goat anti-horse antibody, ranging from 0–750 μg/ml, were sprayed onto nitrocellulose. Signals were generated using 100μl of running buffer containing the carbon-conjugated horse antibody (Figure 4.2.34). A concentration of 500 μg/ml of the goat anti-horse antibody was determined to be the optimal concentration for spraying of nitrocellulose strips. This gave a clear and definite signal for the control line, without unnecessary waste of reagents.

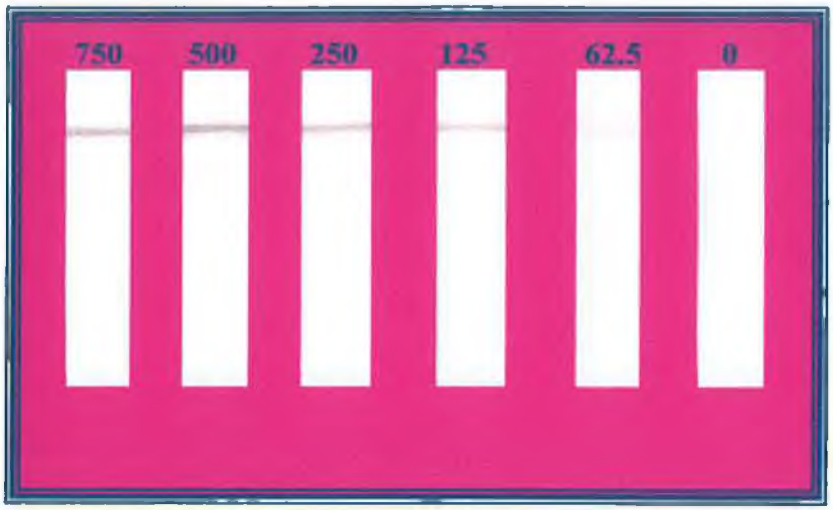


Figure 4.2.34: *Optimisation of capture antibody concentration for generation of control line. Strips were sprayed with 750, 500, 259, 125, 62.5 and 0 μg/ml anti-horse antibody. The signal was generated using 1 μl of carbon-labelled horse antibody. A concentration of 500 μg/ml of anti-horse antibody was determined to be optimal for generation of the test line.*

4.2.6.4 Optimisation of test line for use in an indirect LFIA for M3G

Initially, the concentration of the capture antibody for use in the test line was optimised. This was sprayed onto nitrocellulose, 3 mm below the control line of anti-horse antibody, at 500 μg/ml. Varying concentrations of monoclonal anti-flag antibody, ranging from 0–1,000 μg/ml, were analysed to determine the optimal concentration for test line. The test line signal was generated using a sample droplet containing the anti-M3G scFv and the M3G-OVA-carbon conjugate and the control line signal was generated using a carbon-conjugated horse antibody. It was found that 750 μg/ml of the anti-flag antibody sprayed onto the nitrocellulose was optimal for the development of the test line (Figure 4.2.35).

The concentration of monomeric anti-M3G scFv to be used in the test line generation was then optimised. Nitrocellulose was sprayed with 500 μg/ml anti-horse antibody (control line) and 750 μg/ml anti-flag monoclonal antibody (test line). The sample was prepared by mixing 1 μl of carbon-labelled horse antibody, 1 μl of carbon-labelled M3G-OVA and varying amounts of monomeric scFv, ranging from 10 to 0 μl. Figure 4.2.36 demonstrates that 10 μl of monomeric anti-M3G scFv was the lowest amount of antibody, sufficient to generate an acceptable signal in the test line.

The concentration of carbon-labelled M3G-OVA conjugate to be used in the generation of test line was also optimised. Nitrocellulose strips were sprayed as before, with optimised concentrations of capture antibodies for control and test lines. The sample added included 1 μl of carbon-labelled horse antibody; 10 μl of anti-M3G monomeric scFv and varying dilutions of carbon-labelled M3G-OVA conjugate, ranging from neat to 1 in 16 dilution of the 350 μg/ml stock. A 1 in 8 dilution proved optimal for generation of the test line, as demonstrated in Figure 4.2.37.

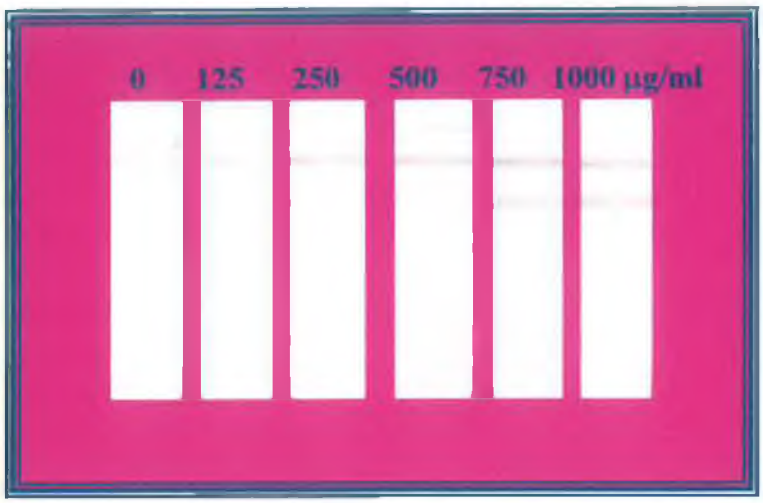


Figure 4.2.35: *Optimisation of capture antibody for generation of test line. Varying concentrations of monoclonal M1 anti-flag antibody, from 0 to 1,000 µg/ml were sprayed onto nitrocellulose, 3 mm below the control line. The signal was developed using carbon-labelled horse antibody (control line) and 10 µl anti-M3G scFv with 1 µl of carbon-labelled M3G-OVA (test line). A concentration of 750 µg/ml of anti-flag antibody was determined to be sufficient as concentration of capture antibody for test line generation.*

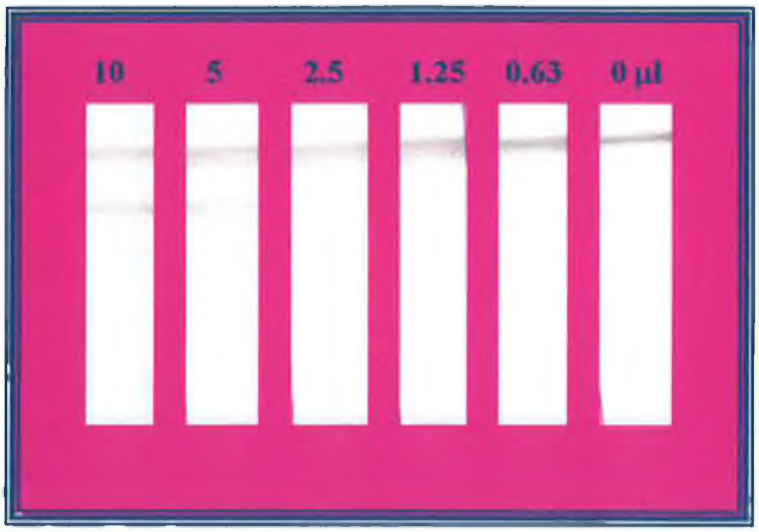


Figure 4.2.36: *Optimisation of the concentration of monomeric anti-M3G scFv to be used in LFIA test line. Control and test lines were sprayed as before. The test line was run using varying amounts of anti-M3G scFv, from 0 to 10 µl. 10 µl of the scFv was determined to be the minimum amount of antibody required to generate an acceptable test signal.*

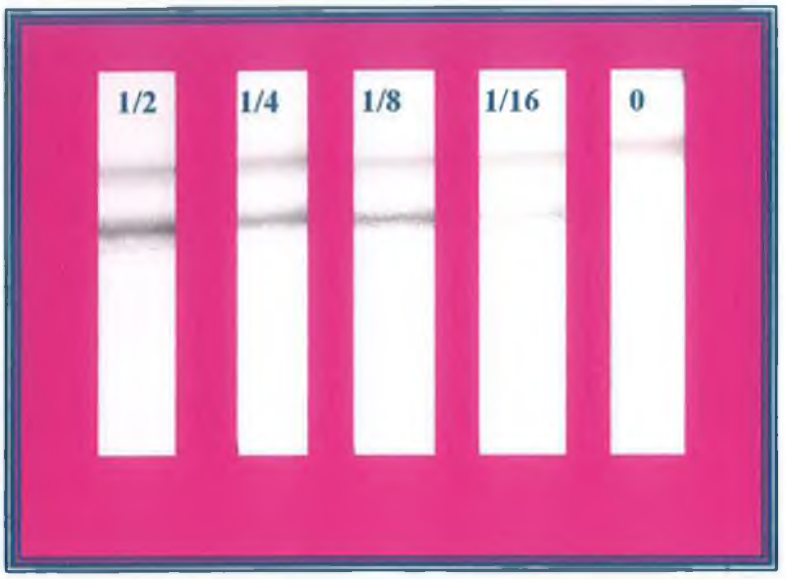


Figure 4.2.37: *Optimisation of carbon conjugate dilution to be used in test line signal generation. Control and test lines were sprayed as before. The test line was developed using monomeric anti-M3G and carbon-labelled M3G-OVA, at varying dilutions, from neat to a 1 in 16 dilution. A 1 in 8 dilution of the carbon conjugate was determined to be optimal, giving the lowest background signal and was chosen for use in further assay development.*

4.2.6.5 Development of an indirect LFIA for the detection of M3G using monomeric scFv

Following optimisation of the various parameters required for the control and test lines, an indirect competitive LFIA for the detection of M3G was developed using the anti-M3G monomeric scFv. Nitrocellulose strips were sprayed with 750 μg/ml of the monoclonal anti-flag antibody (test line), 3 mm below the control line, which consisted of 500 μg/ml of the goat anti-horse antibody. Standards of free M3G, ranging in concentration from 20 ng/ml to 1.25 ng/ml, were prepared in running buffer. 50 μl of each standard was then mixed with an equal volume of running buffer containing the 10 μl of anti-M3G monomeric scFv, 1 μl of a 1 in 8 dilution of the M3G-OVA-carbon conjugate and 1 μl of carbon-conjugated horse antibody. The samples were mixed and allowed travel along the nitrocellulose membrane, for

approximately 15 mins. After this time a clear black control line should be visible. This ensures that the sample has passed above the test line and has been captured by the control capture antibody. Any test result, which does not yield a clear control line, indicates that the result is not valid. The nitrocellulose was then washed with 100 µl of running buffer. The wash step was included to generate clearer images for the purposes of scanning images to be included in this thesis. The visualisation of two lines indicates a negative result, while the presence of the line control line, in the absence of a test line signal is indicative of a positive specimen. The limit of detection of the assay was found to be 20 ng/ml (Fig. 4.2.38).

4.2.6.6 Development of an indirect LFIA for the detection of M3G using an anti-M3G dimeric scFv

An indirect LFIA was also developed using the dimeric anti-M3G scFv. Conditions were optimised as in Section 4.2.6.4. The amount of dimeric scFv required for the generation of the test line was determined to be 1 µl. Drug standards were prepared containing 20, 10, 5, 2.5, 1.25 and 0 ng/ml of M3G in running buffer. The control line was generated using the same concentrations of anti-horse and carbon-labelled horse antibodies used for monomeric scFv (Section 4.2.6.3). 50 µl of each standard was then mixed with an equal volume of running buffer containing the 1 µl of anti-M3G dimeric scFv, 1 µl of a 1 in 8 dilution of the M3G-OVA-carbon conjugate and 1 µl of carbon-conjugated horse antibody. The samples were mixed and allowed travel along the nitrocellulose membrane, for approximately 15 mins. The nitrocellulose was then washed with 100 µl of running buffer. The limit of detection of the assay was found to be 20 ng/ml, as below this concentration a line was generated for the test line, indicating a negative result (Fig. 4.2.39).

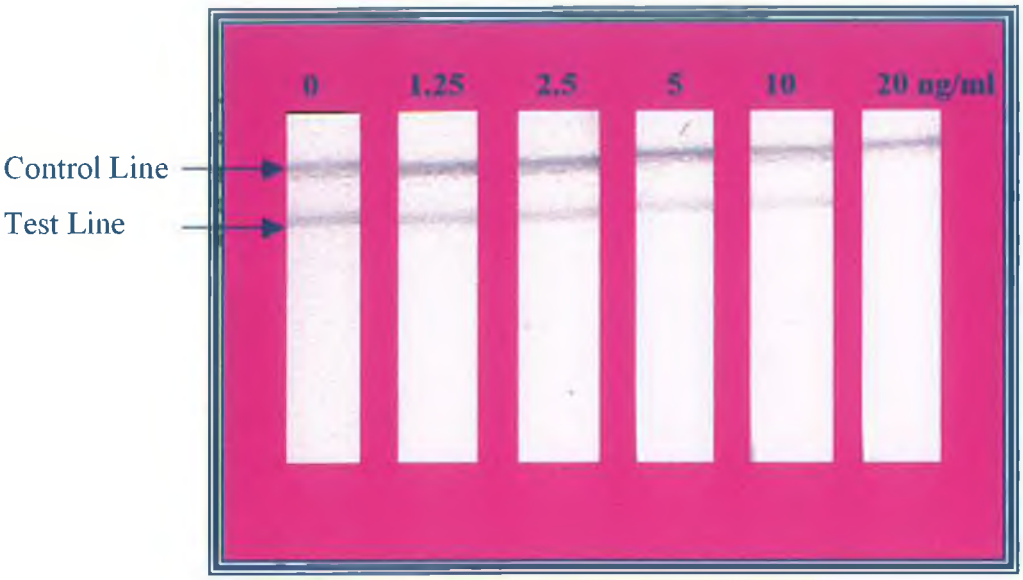


Figure 4.2.38: *Competitive LFIA for the detection of M3G, using an anti-M3G monomeric scFv. The numbers above the strips represent the final concentration of in each the sample (ng/ml). The assay had a limit of detection of 10 ng/ml. A control line, consisting of carbon-labelled horse antibody, captured with an anti-horse antibody is included to validate the assay and ensure that assay had run to completion.*

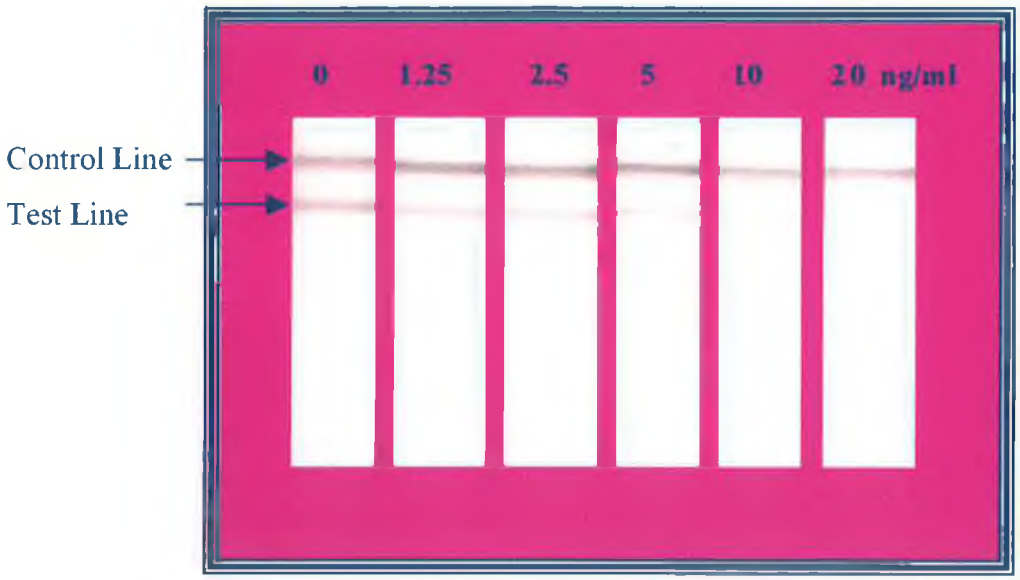


Figure 4.2.39: *Competitive LFIA for the detection of M3G, using an anti-M3G dimeric scFv. The numbers above the strips represent the final concentration of in each the sample (ng/ml). The assay had a limit of detection of 10 ng/ml. A control line, is included to validate the assay*

4.3 Discussion

This chapter follows on from Chapter 3, which describes the production and initial characterisation of genetically-derived scFv antibody fragments. The aim of this chapter was to carry out a case study on the feasibility of using genetically-derived scFv antibody fragments to monitor heroin use by detecting morphine in saliva Initially the affinities of all three antibodies were measured to analyse which scFv was best suited to analysis of morphine in saliva. This was determined using both ELISA and biosensor-based methods of affinity determination. Monomeric scFv was determined to have the best potential for morphine analysis and was therefore applied to both ELISA and BIAcore-based assays for the detection of morphine in saliva. An indirect lateral flow immunoassay was also developed for the detection of M3G using monomeric and dimeric scFvs.

Affinity studies were carried out by two methods. The first method was an ELISA-based method involving equilibrium analysis of antibody:antigen mixtures, described by Friguet *et al.* (1985). This involves quantifying free, unbound, antibody present at each antigen concentration by means of an indirect ELISA. The Friguet method has many limitations. It is not suitable for either very low or very high affinity antibodies. Low affinity antibodies, or those with high K_D values, are not suited to this form of analysis as too much displacement from the solution phase equilibrium occurs during the indirect ELISA. High affinity antibodies, or those with very small K_D values ($<10^{-10}$ M) are also not suitable to be analysed in this manner as the K_D value will be limited by the sensitivity of chromogenic substrate and affinity of secondary antibodies (Neri *et al.*, 1996). The second analysis involved using BIAcore to monitor 'real-time' biomolecular interactions and determine solution phase equilibrium dissociation constants of monomeric and dimeric scFvs. Large to small K_D values have successfully been determined using BIAcore. Nieba *et al.*, (1996) successfully measured K_D values as large as 10^{-4} M using a BIAcore competition on-rate determination assay. Quinn and O'Kennedy, (2001), have successfully measured K_D values as small as 6×10^{-10} M using solution phase equilibrium analysis.

Friguet analysis of monomeric scFv reveals an affinity constant of 3 nM for M3G and 17 nM for morphine. Both curves are a good fit with an $R^2 > 0.99$. Dimerisation of the scFv was shown to decrease the affinity of the monovalent interaction between antibody and M3G. The effect was less significant with morphine. When this was corrected for the bivalency of the antibody, similar affinities for M3G were observed to that of monomeric scFv. No increase in affinity through co-operative binding of the two antigen-binding sites was observed, as previously reported for diabodies produced with a shortened linker (Hollinger *et al.*, 1993). A slight increase in affinity for morphine, from 20 to 7.5 nM, was observed. This model assumes a bivalent interaction. However, from analysis of curve fitting for corrected and uncorrected values, neither curve was an ideal fit. In reality the 'correct' model for a bivalent antibody binding lies somewhere between the mono- and bivalent models shown. The bifunctional scFv was also shown to exhibit dissociation constant in the region of 10 nM for both targets. This illustrates that the dimerisation of the scFv had little effect on the antibody's overall affinity for targets. The production of other derivatives of the antibody had its advantages, however, as illustrated in chapter 3. Dimerisation of the molecule facilitated larger detection ranges and direct immobilisation of antibody through surface absorption (Kerschbaumer *et al.*, 1997), while the inclusion of an enzyme-label decreased both assay time and complexity significantly.

Affinity constant determination by solution phase equilibrium analysis using BIAcore technology was also carried out. Equilibrium dissociation constants were determined for both monomeric and dimeric scFvs to M3G and morphine. Bifunctional scFv, produced with the pAK 600 vector, was not analysed as it could not be successfully purified and hence its concentration could not be determined. Solution phase analysis of monomeric scFv revealed significantly lower association constants than those determined by ELISA. The reason for this is thought lie in the initial determination of antibody concentration, which was performed by measuring nominal (total) protein rather than active antibody concentration. A more accurate method, like quantification by flag tag would be more suitable for use in this form of analysis. Determining the active concentration of antibody would also take into account any antibody that had become denatured during storage. If active concentration of antibody is actually lower than the experimentally determined nominal concentration, the reagent is defined as being 'not completely immunoreactive', and apparent

affinities measured will be lower than the 'real' affinity (Neri *et al.*, 1996). Even though the antibody was deemed to be extremely pure by SDS PAGE analysis (Figure 3.2.13), small impurities may be present, leading to an overestimation of concentration. As BIAcore works by detecting mass changes at the sensor surface, the molar concentration for such a small molecule needs to be large to generate a measurable response, thereby multiplying any over estimation of antibody concentration accordingly. For this reason the ELISA-based affinity determinations, described by Friguet *et al.*, (1985), were deemed to be more definitive for the estimation of affinity constants. The model for the affinity constant determination of the dimeric scFv for M3G did not fit the dataset particularly well. It has previously been observed that solution phase analysis using immobilised conjugate underestimates true affinity by preventing bivalent binding. Fitzpatrick, (2001), showed that by using a directly immobilised chip was rather than a conjugate-coated chip for analysis, the observed affinity constant was one order of magnitude greater. With a directly immobilised antigen, steric hindrance and lower epitope densities inhibit antibody rebinding. Non-specific amine immobilisation of antigen-conjugate may result in a heterogenous population of conjugate on the sensor chip surface leading to a potential source of error and an underestimation of 'true' affinity (Kortt *et al.*, 1997).

Cross reactivity studies (Chapter 3) and affinity analysis revealed that monomeric scFv had the best potential for application to a saliva-based assay for morphine. This form of the scFv exhibited a low equilibrium dissociation constant and sensitive least detectable dose (LDD) for morphine, while displaying limited cross-reactivity to other opiates. The assay was first carried out in the 'ideal' buffer' system of PBS, as this would determine any interferences through matrix effects caused by the saliva sample. The assay also had to be optimised to require as small a sample volume as possible, as previous analysis has shown that drug users have a diminished capacity to provide large volumes of saliva for analysis (Fanning, 2002). Sample volumes as small as 25 µl could be successfully used to detect morphine in this model buffer system. The assay displayed a range of detection of 6.1 to 1,563 ng/ml and a LDD of 20 ng/ml (Figure 4.2.24). The assay was then applied to the detection of morphine in 'spiked saliva' samples. The samples were donated from 10 individuals who were opiate-

free and subjected to a simple 'freeze-thaw' and centrifugation step, prior to analysis. The assay proved to be sensitive and robust for morphine detection, with no negative interferences observed for such a crude biological matrix. The assay exhibited a range of detection of 6.1 to 1,563 ng/ml with an LDD of 12 ng/ml. All % C.V.'s were below 10 % and percentage recoveries were between 92% and 113%.

The competitive ELISA developed for the detection of morphine in saliva was applied to 'real-saliva' samples donated by patients attending Trinity Court Drug Treatment Centre. This was a pilot study carried out to determine the feasibility of using recombinant antibodies for use in morphine analysis in saliva samples using immunoassay technology. 25 µl of each sample was analysed in triplicate. Initial analysis of samples 3, 5, 6, and 7 revealed the presence of very high concentrations of morphine. For this reason, analysis of these samples was repeated using dilutions of saliva from a 1 in 10 to a 1 in 50 dilution. Samples were determined to contain between 22 and 37,000 ng/ml of morphine in patient saliva, following recent heroin use. This correlated very well to levels detected using a more 'conventional', polyclonal antibody immunoassay format, which detected levels from 99 to 39,500 ng/ml of morphine in the same sample set (Fanning, 2002). Patient 6 exhibited the highest levels of morphine in saliva (36,982 ng/ml). This patient had recently both taken heroin and DF118's, a dihydrocodeine-based painkiller that binds to opiate receptors in the brain, mimicking the effects of heroin. It was the first time that this patient had attended the treatment centre and he/she was not receiving any methadone replacement therapy. Patient 3 also displayed a high level of morphine in saliva, this patient had also used heroin within 24 hours. Patients 7,5 and 2 showed decreasing morphine levels in their saliva, all of these patients had used heroin in the past 24-48 hours. Patients 1 and 4 showed the lowest levels of morphine, patient 1 was receiving a high dose of methadone replacement therapy and was deemed to be of low risk of relapse. This patient had used heroin sometime within a 5-day window. All results obtained, with the exception of patient 2, correlate very will with how recently heroin was used. Patient 2 admitted to taking heroin within 15 to 20 minutes of the interview. This was the most recent sample obtained following heroin use. Although this patient did not exhibit the highest levels of morphine, it is clear that high levels of morphine (342 ng/ml) could still be detected, even after such a short period of time. It

must also be taken into account that the dose of heroin taken will determine the concentration of morphine detected in saliva, the higher the dose of heroin taken, the higher the concentration of morphine that will be detected. The route of administration will also affect the pharmacokinetics of the drugs. Patients were very vague and confused in their accounts of the timeframe in which they had last used heroin. The questionnaire was used to give a general indication of previous drug use. There is a strong possibility of inaccuracies in patients' accounts as to how recently each drug was used. Unless a controlled study was carried out in which the heroin was administrated to patients at intervals between the collection of saliva samples, no exact timeline of drug use can be extrapolated. This study, however, proves that heroin use can be detected within minutes and for a period of days by monitoring morphine levels in saliva.

A BIAcore inhibition assay was also developed for the detection of morphine in saliva. In the past several difficulties have been encountered during the development of BIAcore assays using saliva as a detection matrix. Problems have been encountered due to the 'stickiness' of the sample, which varied between individuals, making the assay irreproducible (Fanning, 2002). An inhibition assay for morphine was successfully developed in HBS buffer, with a range of detection from 781,250 to 381 pg/ml, and a LDD of 1,200 pg/ml. This was used as a calibration model to examine matrix effects of saliva and investigate how best to eliminate these interferences.

The heterogeneous nature of saliva samples and complexity of the biological matrix was seen to contribute to a substantial amount of non-specific binding to the sensor chip surface. A number of strategies were investigated to minimise the overall effect of this on the assay. The effect of ionic strength and length of dextran layer has been previously investigated and found not to eliminate non-specific binding responses (Fanning, 2002). Reference subtraction of sample binding to an OVA surface proved to significantly decrease the sensitivity of the assay. Binding responses of the sample to the OVA surface proved to be greater than to the conjugate immobilised surface, particularly in the absence of antibody. The specificity of the antibody binding response was therefore shown to decrease the overall non-specific interaction of sample and surface. For this reason it was decided to minimise the contact time

between the sample and the surface and to increase the flow rate of the assay, in an attempt to minimise these interferences. Flow rate for the assay was increased from 2 µl to 5 µl and surface contact time was reduced from 4 minutes to 2 minutes 20 seconds. This decreased any non-specific interaction with the chip surface to below 50 response units for a negative control. Using these conditions an inhibition assay was successfully developed for morphine in 'spiked saliva' samples. The assay had a detection range of 1,562,500 to 763 pg/ml, with a LDD of 2.5 ng/ml. All % C.V.s were below 14% and percentage recoveries were within ± 25%, above the LDD level. This represented only a slight decrease in sensitivity from the inhibition assay developed in a 'model' buffer system and a 20-fold increase in the detection range sensitivity over ELISA analysis. These statistics fulfill the SAMHSA criteria for accreditation, which state that the quantitative value for at least 80% of samples must be within ± 20 percent of the calculated reference group mean. These detection ranges for morphine are also well below the 40 ng/ml morphine cut-off concentrations proposed in SAMSHA guideline for oral fluid analysis.

The detection limits achieved compare favourably with previously published immunoassay techniques. Beike *et al.* (1999), have reported an immunoaffinity–based extraction method for the detection of morphine in blood with a detection limit of 3 ng/ml and a quantitation limit of 10 ng/ml for morphine. A radioimmunoassay developed by Steiner and Spratt (1978), exhibits a sensitivity of 500 ng/L of serum for morphine and a similar technique reported by Spector (1971), exhibits a detection limit between 50 and 100 pg of morphine in serum. Usagawa *et al.* (1993), have reported an immunoassay using a monoclonal antibody directed against morphine capable of detecting concentrations as low as 100 pg/ml. Speckl *et al.* (1999), have employed GC – MS to monitor the presence of opiates in the saliva and urine of subjects participating in a drug withdrawal programme. The detection limits for opiates in saliva were found to be 10 ng/ml. This assay developed for morphine in saliva, using recombinant assay techniques, compares favourably with these detection limits, with minimal sample preparation, requiring only a simple freeze - thaw step, followed by centrifugation.

The development of a lateral flow immunoassay (LFIA) for M3G was also investigated using both monomeric and dimeric scFvs. Initial attempts to develop a direct assay suffered from low levels of sensitivity. Daly[B] *et al.*, (2001), reported similar findings, with an indirect LFIA proving more sensitive for detection of aflatoxins. It was therefore decided to use a monoclonal anti-flag antibody to capture the scFv on the LFIA strip. The scFv antibody, free M3G and carbon-labelled M3G-OVA were mixed and applied to the strip. For the successful development of a LFIA, a control line must be incorporated into the assay. This validates the correct running of the assay and ensures the sample has reached the capture line before a valid result is obtained. For a commercial lateral flow device, a preferred control line would detect that all analytes had been included in the sample. This is would be required internal control measure to ensure that all analytes were included in the device sample pad. The use of an anti-OVA or anti-M3G antibody in this assay would validate the presence of the labelled M3G-OVA tracer and that the sample had indeed passed above the test line capture zone. As neither of these antibodies was available, a compromise was made and a number of other antibody pairs were investigated for the generation of the control line signal. Preliminary optimisation involved testing a number of antibodies to find a suitable antibody pair for use in the generation of an acceptable control line signal, without interfering in the signal of the test line. Many commercially bought antibodies showed cross reactivity with the BSA in the preservative solution of the monoclonal anti-flag antibody. Other 'in-house' rabbit antibodies tested bound non-specifically to the OVA moiety of the M3G conjugate. A goat anti-horse and horse antibody pair proved the most specific for generation of the control line signal. The assay was proven to be sensitive and specific for the detection of M3G, with a detection limit of 20 ng/ml with each scFv, both monomeric and dimeric. The assay could be carried out in approximately 15 minutes and was shown to be reproducible over four different days. The assay was only slightly less sensitive than ELISA analysis (LDD = 5 ng/ml) and could be carried out in a fraction of the time.

This chapter focussed on the applications of genetically-derived scFv antibody fragments in various assay formats. Equilibrium dissociation constants of monomeric, dimeric and bifunctional antibodies for M3G and morphine were assessed to investigate which scFv would be most suited to analysis of morphine in saliva.

Monomeric scFv was determined to be the applicable for this type of analysis. A competitive ELISA assay was successfully developed to detect morphine in saliva. This was applied to monitoring recent heroin use in patients attending a drug rehabilitation clinic. The scFv could successfully detect both high and low concentrations of morphine in the saliva of these patients following heroin use. An inhibition BIAcore assay was also developed to detect morphine in saliva. The sensitivity of the assay was improved 20-fold from that of ELISA analysis. Percentage recoveries were all within ± 25% within the range of quantification, despite the complexity of the biological matrix on a system as sensitive as BIAcore. An indirect lateral flow immunoassay for the detection of M3G was successfully developed. The assay was capable of detecting 20 ng/ml M3G in an analysis time of 15 mins. This chapter focused on the wide variety of applications possible with recombinant antibodies. Saliva was found to be a suitable matrix for monitoring drug use and the application of recombinant antibodies to 'real-life' analysis was proven to be possible. The success of immunology-based tests for drugs of abuse is reliant on the quality of the antibodies used. The results illustrated in this chapter demonstrate that recombinant antibody technology offers an excellent source of such designer antibodies, with defined affinities and specificities.

Chapter 5: Isolation of Recombinant scFv Antibodies against Tetrahydrocannabinol

5.1 Introduction

5.1.1 History of cannabis

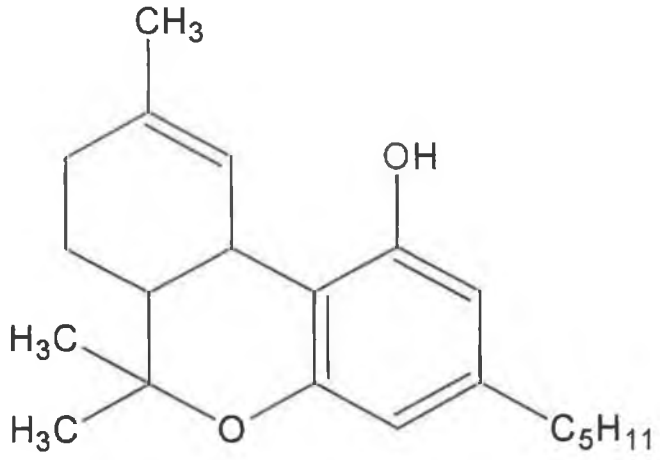


Figure 5.1.1: *Structure of delta-9-tetrahydrocannabinol, the pharmacologically active constituent of cannabis.*

Delta-9-tetrahydrocannabinol (Figure 5.1.1) is the pharmacologically active component of cannabis (also known as marijuana, pot, hashish and grass). Cannabis is the mood-altering product produced as a resin by the hemp plant *Cannabis sativa*, subspecies, indica. The highest concentration of psychoactive substance is present in the flowering buds of the female plant, followed by the leaves (Adams and Martin, 1996). Small amounts are found in the stem and root, while no traces are present in the seeds. Cultivation conditions of cannabis plants vary greatly and determine the cannabinoid content of the plant. Over the last decade, advances in indoor cultivation (hydroponics) and breeding have increased cannabinoid content of plants over 10-fold (Ashton, 2001). Marijuana refers to two types of plant preparation, ganja and hashish. Ganja is obtained from the drying the leaves and plant tops, while hashish is prepared from the resin of the plant and contains a higher cannabinoid content.

Cannabis use leads to a wide range of behavioural affects. The initial 'high' gives a sense of relaxed euphoria. Reaction times are decreased and senses become enhanced; there is a stimulation of appetite and an altered perception of time and space. This impairment of motor skills makes cannabis a major concern in driving

while under the influence of drugs ('drug driving'). Albery *et al.* (2000), found that cannabis was second only to alcohol in its prevalence for abuse in a group of out-of-treatment drug users while driving.

The use of cannabis, for medicine, food, and fibres, dates back to Neolithic periods. In the Western world cannabis was widely used in medicinal circles in the nineteenth century, with Queen Victoria being a famous recipient of the medication. It was with the invention of the syringe that medical use of cannabis decreased, as other drugs such as, aspirin and morphine, became popular because they could be administered intra-venously. Following the second international meeting on drugs, cannabis was outlawed in the UK in 1928 with the implementation of the 1925 Dangerous Drugs act. The US also outlawed the drug in 1937. Recreational use of cannabis continued, however, through the arrival of immigrants from the Caribbean in the 50's and became associated with many famous musicians. The 1960's saw a dramatic increase in popularity of the drug, with the onset of the 'flower-power years'. Cannabis remains one of the most frequently abused drugs in the USA today, although its popularity is decreasing.

5.1.2 Administration of cannabis

Cannabis is most commonly administered by smoking or ingestion. I. V. administration is difficult as the substance lacks a nitrogen molecule, rendering it insoluble. Smoking is the most common form of cannabis administration. The drug is commonly smoked alone or mixed with tobacco leaves for increased potency. A typical 'joint' contains 0.5 to 1 g of plant matter (Adams and Martin, 1996). A dosage between 2 and 22 mg is required to produce pharmacological effects in humans (Martin, 1986). About 50% of the THC in a joint of herbal cannabis is inhaled in mainstream smoke (Ashton, 2001). The onset of behavioural effects become apparent within minutes of administration and dissipate rapidly, usually within an hour. Cannabis may also be chewed or eaten. The onset of symptoms is slower when administered orally (0.5-2 hours), with a bioavailability of 25-30% less than when smoked.

5.1.3 Metabolism of cannabis

Following inhalation, delta-9-THC levels in the blood rapidly increase. THC is metabolized to 11-hydroxy-THC (11-OH-THC) by the lungs and liver. This can readily cross the blood brain barrier, making it a more potent metabolite. It is then converted to 11-nor-delta-9-THC-9-carboxylic acid (THCCOOH), an inactive metabolite, by the liver. This is the most predominant form of THC detected in the blood and urine of cannabis users and remains elevated for an extended period of time (Adams and Martin, 1996; Cone, 1998). Following oral administration, THC is metabolised in a similar manner. However, THC levels rise more gradually over a period of 4-6 hours. THCCOOH is also found in higher concentrations in the blood.

The time dependence of delta-9-THC levels mirrors the behavioural effects of cannabis and hence, it is the ideal metabolite to target in an assay system designed to detect drug impairment. Plasma levels of the metabolite rise and fall with the symptoms of drug use. THCCOOH, however, may remain in the urine for up to three days (Adams and Martin, 1996). Urinary analysis of this metabolite is only indicative of past drug use and not of physical impairment.

5.1.4 Difficulties associated with THC analysis

THC immunoanalysis has proved problematic in the past (Cook *et al.*, 1976). A prerequisite for any immunoassay is the production of sensitive and specific antibodies against the target antigen. Antiserum raised against THC has proved to lack the desired specificity and be of low concentration (Cook *et al.*, 1976). The lipophilic nature of the molecule leads to problems when an aqueous media is chosen for analysis. THC proves insoluble at high concentrations and also adsorbs to glass and plastic surfaces. These authors demonstrated the loss of 41% of tritiated THC, dissolved in aqueous solution, due to adsorption of the drug to the surface of a glass container. The preparation of THC samples in organic solvent or in a strong basic solution is reported to alleviate the problem (Joern, 1987). This approach is appropriate for chromatographic analysis. However, in the case of immunoassays,

these solutions could seriously affect the activity of the detection antibody, rendering the approach unsuitable. Cannabis was also reported to lose potency and lack stability following storage (Fairbairn *et al.*, 1976). These authors demonstrated that exposure to light had a deleterious effect on cannabinoids. Exposure to air was also found to adversely affect cannabinoid concentrations to a lesser extent. Romberg and Past (1994,) also reported an average of a 25% decrease in the THCCOOH concentrations detected in urine by GC-MS, following storage at –20°C for an average of 2.3 months. Anomalies have been reported in THC levels detected depending on the choice of container used for sample storage. Christophersen (1986), observed no significant decrease in THC levels found in blood, following storage in glass containers at –20°C. Aliquots of the same samples stored in plastic containers were seen to lose 60 to 100% of their THC content. Repeated freeze-thawing of saliva samples, stored in plastic containers was also shown to lead to significant losses of THC (Fanning, 2002). High-density polyethylene containers have been shown to be suitable for urine storage, prior to analysis for THCCOOH, where the use of glass for high-density sampling is inconvenient (Giardino, 1996).

5.1.5 THC analysis

Cannabis metabolites have been successfully detected in the blood (Moeller *et al.*, 1998), urine (Wenning *et al.*, 1998), saliva (Fanning, 2002), sweat (Kidwell *et al.*, 1998) and hair (Spiehler, 2000). Gas-chromatography coupled to mass spectrometry is the 'gold-standard' for THC identification and quantification by analytical laboratories within the European Union (Badia *et al.*, 1998). Chromatographic analysis of biological samples requires extensive sample preparation. For this reason, a preliminary screening assay is usually carried out. Screening analysis is primarily carried out using one of the commercially available rapid immunoassay kits (detailed in Chapter 1) that are capable of detecting multiple drug residues. Once THC has been positively identified in the sample, quantification may sometimes be carried out by GC-MS. Chromatographic methods for the detection of cannabis metabolites in biological matrices have been reviewed in the literature by Simpson *et al.* (1997) and more recently by Drummer (1999).

Research into immunoassay-based assays for the detection of cannabinoids has decreased in recent years, presumably due to the difficulties associated with THC analysis discussed earlier. In fact Moeller *et al.* (1998), who reviewed drug determination in blood, state that there were no reports of direct immunological detection methods for cannabinoids in blood detailed in the literature. Contrary to this claim, reference to radioimmunoassays for cannabinoid metabolites has been found in earlier literature. Gross *et al.* (1974) describe a radioimmune technique for the detection of delta-9-THC in blood, using a polyclonal THC antiserum with a detection limit of 25 ng/ml for THC. They later applied this to a radioimmunoassay, which was used to detect delta-9-THC and THCCOOH in blood and 11-nor-9-carboxy-delta-9-THC in urine (Gross and Soares, 1976). They found plasma levels of delta-9-THC peaked shortly after cannabis administration and dissipated within 2 hours. THCCOOH levels, however, persisted in both matrices and were therefore not indicative of recent drug use. Teale *et al.* (1974), also describe a radioimmunoassay for THC in blood and urine. They produced a polyclonal antisera raised against THC in sheep and used this along with a competing tritiated THC tracer to analyse THC levels. THC levels in animals and volunteers were monitored and plasma levels of delta-9-THC were found to correlate well with subjective effects experienced by volunteers. The assay was capable of detecting THC following smoking a single 'joint', impregnated with 5 mg of pure THC, in less than 1 ml of blood. Sensitivity of the assay was further improved to detect 7.5 ng/ml THC in plasma (Teale *et al.*, 1975).

Cook *et al.* (1976) detail how they tried to address some of the difficulties associated with THC assay development. Using optimized buffer composition, along with an improved THC conjugate for antisera generation, they developed a radioimmunoassay with a standard curve from 5-100 ng of delta-9-THC in spiked plasma samples. Enzyme-based immunoassays have also proved successful for detecting THC. A competitive ELISA assay with a range of detection from 96-25,000 ng/ml for THC in spiked saliva samples was also capable of detecting recent cannabis use in volunteers attending a drug treatment centre (Fanning, 2002). A biosensor assay was also developed using BIAcore, an optical-based biosensor, which exhibited enhanced sensitivity for THC, with a detection limit of 12.2 ng/ml in a buffer matrix.

5.1.6 Aims

The aim of this chapter was to generate recombinant antibody fragments specific to THC. The main advantage of recombinant antibody technology over traditional monoclonal and polyclonal antibody techniques is the possibility of altering the antibody's affinity, specificity and physical characteristics with relative simplicity. This may eliminate the lack of specificity associated with polyclonal anti-THC antibodies previously developed (Cook *et al.*, 1976). Recombinant antibodies are expressed in bacteria, and this facilitates the elimination of problems associated with low serum titres. The developed antibody will be applied to an ELISA assay format for THC detection. Immunoassays are proven to be a rapid, sensitive and specific method of analysis (Fitzpatrick *et al.*, 2000), particularly suited to rapid analysis of biological matrices for THC residues.

A variety of strategies for antibody generation were employed, however the generation of an antibody specific to THC proved problematic. The biopanning of a semi-synthetic naïve human library led to the isolation of an scFv specific for THC. The antibody was expressed in high levels in a scAb format and purified by IMAC. It was later found that the antibodies were spontaneously aggregating, leading to a loss in assay sensitivity. However, the phage-displayed scFv antibodies proved an ideal reagent for THC detection by competitive phage ELISA.

5.2 Results:

5.2.1 Production of a recombinant antibody library against tetrahydrocannabinol

The Krebber system of phage display (Krebber *et al.*, 1997) was used to produce single chain antibodies to tetrahydrocannabinol. This system of phage display has been optimised for robustness, vector stability, control of scFv-Δ gene III expression, primer usage, scFv assembly and directional cloning. In order to create an extensive panel of antibodies, with the highest affinity to THC possible, it was decided to develop an immunised rather than naïve library. The immune system of a mouse was primed by sub-cutaneous immunisation with a THC-BSA conjugate, over a 12-week period.

5.2.1.1 Antibody titre of mouse used for recombinant antibody library

Mice were immunised according to the schedule outlined in Section 2.3.2. Tail bleeds were performed 7 days post-immunisation, the blood collected and the serum recovered. A direct ELISA was carried out as per Section 2.3.2.2 to determine the titre of antibodies raised against the antigen. The serum, which was raised against THC–BSA, was screened against THC–BTG to eliminate binding of antibodies raised against the carrier protein. Serum samples were also pre–incubated with 1% (w/v) BSA to prevent any non–specific interactions. As can be seen from Figure 5.2.1, the serum titred out at approximately a 1 in 100,000 dilution.

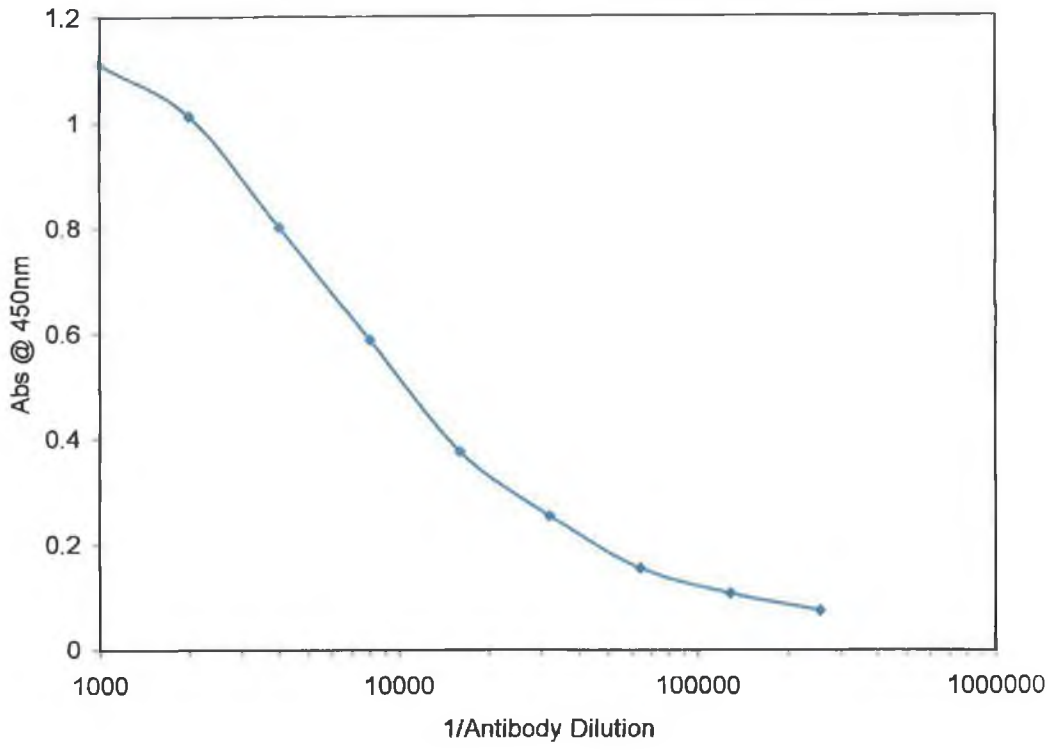


Figure 5.2.1: *Titre of serum from mouse immunised with THC–BSA as described in Section 2.3.2.2. The serum was titred against THC–BTG with 1% (w/v) BSA in the diluent, to remove any antibodies raised against the carrier protein. Bound antibodies were detected using a peroxidase-labelled rabbit anti-mouse secondary antibody, followed by o-PD substrate. A sufficient antibody titre (1/100,000) was obtained to ensure that a high proportion of the antibodies produced was directed against THC. The spleen was used to extract RNA for the generation of a recombinant antibody library against THC.*

5.2.1.2 Reverse transcription of RNA

The spleen was removed and the RNA extracted as per Section 2.4.1. Reverse transcription of extracted murine RNA to cDNA was carried out using random primers as described in Section 2.4.2 using a reverse transcription kit (Promega). The cDNA generated in Figure 5.2.2 was used as a template for the amplification of variable heavy (V_H) and variable light (V_L) genes.

5.2.1.3 Amplification of variable heavy and light chain antibody genes

Variable heavy (V_H) and light chain (V_L) antibody genes were amplified using the list of primers detailed in Section 2.4.4.1. This primer set described by Krebber *et al.* (1997), incorporates all mouse V_H, V_λ and V_κ genes and assembles the fragments in the V_L-$(Gly_4Ser)_4$-V_H orientation. A primer mix was prepared according to the degeneracy (D) of each primer, 1 µl for all non-degenerate primers, 2 µl for D=2-4, 3 µl for D=6-9 and 4 µl for D=12-16. The term 'back (B)' is used to describe primers that amplify toward the 3′ direction and the term 'forward (F)' describes amplification in the 5′ direction. For amplification of variable light chain genes (V_L), 1 µl of L_F and 1 µl of L_B mix was added to the PCR reaction. PCR conditions for V_L were optimised to include an annealing temperature of 55°C and a final buffer concentration of 60 mM Tris-HCl, 15 mM ammonium sulphate, 2.5 mM $MgCl_2$, pH of 8.5. For amplification of V_H genes, a H_F and H_B primer mix was prepared. 1 µl of each was added to the reaction. The optimised PCR conditions included an annealing temperature of 55°C and a final buffer composition of 60 mM Tris-HCl, 15 mM ammonium sulphate, 2.5 mM $MgCl_2$, pH 9.0, supplemented with 2.5 % (v/v) DMSO. Both PCR products were separated by electrophoresis on a 1% (w/v) agarose gel. Figure 5.2.3 demonstrates that the expected bands of 375-402 bp for V_L and 386-440 bp for V_H were observed. Both PCR products were purified from the gel using a Perfectprep gel purification kit. Purified products were then quantified prior to annealing PCR.

5.2.1.4 Linking of heavy and light chain genes by splice by overlap extension (SOE) PCR

V_H and V_L genes were annealed together using a splice by overlap extension (SOE) PCR reaction. Once buffer composition (60 mM Tris-Cl and 15 mM ammonium sulphate, with a magnesium concentration of 1.5 mM $MgCl_2$, pH 9.0) was optimised for the reaction, a titration of V_H (approx 30 ng/µl) and V_L (approx 40 ng/µl) concentrations was performed. This ensured that V_H and V_L genes were present in approximately equal concentrations. Figure 5.2.4, shows that a ratio of 0.5 µl V_H and 0.5 µl V_L proved optimal for a specific band at 800 bp to be observed.

5.2.1.5 Digestion of pAK 100 vector and scFv insert with Sfi 1

The pAK 100 plasmid was isolated from XL-1 Blue cells using a Promega miniprep kit. This was digested with *Sfi 1* as described in Section 2.4.6.2. The restricted plasmid was separated on a 1% (w/v) agarose gel by electrophoresis and the 4,425 bp fragment purified from the gel (Figure 5.2.6). The SOE PCR product was restricted with *Sfi 1*, directly from the PCR reaction, as described in Section 2.4.6.2. The digested product was run on a 0.7% (w/v) gel (Figure 5.2.5) and purified using a Perfect prep gel purification kit. Both purified products were quantified using a quantification marker and ligated at a vector to insert ratio of 1.5:1, using T4 DNA ligase overnight at 16°C.

5.2.1.6 Electroporation of E. coli XL-1 Blues cells with cloned library

High efficiency electro-competent XL-1 Blue cells were prepared, as described in Section 2.4.6.4. 1 µl of ligation reaction was used to transform 50 µl of cells. This produced a recombinant antibody library of approximately 3×10^4 cells.

Figure 5.2.2 *Reverse Transcription of RNA to generate cDNA. Lane 1: Gibco 1 kb plus DNA ladder. Lane 2: Extracted RNA. Lane 2-13 cDNA reverse transcribed from RNA isolated from a mouse immunised with THC-BSA.*

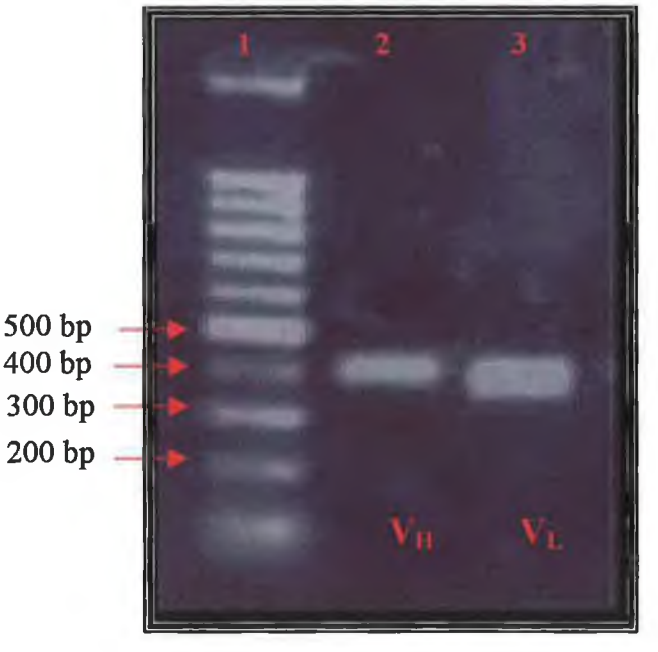


Figure 5.2.3: *Amplified variable heavy (V_H) and light chain (V_L) genes. Lane 1: 1 kb DNA ladder. Lane 2: amplified heavy chain DNA with the expected band of 386-440 bp. Lane 3: amplified light chain DNA with expected band at approx. 375-402 bp.*

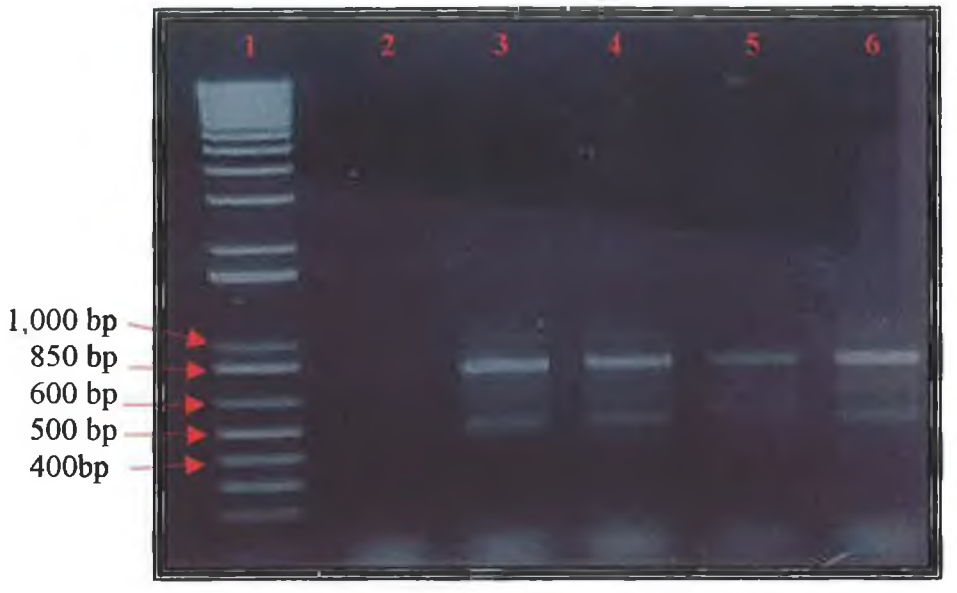


Figure 5.2.4: *1% (w/v) agarose gel showing a concentration titration of V_H and V_L for SOE PCR. The final $MgCl_2$ concentration in the reaction was optimised to yield a level of 1.5 mM $MgCl_2$, pH 9.0. Varying concentrations of V_H and V_L were included in the reaction mix, as shown in Table 5.2.1. 0.5 µl of both V_H and V_L proved optimal for the production of a specific 800 bp band.*

Table: 5.2.1: *Table illustrating the volumes of V_H (approx 30 ng/µl) and V_L (approx 40 ng/µl) used in concentration titration for SOE PCR, shown in Figure 5.2.4.*

Lane No	V_H µl	V_L µl
2	0.6	0.45
3	0. 6	0.35
4	0.5	0.3
5	0.5	0.4
6	0.5	0.5

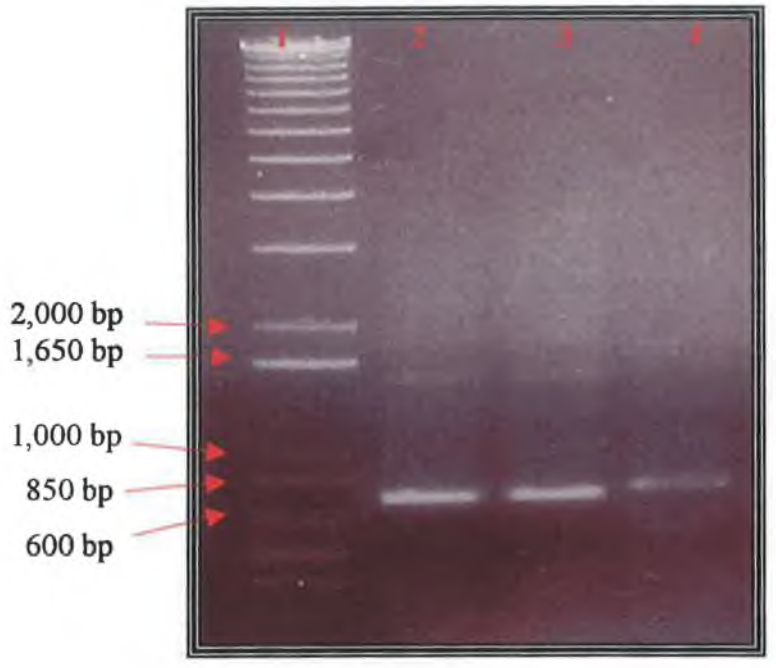


Figure 5.2.5: *Lane 1: Gibco 1 kb plus DNA ladder. Lane 2 + 3: Sfi 1digested SOE product. Lane 4: Purified SOE product of approx. 800 bp.*

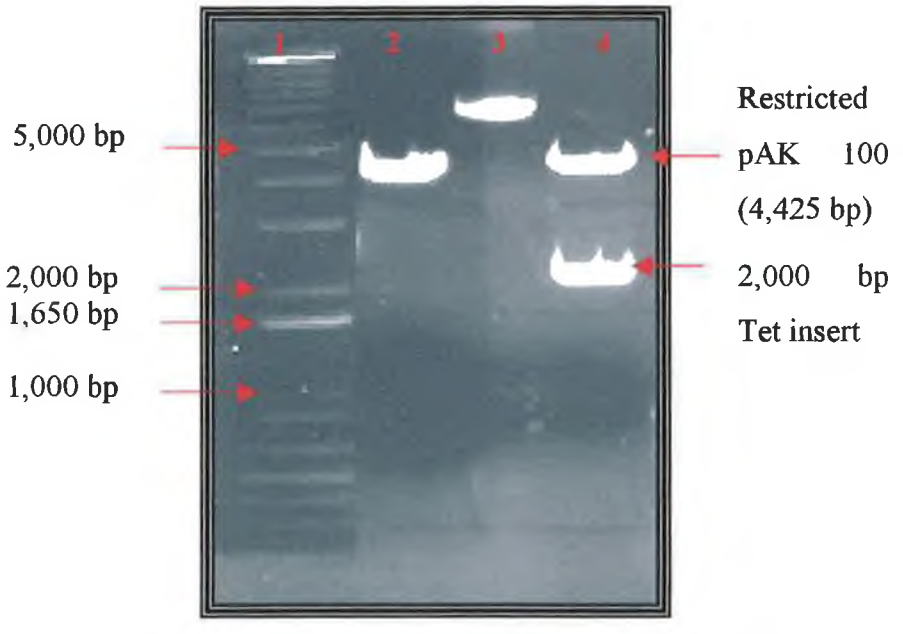


Figure 5.2.6: *Lane 1: Gibco 1 kb plus DNA ladder. Lane 2: Purified Sfi 1 digested pAK 100. Lane 3: unrestricted pAK100, harbouring 2,000 bp tetracycline insert, yielding a vector size of 6,425 bp. Lane 4: Sfi 1 restricted pAK 100 (4,425 bp) minus tetracycline insert.*

5.2.2 Isolation of scFv antibodies to THC from a murine library

In order to enrich the constructed library for antibodies specific to THC, a series of selections on coated-immunotubes were performed. This process is known as biopanning. Phage displaying scFv's were rescued from the library at 26°C overnight as described in Section 2.5.1. The lowering of temperature was employed to increase functional antibody production and aid folding. Phage were concentrated 100-fold using a PEG precipitation. Phage were re-suspended in sterile PBS and pre-blocked in 4% (w/v) MPBS. Phage were then added to a blocked immunotube containing the antigen of interest, in the form of THC-protein conjugate, immobilised on the tube surface. ScFv-displaying phage were added to the immunotube and incubated with end-over-end rotation for 2 hours. Non-specific binders were removed using a series of stringent wash steps with PBST and PBS. Bound antibodies were eluted using an acidic shock with 0.1 M glycine, pH 2.2. Phage were removed from the immunotube and neutralised with 2 M Tris, pH 7.4. The phage were then enriched by reinfection into *E. coli* cells, amplified overnight, concentrated by PEG precipitation and subjected to further rounds of selection.

Phage underwent a total of four selection rounds. In rounds 2 and 4, a subtractive selection approach was employed. This involved pre-incubating phage in an immunotube coated with BSA and MPBS, prior to exposure to target antigen, thereby eliminating any antibodies specific to the carrier protein, BSA. 95 clones from each round of panning were analysed for specific antigen binding, no antibodies specific for THC were isolated (Table 5.2.2.). At this stage it was decided to try alternative panning strategies. One such strategy involved passive elution using male *E. coli* cells, followed by an alkaline shock. Bacterial elution should, in theory, isolate lower affinity binders, with any remaining higher affinity binders being eluted using 100 mM triethylamine, pH 12 (Wind *et al.*, 1997). Elution time was increased to 15 mins as de Bruin *et al.*, (1999) had shown that more stringent elution conditions lead to the isolation of scFvs that could not be isolated using a 10 min acidic shock. Phage output titres from two rounds of selection can be seen in Table 5.2.3. Phage were analysed by polyclonal phage ELISA to monitor overall enrichment of phage population. The binding profile of the phage population showed a general enrichment

in specificity for the THC-BTG conjugate, as can be seen in Figure 5.2.7. Phage were then analysed in a monoclonal ELISA format. However, no scFvs specific to THC were identified.

Table 5.2.2: *Results of panning of an immunised murine library against Tetrahydrocannabinol. Phage were eluted using 100 mM glycine, pH 2.2, and neutralised with 2 M Tris-HCl, pH 7.4. Eluted phage were then re-infected into XL-1 Blues to calculate the phage titre.*

Pan No	Selection Conditions	Phage input titre cfu/ml	Phage output titre cfu/ml	No of positives
1	10 μg/ml THC-BSA	2×10^{10}	1.2×10^{5}	0/95
2	10 μg/ml BSA subtractive pan followed by 10 μg/ml THC-BSA	4×10^{10}	1.0×10^{3}	0/95
3	10 μg/ml THC-BTG	8.6×10^{10}	1×10^{5}	0/95
4	10 μg/ml BSA subtractive pan followed by1 μg/ml THC-BSA	1×10^{10}	1.4×10^{5}	0/95

Table 5.2.3: *Results from two rounds of panning of an immunised murine library against tetrahydrocannabinol using bacterial/alkali elution method. Eluted phage were re-infected into XL-1 Blue cells to calculate phage titre.*

Pan No	Conditions	Phage Input titre cfu/ml	Bacterial elution Phage output titre cfu/ml	Alkali elution phage output titre cfu/ml	No of positives
1	10 μg/ml THC-BTG	4.4 x 107	6.0×10^{2}	5.4×10^{2}	0/95
2	10 μg/ml THC-BTG	5×10^{5}	4.7×10^{3}	1.8×10^{3}	0/95

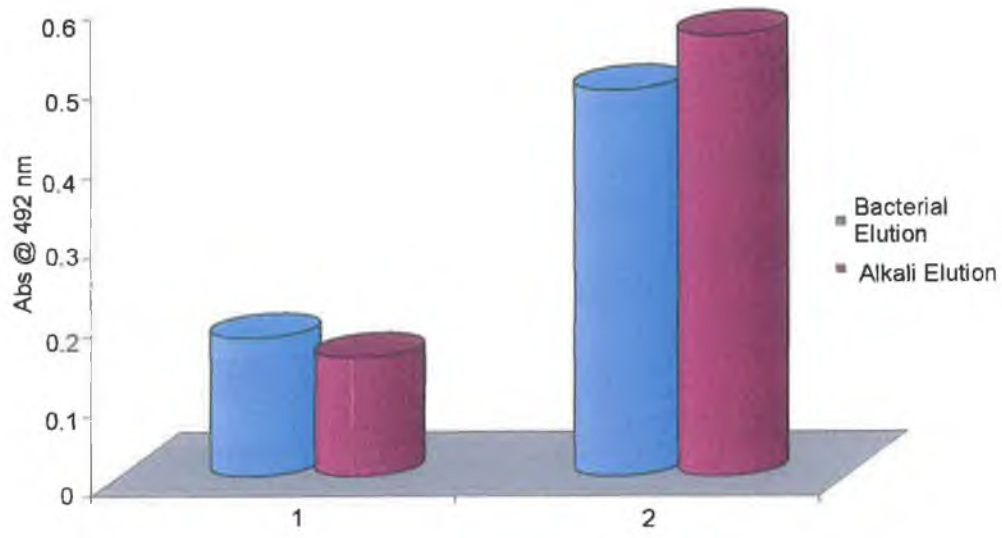


Figure 5.2.7: *Results of polyclonal phage ELISA following bacterial and alkaline elutions. Plates were coated with THC-BTG and blocked with 4% (w/v) MPBS. 10 μl of polyclonal phage from each round of panning were added to each well in 100 μl 4% (w/v) MPBS. Phage were detected using anti-fd Bacteriophage antibody, followed by a peroxidase-labelled anti-rabbit antibody.*

5.2.3 Selection of scFvs from a natural naïve human library

It was decided to pan a naïve human library with a diversity of 1.4 x 10^{10} clones (Vaughan *et al.*, 1996), donated by Cambridge Antibody Technology. This library represented two major advantages over the immune library produced. Immune libraries suffer from one major drawback, the response of the immune system to difficult antigens, such as THC, can be very unpredictable and uncontrolled. The naïve human library also represented a large increase in repertoire diversity. The panning strategy employed with this library involved eluting scFvs by free antigen elution. Bound phage were incubated with increasing concentrations of free THC for a period of 30 mins for each concentration. Each batch of phage was re-infected into *E. coli* for and enriched by phage rescue followed by PEG precipitation. The phage output titres from each round of panning are shown in Table 5.2.4. Although several scFvs specific for the conjugate THC-BSA were isolated (Figure 5.2.8), these showed no binding inhibition when presented with free THC, indicating that they were specific for the hapten-protein conjugate rather than the free drug itself (Figure 5.2.9).

Table 5.2.4: *Phage titre results of panning of a naïve human library donated by Cambridge Antibody Technology. In order to isolate high affinity binder, phage-displaying antibodies were selected using elution with increasing concentrations of free antigen. In round 3 a concentration of 100 µg/ml THC was used to elute antibodies to free THC. In round four, a step-wise elution strategy was employed. This involved incubating bound phage with an initial THC concentration of 1 µg/ml for 30 mins. This was increased to 10, 50 and 100 µg/ml, each for 30 mins. Antibodies with the highest affinity should elute at lower THC concentrations. Phage eluted at each concentration, were then re-infected into TG1 cells, and enriched prior to further rounds of selection.*

	Round 1	Round 2	Round 3	Round 4
Input titre (cfu/ml)	1×10^{13}	8×10^{4}	6×10^{6}	3×10^{6}
Competitive Output titre (cfu/ml)	N/A	N/A	6×10^{2}	
Alkali output titre (cfu/ml)	3×10^{1}	4×10^{3}	1×10^{2}	6×10^{2}

1 µg/ml THC	3.5×10^{4}
10 µg/ml THC	2.6×10^{4}
50 µg/ml THC	1.3×10^{4}
100 µg/ml THC	2.4×10^{4}

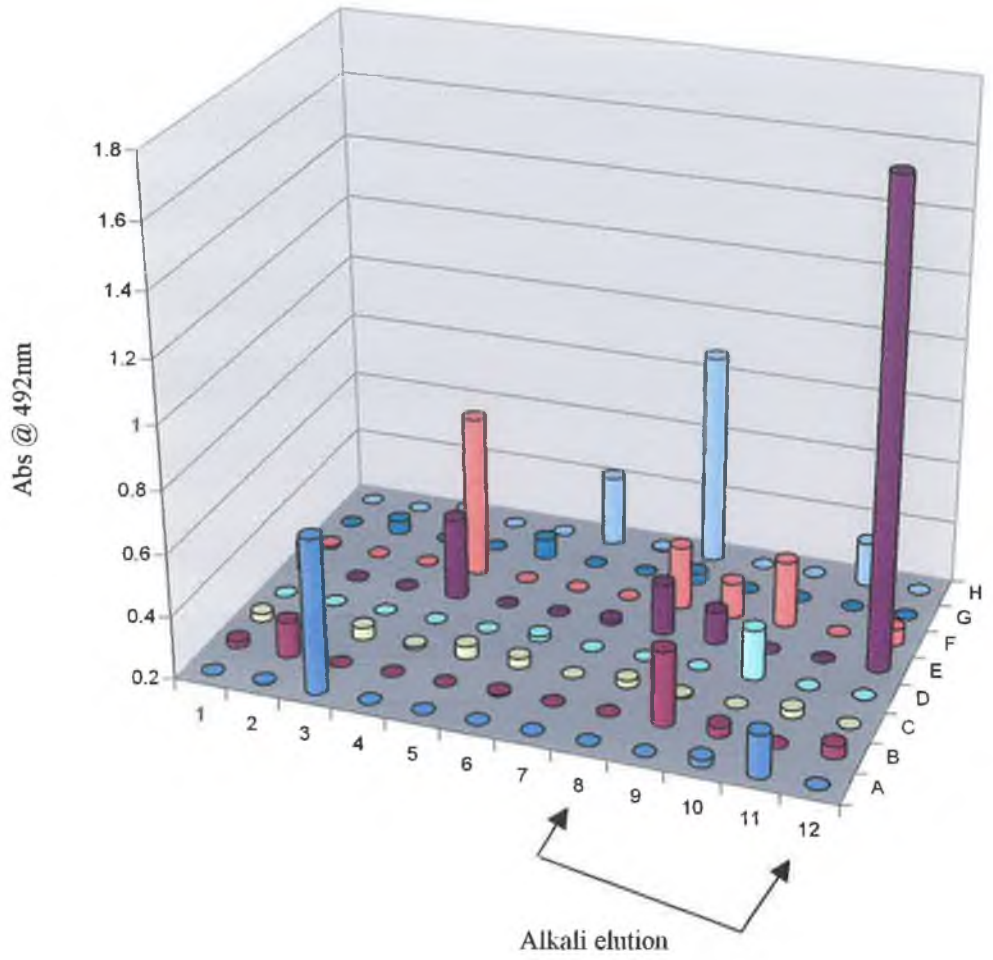


Figure 5.2.8: *Monoclonal phage ELISA following four rounds of biopanning. Phage were screened against THC-BSA conjugate. All clones gave a background absorbance of < 0.2 a.u. against the carrier protein BSA.*

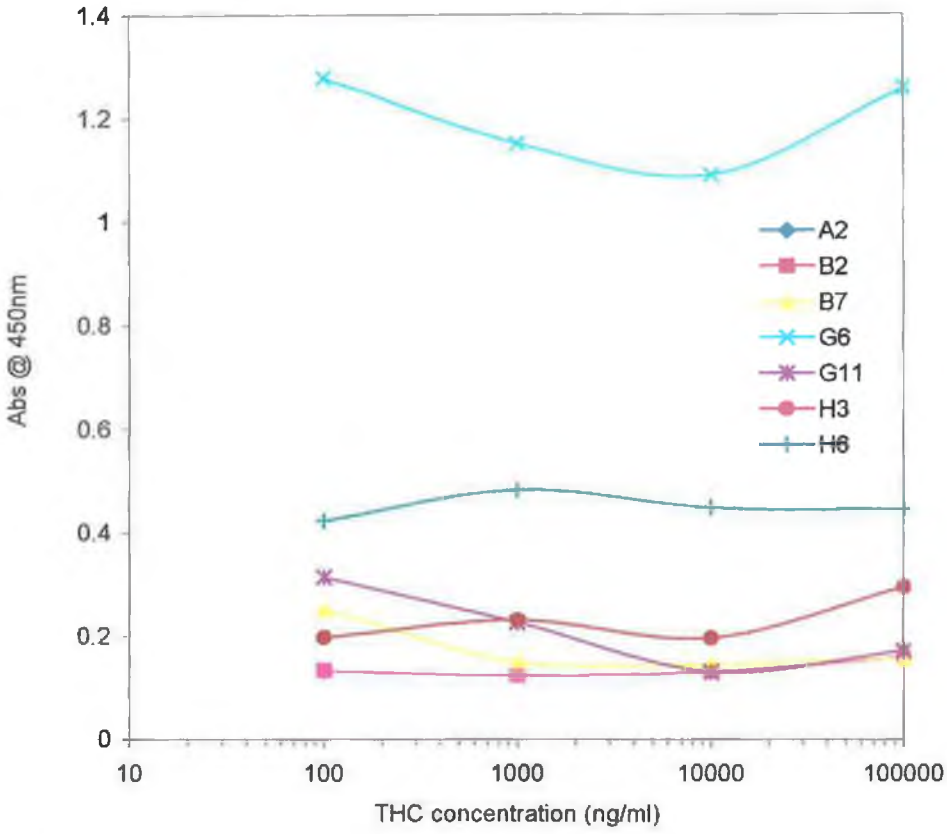


Figure 5.2.9: *Competitive phage ELISA using positive phage clones isolated in monoclonal phage ELISA. No inhibition was seen when phage-displayed scFvs were presented with free THC. This indicated that all clones were specific for the protein-hapten conjugate rather than free THC.*

5.2.4 Selection of scFvs against Tetrahydrocannabinol from the Griffin.1 semi-synthetic naïve human library

The Griffin.1 library was donated to the University of Aberdeen, by the MRC Centre for Protein Engineering, Cambridge. The Griffin.1 library is a scFv phagemid library made from semi-synthetic V-gene segments, with a diversity of 1.2 x 10^9 clones (Griffiths *et al.*, 1994). The library is derived from genuine human germline sequences, in which CDR L3 loops have been partially randomised to mimic natural diversity. This represents a more 'natural' source of antibody than a naive library, derived from IgM, which may suffer from somatic mutations (Hoogenboom, 1997). 1 x 10^{12} phage were used for the first round of biopanning. The phage input titres and panning conditions are detailed in Table 5.2.5. The initial two rounds of selection were against decreasing concentrations of THC-BTG, as no other conjugate was available at this time. In Table 5.2.5, it can be seen that after an initial decrease in phage numbers, the library was enriched for THC-BTG binders. The third round of panning involved elution by free antigen. Phage titres were seen to decrease at this stage as phage specific for the conjugated-form of THC were removed. In the fourth round of selection, the coating conjugate was changed to THC-BSA, to further ensure that all BTG binders were removed. Elution was by means of a decreased concentration of free THC. In the fifth round of panning, the concentration of THC elution concentration was 1 nM in order to isolate higher affinity THC binders.

All phage were re-infected into TG1 cells and analysed in a monoclonal format. A total of 92 clones from pans 2-4 were analysed by monoclonal phage ELISA, as illustrated in Figure 5.2.10. A further 95 clones from pan 5 were also analysed (Figure 5.2.11). A high degree of enrichment for positive binders can be seen between earlier selection rounds and the fifth round, as shown in Table 5.2.6. All phage clones that gave a positive binding response to both THC-BSA and THC-BTG conjugates were re-analysed using a competitive phage ELISA. Figure 5.2.12 illustrates the competition observed between two of these phage-displayed antibodies and free THC in solution. Upon sequencing of 7 positive binders, it could be seen that the same antibody sequences were being amplified and enriched so that they were present in higher numbers (Figures 5.12.13 to 5.2.15). For example, clone 3D8,

isolated in round 3 of panning was also isolated in rounds 4 and 5, as clones 4A5, 5C5 and 5G4. This demonstrates the efficiency of the panning process, increasing the proportion of specific binders to non-specific binders in the general phage population.

Table 5.2.5: *Phage titre results following five rounds of biopanning of Griffin.1 Library against THC.* 1×10^{13} *phage were used for initial selection and bound phage eluted using 1ml of 100 mM triethylamine, pH 12. Competitive elution using decreasing free antigen concentration was employed in rounds 3-5.*

Round of Panning	Conditions	Phage Output Titre (cfu/ml)
1	50 µg ml THC-BTG	3.8×10^3
2	10 µg ml THC-BTG	3×10^5
3	10 µg ml THC-BTG 1 µM THC elution	6×10^4
4	1 µg ml THC-BSA 100 nM THC elution	1×10^3
5	1 µg ml THC-COOH-BSA 1 nM THC elution	8.8×10^3

Table 5.2.6: *Table showing the enrichment of library with positive antibodies to THC during each rounds of selection. All clones were analysed by monoclonal phage ELISA. Results can be seen in Figure 5.2.10 and 5.2.11.*

Round of Panning	No. of clones screened	No. of positive clones	% Positives
2	36	3	8.3
3	35	9	25.7
4	24	6	25
5	92	51	55.4

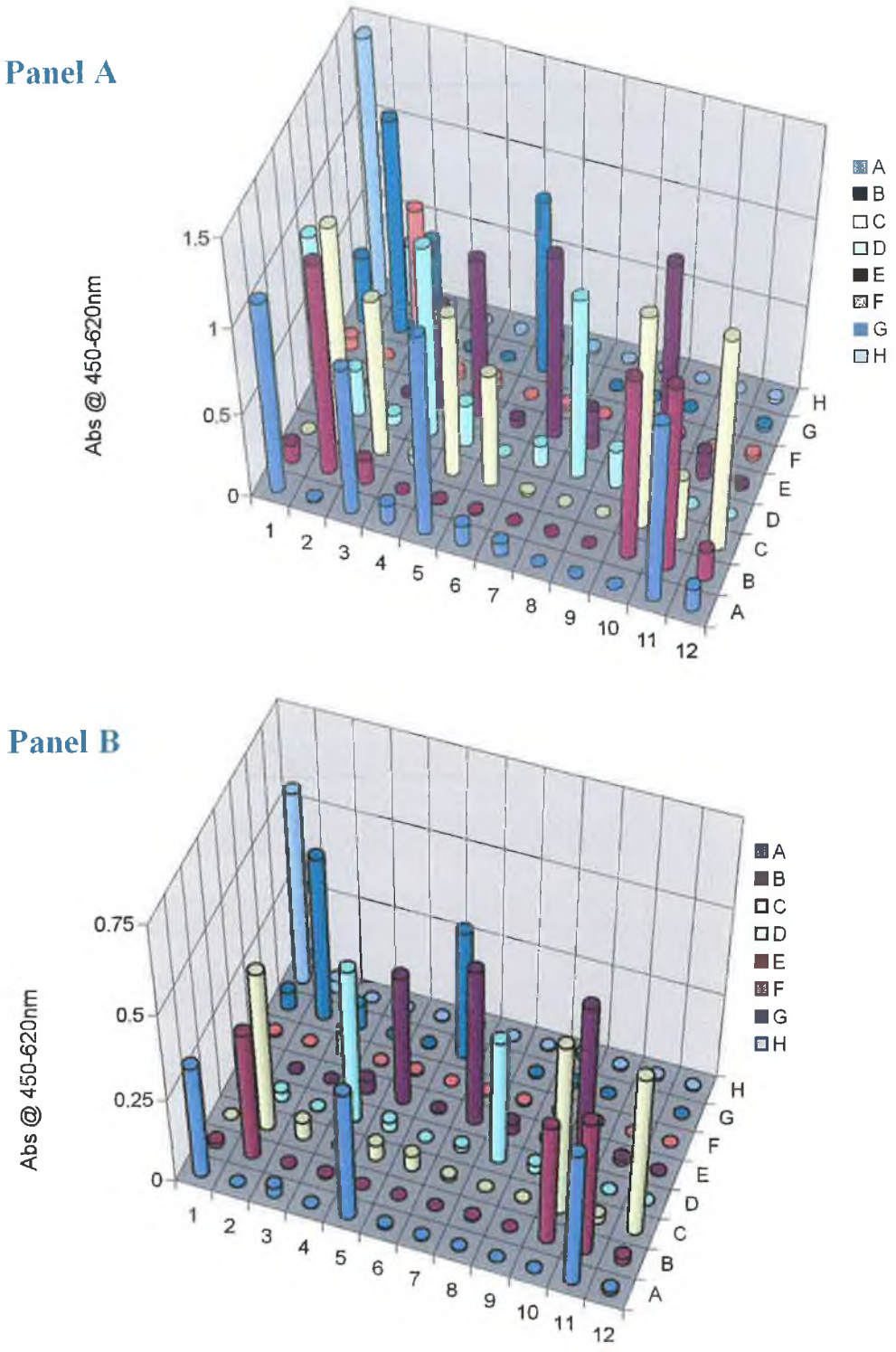


Figure 5.2.10: *Monoclonal phage ELISA results illustrating absorbance seen from a 96-well ELISA plate, containing phage from pan 2-4. Rows A and B represent clones from fourth round, rows C, D and E represent clones from round 3 and rows F, G and H, contains clones isolated from round 2. Results from panel A show binding to plate coated with 10 μg/ml THC-BTG and panel B, shows binding to a 10 μg/ml THC-BSA-coated plate.*

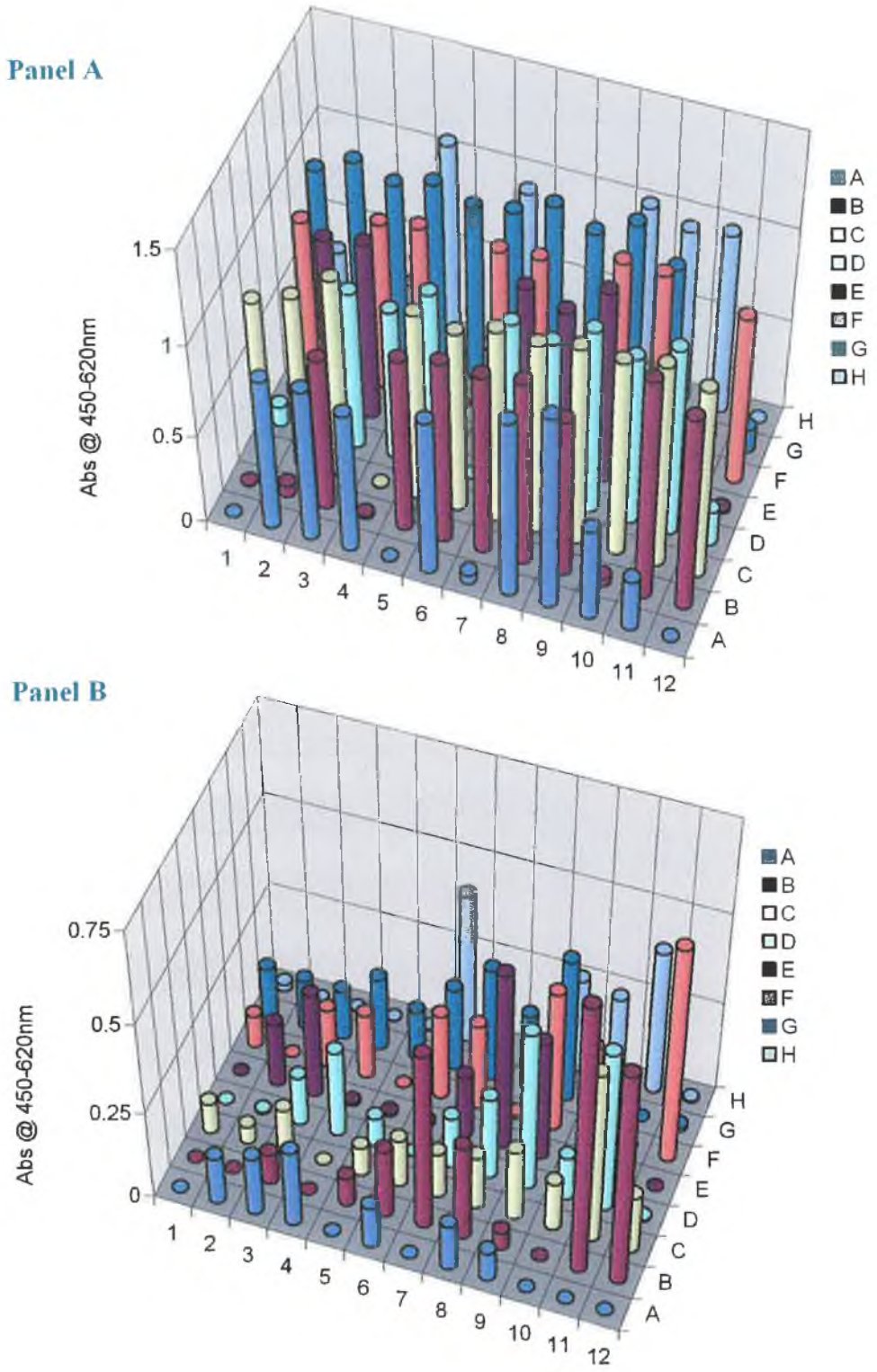


Figure 5.2.11: *Monoclonal phage ELISA results illustrating absorbance seen from a 96-well ELISA plate, containing phage from pan 5. Results from panel A show binding to plate coated with 10 μg/ml THC-BTG and panel B, show binding to a 10 μg/ml THC-BSA-coated plate.*

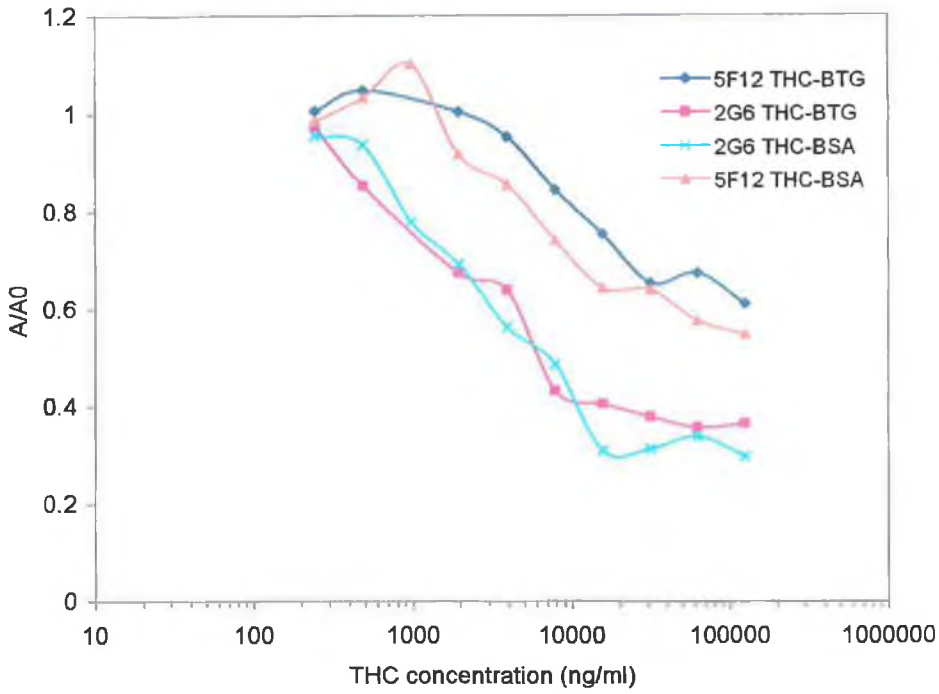


Figure 5.2.12: *Competitive phage ELISA showing two different clones isolated against THC. Plates were coated with 1 µg/ml of THC-BTG or BSA conjugate. 50 µl of phage were added to each well containing 50 µl of free THC in 2% (w/v) MPBS. Absorbance was read at 450-620nm and normalised by dividing the absorbance at each THC concentration by absorbance with no THC (A0).*

5.2.4.1 Genetic analysis of scFv genes encoding antibodies capable of THC detection

A total of seven scFv clones, that showed the highest level of inhibition in ELISA when presented with free THC, were sent for sequencing. In order to generate high quality DNA for sequencing, plasmid DNA from each clone was transformed into XL-1 Blue cells. A single colony of each clone was grown overnight in LB media. The pHEN 2 vector, containing scFv gene of interest, was isolated by miniprep, as described in Section 2.4.6.1. Plasmid DNA was sent to the Institute of Medical Sciences, University of Aberdeen for sequencing, using the primers AH1 reverse (5'–AAATACCTATTGCCTACGGC-3') and gene III forward (5'-GAATTTTCTGTATGAGG-3'). Raw DNA sequences in each direction were analysed using CHROMAS sequence analyser software. The DNA sequences were exported and translated using the DNA to protein translation package on the Bio.lundberg sequence analysis server. Amino acid residues were exported to ClustalW for alignment and to Genedoc for manipulation. This identified homologous residues in the sequence alignments. The Kabat identification scheme was used to identify antibody complementarity determining regions (CDR's), which are highlighted as blue or red, depending on whether they are of heavy or light chain origin.

5.2.4.2 Alignment of sequences of seven scFv genes that showed specificity for free THC

Of the seven clones sequenced, only three were found be genetically different. This illustrated how the bio-panning process enriched the percentage of positive binders, as each sequence identified in early rounds of panning was present in later rounds. It should be noted, however, that clone 5F12 was not isolated until the fifth round of biopanning. Figure 5.2.13 demonstrates that clone 2G6, isolated in the second round of panning, was isolated again as clone 5B3 in the fifth round. The enrichment becomes more apparent, however, in Figure 5.2.14, where clone 3D8, isolated in the third round of panning was isolated as clone 4A5 in the fourth round, and as clones 5C5 and 5G4 in the fifth round. Figure 5.2.15, illustrates the variations in the amino acid sequences of all three non-identical clones, 2G6, 4A5 and 5F12. All amino acids

that are conserved between the three sequences are highlighted in black, while residues conserved in two out of three clones are shown in grey. Complementarity Determining Regions (CDRs) were identified using the Kabat rules and highlighted accordingly. Heavy chain CDR regions are written in red, while light chain CDR regions are written in blue. The 13 amino acids encoding the linker region are written in yellow. All antibodies were found to be of the κ1 light chain subgroup but varied in their heavy chains. Both clones 4A5 and 5F12 were found to be human subgroup III heavy chains, while clone 2G6 was found to be derived from the human subgroup II heavy chain. All three clones show a significant degree of variation in framework and CDR regions. The composition and length of the third CDR of antibody heavy chains is thought to play an important role in the formation of the hapten-binding pocket, which confers specificity to the molecule (Strachan *et al.*, 2002). CDR H3 lengths were seen to vary from 10 amino acids in clone 4A5 to 9 in clone 5F12 and 8 in clone 2G6. Recent evidence suggests that anti-hapten antibodies bind in a pocket positioned between heavy and light chain variable domains, whereas anti-protein antibodies have a more flat or planar binding site (Johnson and Wu, 1998: Strachan *et al.*, 2002). The formation of this binding pocket is thought to be influenced by CDR H3 length, as longer CDR H3's (9-10 amino acids) have been observed in more sensitive anti-hapten scFvs.

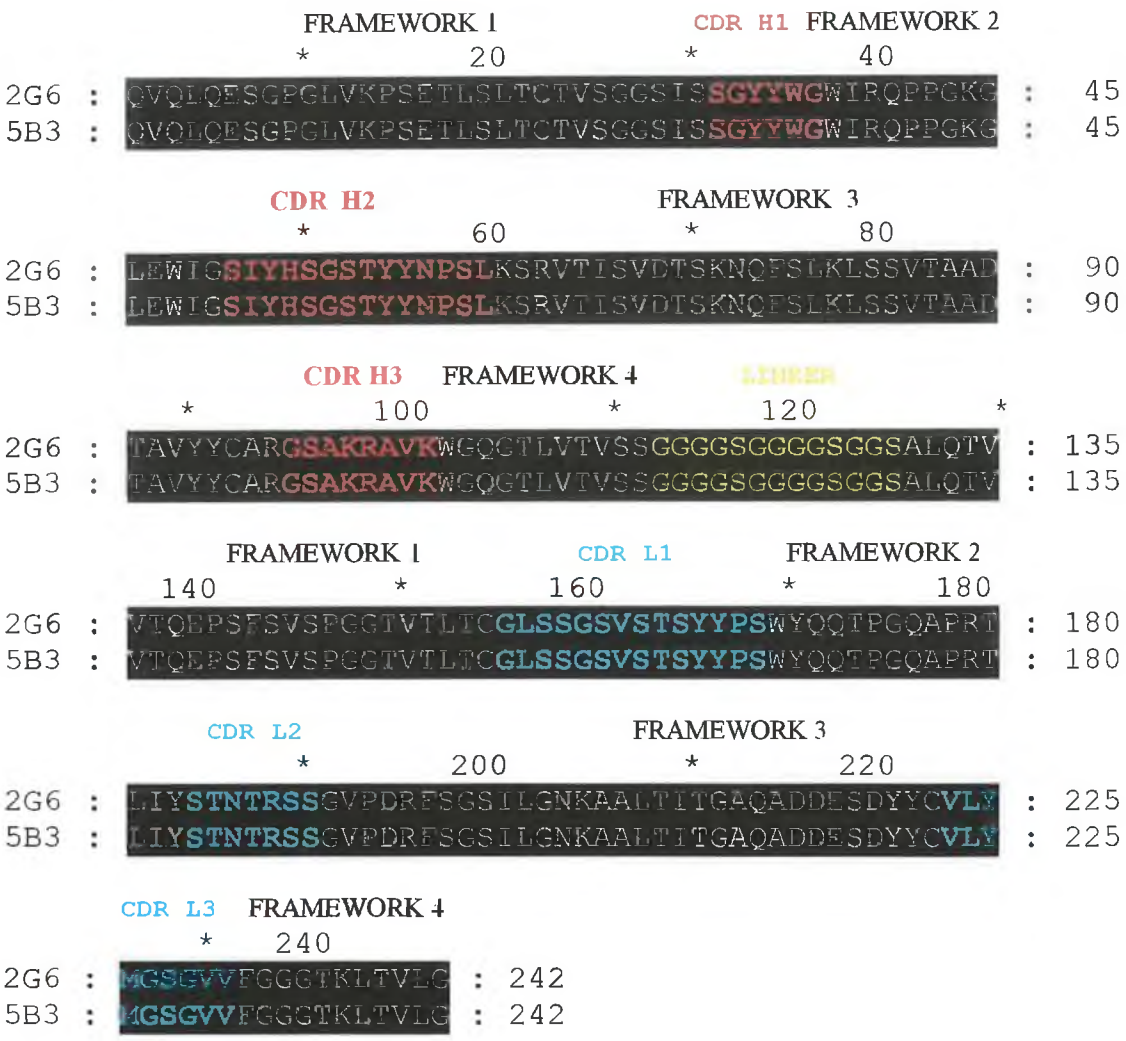


Figure 5.2.13: *Alignment of phage-displayed scFv clones 2G6 and 5B3 isolated from Griffin.1 Library against THC. Both clones show 100% homology as highlighted in black. Heavy chain CDR regions are written in red and light chain CDR regions in blue. The linker region sequence is written in yellow.*

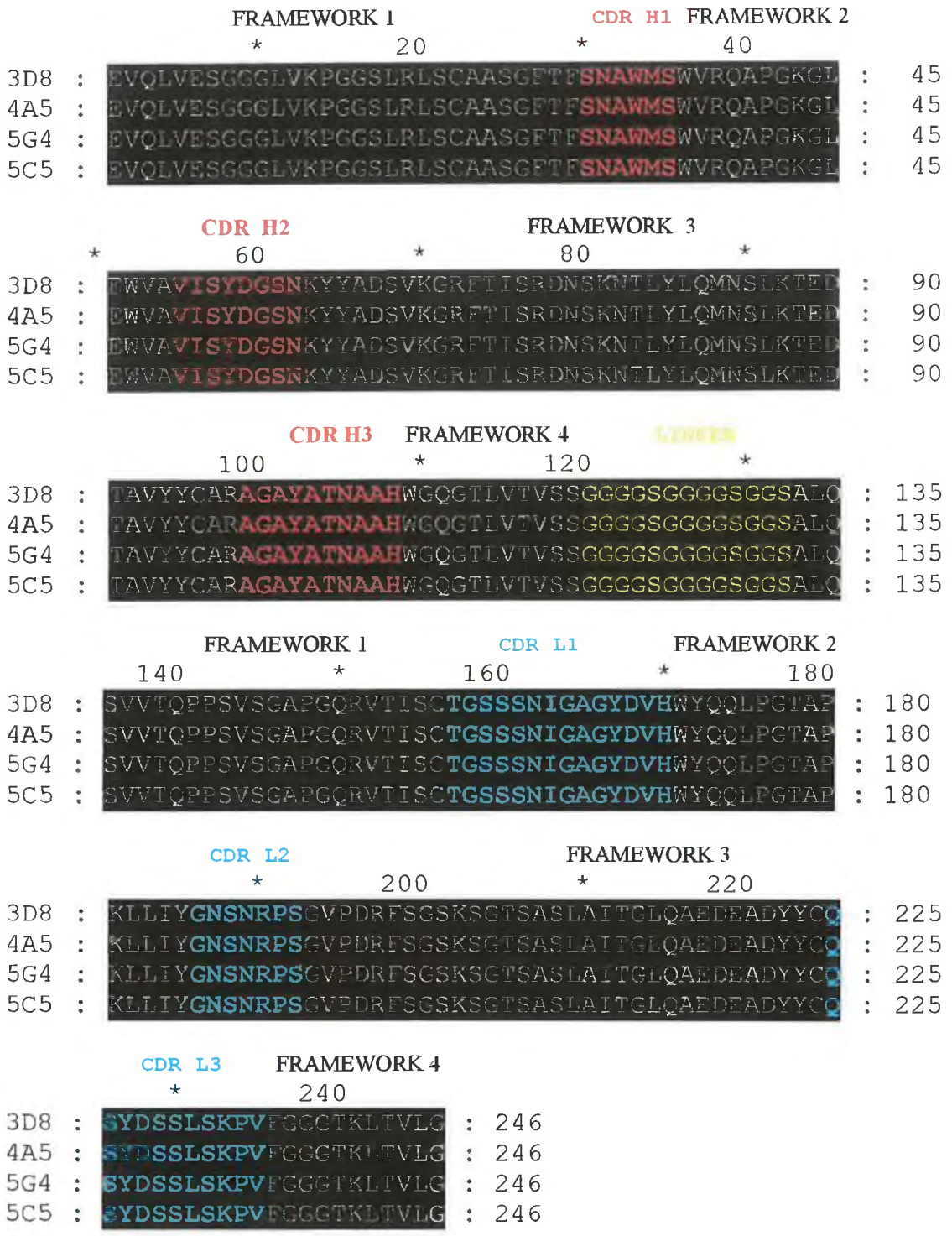


Figure 5.2.14: *Alignment of phage-displayed scFv clones 3D8, 4A5, 5C5 and 5G4 isolated from Griffin.1 Library against THC. All clones show 100% homology as highlighted in black. Heavy chain CDR regions are written in red and light chain CDR regions in blue. The linker region sequence is written in yellow.*

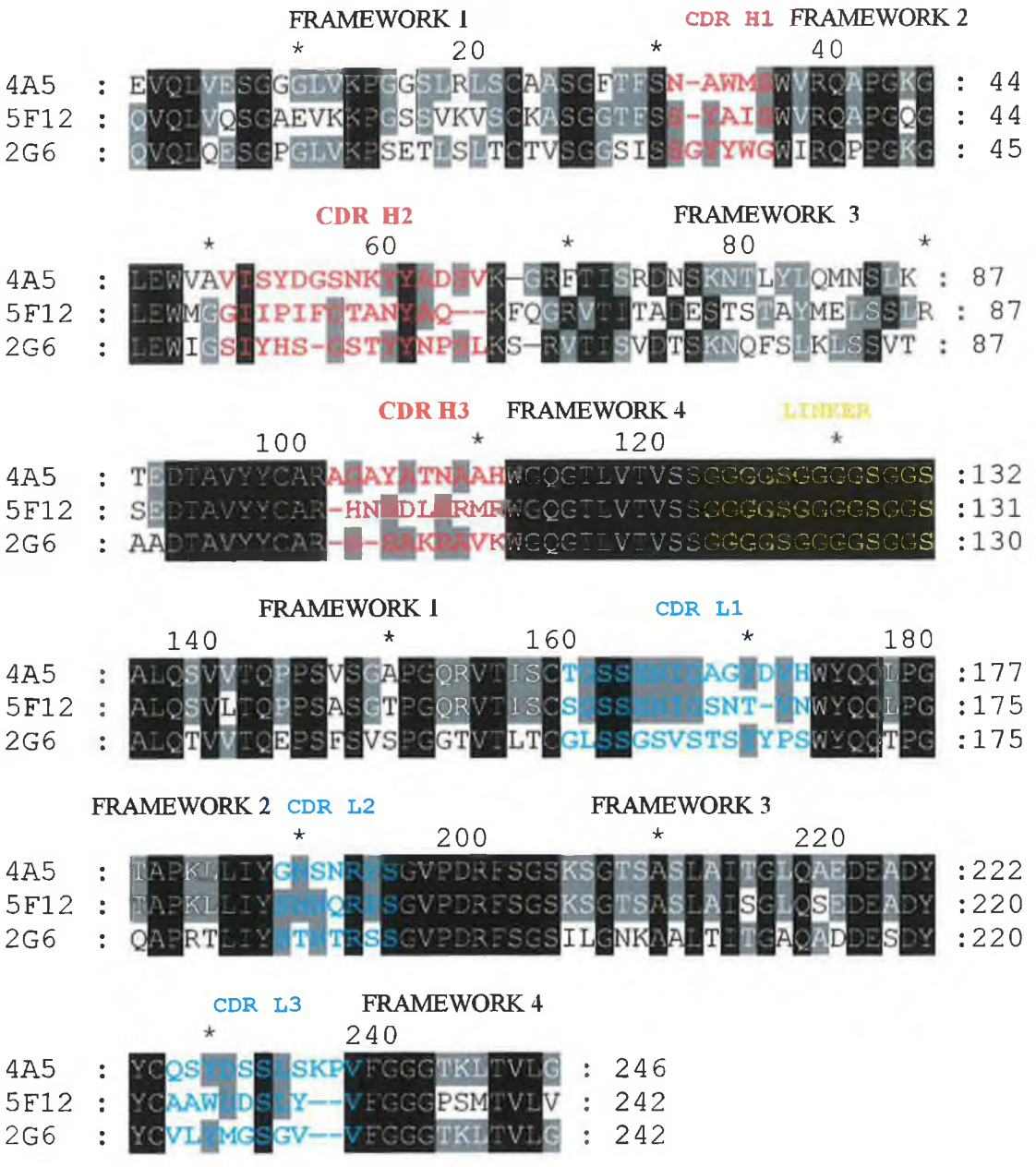


Figure 5.2.15: *Alignment of all phage-displayed scFv clones Griffin.1 Library against THC that displayed sequence variation. The sequences highlighted in black show 100% identity and the sequenced highlighted in grey show identity in 2 out of 3 clones. Heavy chain CDR regions are written in red and light chain CDR regions in blue. The linker region sequence is written in yellow.*

5.2.4.3 Homology modelling of scFv sequences using Swiss-Model and DS Viewer Pro

Antibodies were modelled using Swiss-Model, a fully automated protein structure homology-modelling server (Schwede *et al.*, 2003). Swiss-Model is a server for automated comparative modelling of three-dimensional (3D) protein structures. Comparative modelling can reliably generate a 3D model of a protein target from its amino acid sequence. The server operates by comparing the given amino acid sequence to all experimentally solved 3D protein structures held at the Protein Data Bank (PDB). This identifies a solved protein structure with the greatest sequence similarity to the target sequence. The degree of identity between sequences is calculated and a theoretical model for the target sequence extrapolated. Modelling results were exported to Swiss-PDB Viewer and DS Viewer Pro for visualisation.

Figure 5.2.16 shows a ribbon illustration of scFv antibody (clone 2G6) modelled using DS Viewer Pro software. Heavy chain CDRs are shown in red and light chain CDRs are shown in blue. The linker region is shown in yellow. Figure 5.2.17 shows two schematic illustrations of scFv antibody (clone 2G6) generated using Swiss-PDB Viewer software. In this model arrows pointing in the direction of the C-terminus represent beta sheets and coils are represented by a tubular structure. Each variable domain consists of nine beta sheets connected via a synthetic Gly_4Ser linker, which is clearly visible as a yellow tube. The 6 hypervariable loops that comprise the antibody's CDR regions give rise to a deep antigen binding cavity, associated with hapten-binding. A long CDR L1 loop and an accessible CDR H3 loop are also associated with 'haptogenic' binding.

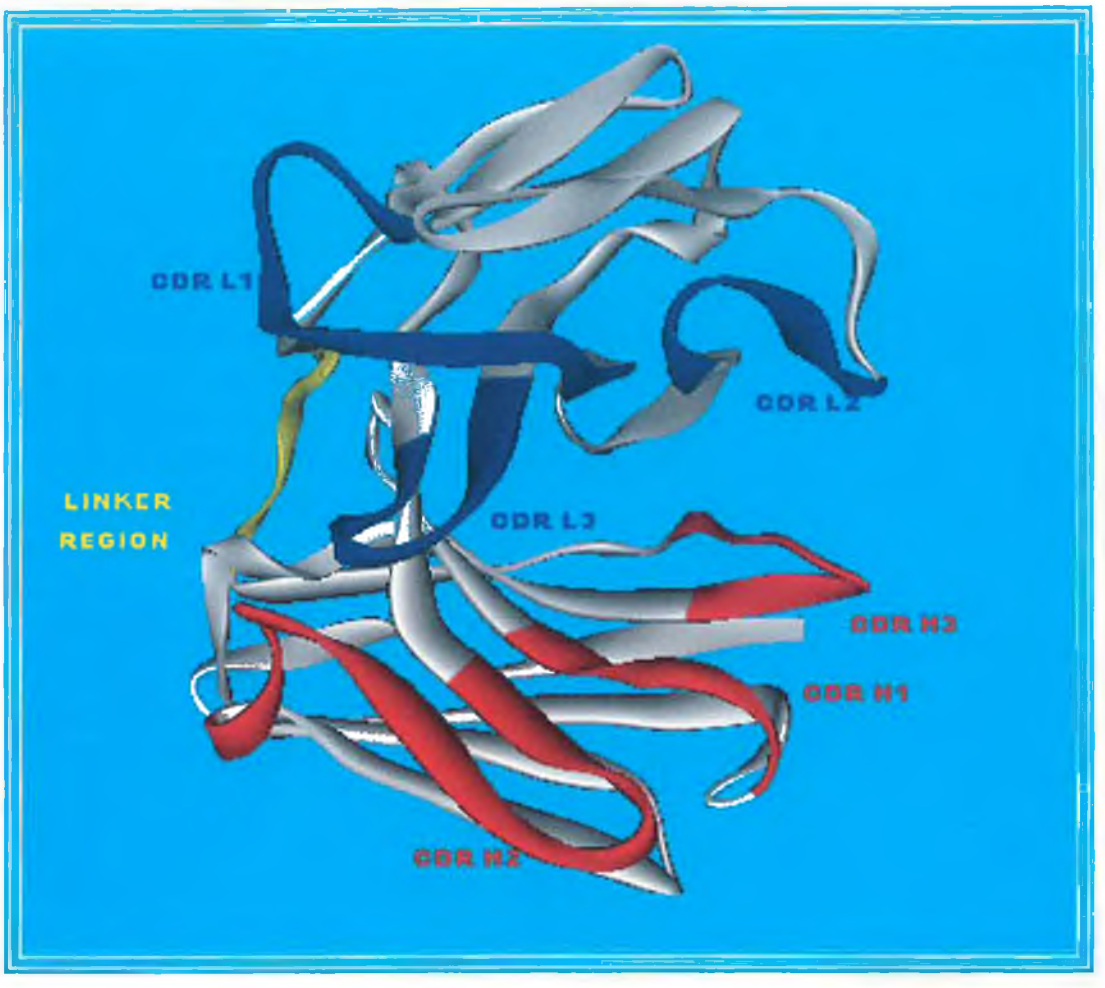


Figure 5.2.16: *Ribbon illustration of anti-THC scFv antibody (clone 2G6). The antibody structure was modelled using DS ViewerPro modelling software. Heavy chain CDRs are shown in red and light chain CDRs are shown in blue. The linker region is shown in yellow.*

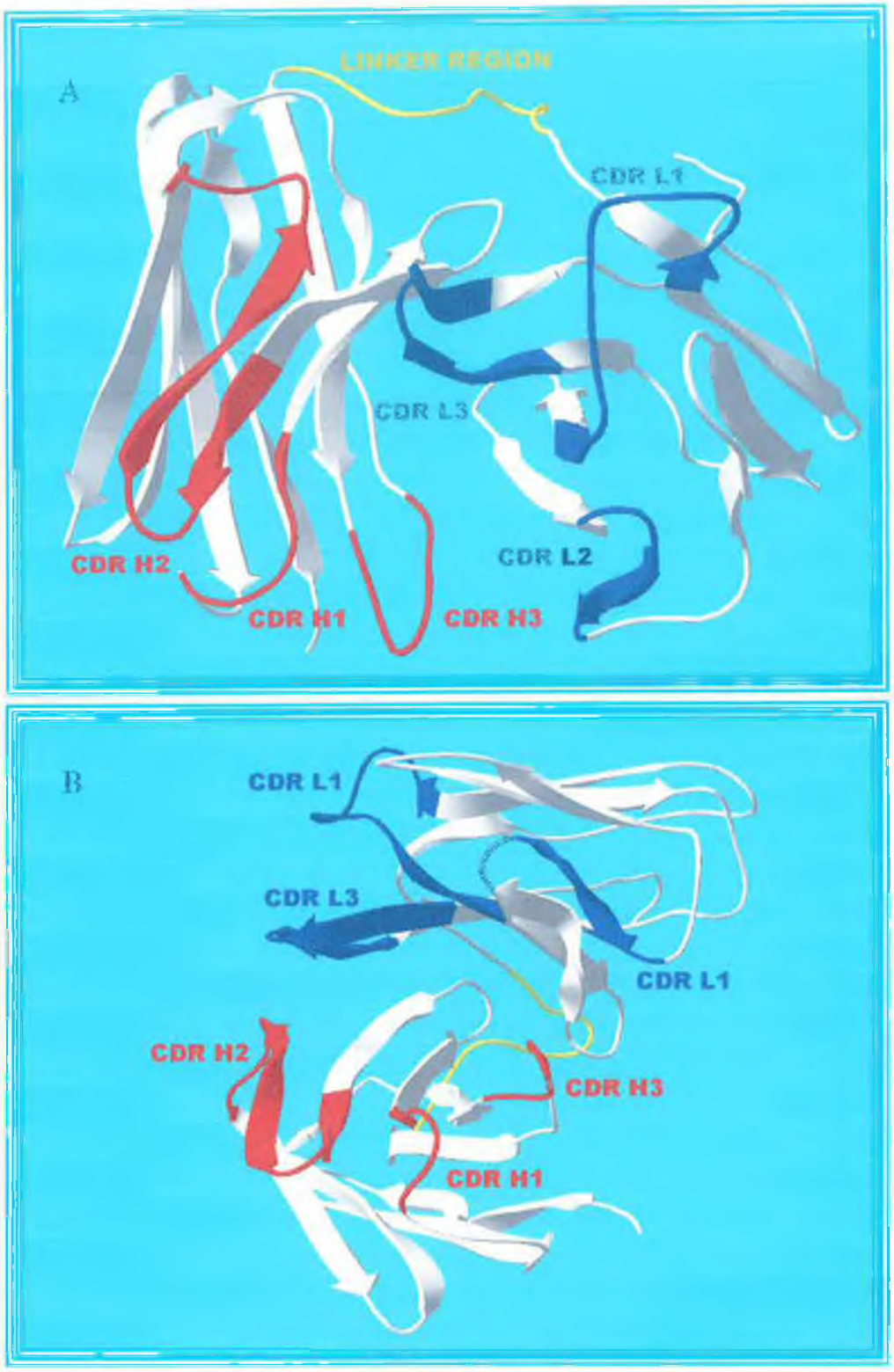


Figure 5.2.17: *Schematic homology model of anti-THC scFv (clone 2G6). Panel A illustrates a front view of the antibody, looking into the antigen-binding pocket and panel B represents a top view, looking down into the antigen-binding pocket. Beta sheets are shown as arrows and coils are represented as tubes. Heavy chain CDRs are shown in red and light chain CDRs are shown in blue. The linker region is shown in yellow.*

5.2.5 Expression of soluble scFv as a scAb in the pIMS 147 expression vector

In order to produce soluble antibody in a single chain format (scAb), the scFv insert was recloned into an enhanced expression vector, pIMS 147, produced by the Molecular and Cell Biology Group, University of Aberdeen. The pIMS 147 vector expresses scFv's in a scAb format, by encoding the human kappa light chain constant gene, at the C-terminus of the scFv gene, resulting in the expression of a single chain antibody.

The pHEN 2 vector, containing the scFv insert, was isolated by minipreping the plasmid DNA. The scFv insert was then isolated from the vector using the *Nco 1* and *Not 1* restriction sites flanking the region. An agarose gel showing doubly digested pHEN and pIMS 147 vectors is shown in Figure 5.2.18. The scFv inserts were gel-purified as described in Section 2.4.4.4, quantified, and ligated into pIMS 147. The plasmid was then transformed into heat-shock competent XL-1 Blues for scAb expression. Antibody clones were grown in TB media, as described in Section 2.6.10, and expression was induced using 1 mM IPTG. Cells were pelleted and subjected to an osmotic shock to lyse the bacterial periplasm. The soluble fraction was harvested and used directly for purification. All scAbs were purified via Ni^{+2}-charged immobilised metal affinity chelate chromatography (IMAC), which utilises the hexahistidine tail of the antibody as a tag for purification. The presence of scAb was detected via the human kappa light chain constant domain (Cκ), encoded in the vector. The purity of all scAbs was monitored by SDS-PAGE. A corresponding gel was also transferred to nitrocellulose and probed with a peroxidase-labelled anti-human kappa light chain antibody (Figure 5.2.19).

The concentration of scAb was determined via capture ELISA as described by McGregor *et al.* (1994). A standard curve of human IgG of known concentrations was plotted against absorbance observed. This could then be corrected for differences in molecular weight between whole IgG (150kDa) and scAb (40kDa). Figure 5.2.20 shows that whole IgG gives the expected sigmoidal curve, the linear portion of which can be used for concentration determination. All scAbs, however, exhibit a flatter shaped capture ELISA curve, with no linear portion. This suggests that the antibody

fragments are aggregating to form multimers. In an attempt to dissociate antibodies into monomeric fragments for quantification, the capture ELISA was repeated, incorporating a low concentration of free drug. The presence of free antigen should prevent any aggregation occurring in the region of the antigen-binding site, as area should preferentially bind antigen. 10 μg/ml of THC was added to each antibody dilution and incubated for 1 hour to reach equilibrium. This was shown to improve antibody aggregation but was still not sufficient to separate antibodies so that a linear region of the sigmoidal curve could be observed, as can be seen in Figure 5.2.20. From SDS-PAGE analysis, it can be seen that the antibodies were sufficiently pure to quantify by total protein concentration, although this may slightly over estimate the expression levels of the antibody. A Bradford assay was performed using a Coomassie protein determination kit. The concentrations of each antibody are shown in Table 5.2.7. The results show that clone 2G6 proved to be the best-expressed antibody. However, clone 5F12 was the most concentrated. It was, therefore, unusual that clone 4A5 appeared to form a visible insoluble precipitate upon storage in PBS.

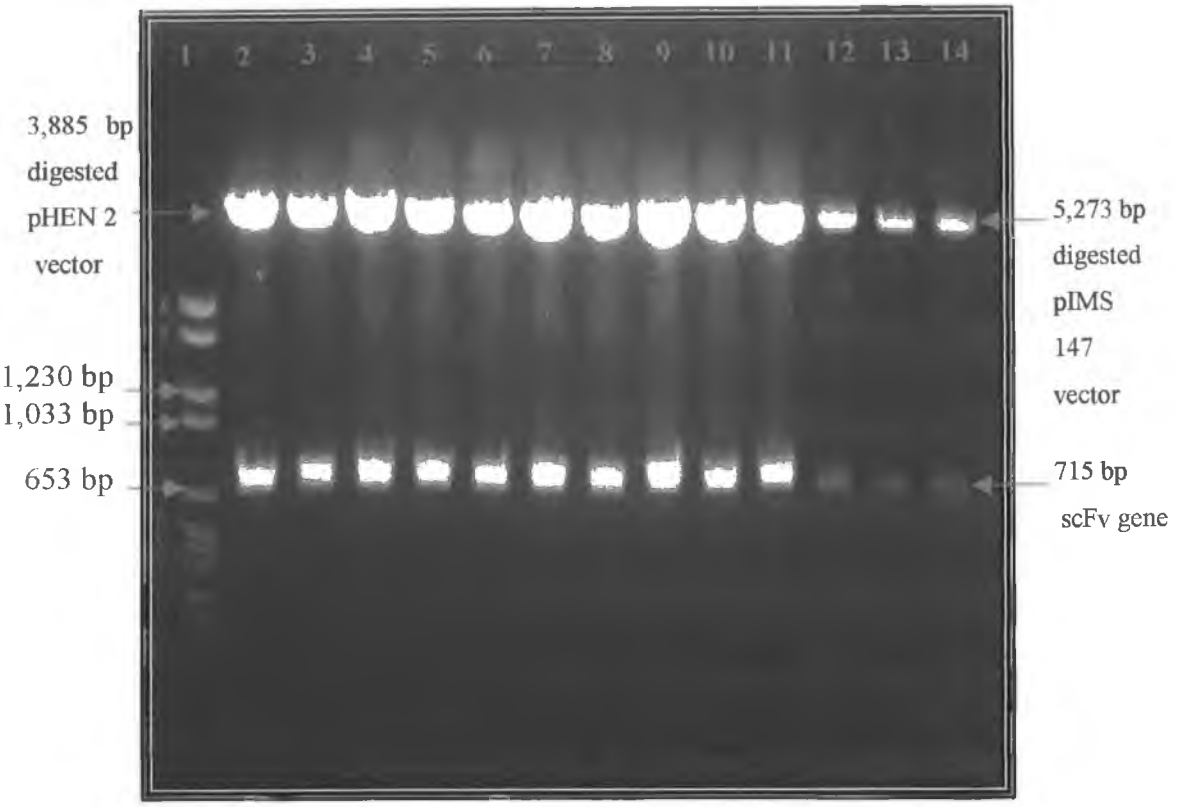


Figure 5.2.18: *Nco 1, Not 1 digest of selected pHEN 2 clones. Lane 1: Roche DNA ladder. Lane 2-11: pHEN 2 clones containing scFv inserts, digested using Nco 1 and Not 1 restriction enzymes, resulting in cut vector of approx 3,900 bp and scFv gene of approx 715 bp. Lanes 12-14: pIMS 147 vector digested with Nco 1 and Not 1 restriction, resulting in cut vecor of approx 5,270 bp and scFv gene of approx 715 bp. The scFv genes from pHEN 2 clones were purified and ligated into the pIMS 147 vector digested with Nco 1 and Not 1.*

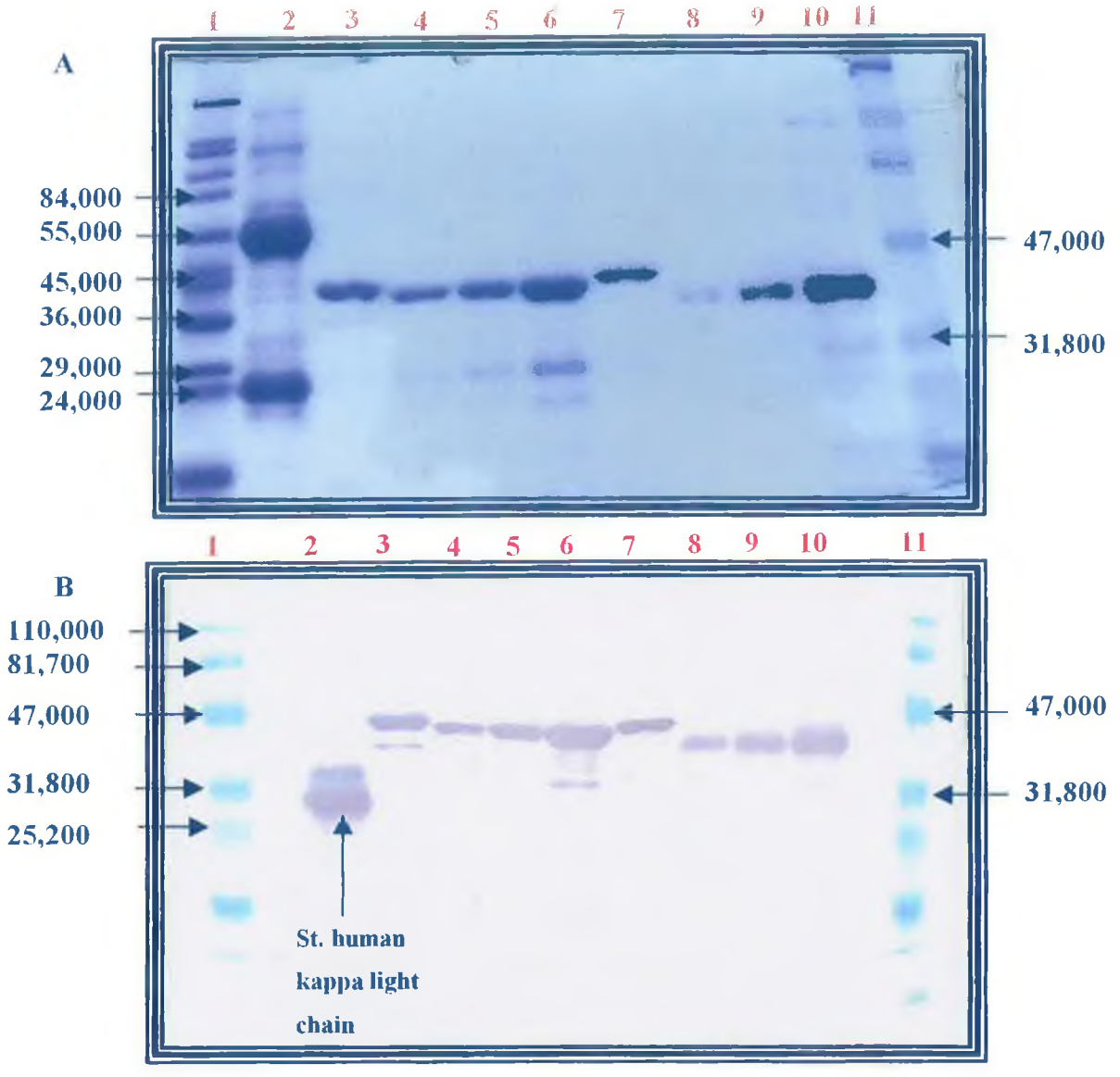
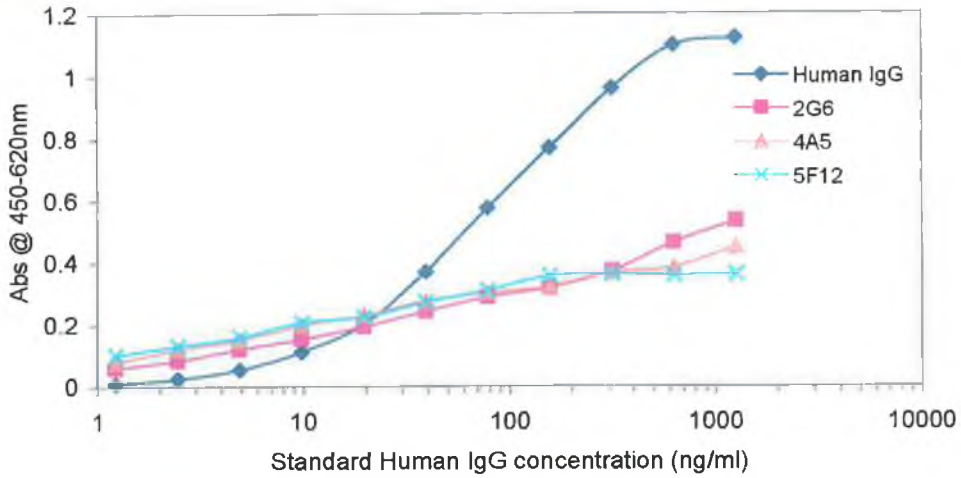


Figure 5.2.19: *Purification of anti-THC scAb clones 2G6, 4A5, 5F12 by IMAC. Panel A shows SDS-PAGE analysis, while panel B shows Western blot analysis of purified scAbs. Antibodies were detected in Western blotting using a peroxidase-labelled anti-human kappa constant light chain domain antibody and visualised using TMB substrate. Lane 1: Molecular weight markers, Lane 2: Standard human IgG at 520 µg/ml, Lane 3: Positive control scAb (clone CSBD9 against microcystin) at 300 µg/ml, Lane 4: Purified scAb clone 5F12 at a 1 in 10 dilution, Lane 5: Purified scAb clone 5F12 at a 1 in 5 dilution, Lane 6: Purified scAb clone 5F12 Neat, Lane 7: Purified scAb clone 4A5 neat, Lane 8:Purified scAb clone 2G6, 1 in 5 dilution, Lane 9: Purified scAb clone 2G6, 1 in 2 dilution, Lane 10: Purified scAb clone 2G6, neat, Lane 11: Prestained molecular weight markers.*

Panel A



Panel B

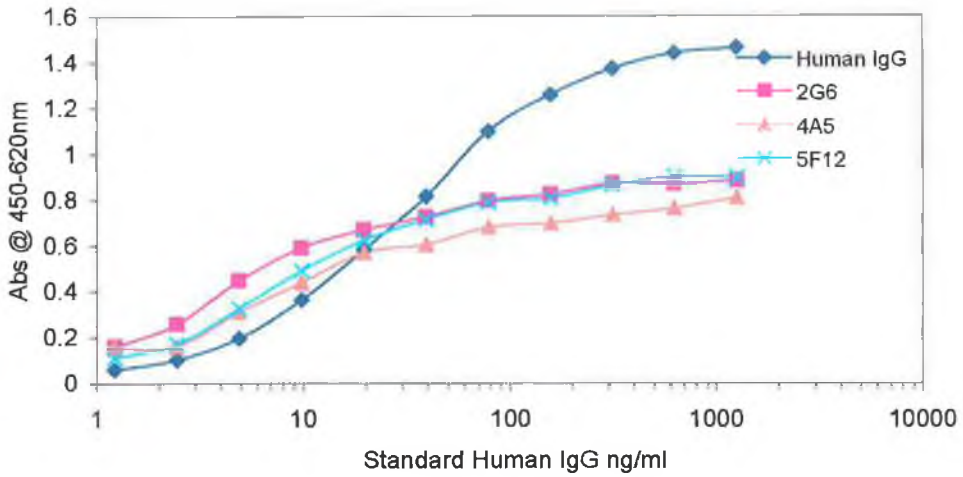


Figure 5.2.20: *The concentration of antibody was determined via capture ELISA as described by McGregor et al. (1994). The human Cκ tag was used to estimate scAb concentration. A standard curve of human IgG of known concentrations was plotted against absorbance observed. Doubling dilutions of each scAb from 1 in 10 to 1,280 were plotted also against absorbance on the same scale. Curves should exhibit a sigmoidal curve, with parallel slopes. ScAb concentration could be calculated from the slope of standard curve and could then be corrected for differences in molecular weight between whole IgG (150 kDa) and scAb (40 kDa). Panel A show the results from a capture ELISA performed in PBS and panel B shows capture ELISA results when performed in PBS, containing 10 μg/ml THC. The scAbs all appeared to form aggregate multimers away from the antigen-binding site as no linear portion of the sigmoidal curve was observed.*

Table 5.2.7: *Results of Bradford assay to determine total protein expression levels for each antibody clone. Varying dilutions of each purified antibody was compared against a known concentration of fully characterised scAb, clone CSBD9. Proteins were quantified using Coomassie reagent and absorbance read @ 562nm.*

Clone	Concentration of purified scAb	Original Culture Volume (ml)	Volume Purified scAb obtained	Total expression level per litre of culture
2G6	477 µg/ml	600	25ml	19.9 mg
4A5	280 µg/ml	600	4 ml	3.7 mg
5F12	1.4 mg/ml	600	5ml	12.0 mg

5.2.6 ELISA analysis of anti-THC scFvs

For the development of a competitive ELISA assay for the detection of THC, the optimal conjugate coating concentration of THC-BTG and optimal antibody dilutions were determined by indirect checkerboard ELISA for each antibody clone. Figure 5.2.21 show results obtained for clone 2G6. A conjugate coating concentration of 1.25 μg/ml THC-BTG along with a 1 in 1,000 (11.35 nM) scAb was chosen as optimal. This scAb dilution gave an absorbance of approximately 0.5, which is considered to be in the most sensitive, linear region of the curve. Figure 5.2.22 shows checkerboard analysis of clone 4A5. A conjugate coating concentration of 2.5 μg/ml and a scAb dilution of 1 in 100 (66.7 nM) were chosen as optimal. Figure 5.2.23 illustrates determination of optimal conditions for clone 5F12. These were chosen as 2.5 μg/ml THC-BTG coating and a 1 in 500 (68.58 nM) scAb dilution. The results highlighted the highly avid nature of clone 2G6 as this antibody could be used at the highest dilution factor, although this was not the most concentrated antibody.

Due to the hydrophobicity of THC, it was decided to optimise competitive ELISA conditions with varying percentages of ethanol. A competitive ELISA was performed using each scAb against varying concentrations of THC, prepared in increasing concentrations of ethanol. Results shown in Figures 5.2.24 to 5.2.26, illustrate ELISA analysis with each clone using THC, prepared in final ethanol percentages of 0% (v/v) to 4% (v/v). This involved making THC standards in ethanol concentrations of 8% (v/v) to 0% (v/v) and mixing them with scAb. The use of higher ethanol concentrations was also investigated, however the organic solvent was found to severely affect antibody activity. The percentage ethanol for use in competitive ELISA was determined to be 2% (v/v) for clone 2G6, 4% (v/v) for clone 4A5 and 0.5% (v/v) for clone 5F12. These parameters were employed in a competitive ELISA format to detect free THC. The optimised assay can be seen in Figure 5.2.27. Clone 2G6 exhibited the smoothest curve with the lowest limit of detection. All scAbs however, proved to be very problematic. The assay suffered from a lack of reproducibility with all soluble antibodies, in which detection limits varied by up to a factor of 10 in free THC concentrations detected, when repeated in an interday format. The standard smooth sigmoidal curve theoretically observed in competition ELISA

proved difficult to obtain. Instead, the standard curve was not robust, with points appearing to zig-zag up and down. This phenomenon was not observed when the antibodies were employed in a phage ELISA format. One possible explanation for this was that genetic mutations had occurred in the scFv genes during subcloning into pIMS 147. To eliminate this possibility all scFv genes were re-sequenced in pIMS 147 using the primers AH1 reverse and Human Cκ forward. All sequences confirmed that no mutations had occurred in the genetic sequence of antibodies. It was also decided to express antibodies in a soluble format directly from the original phagemid vector, pHEN 2. In this format, antibodies are expressed as scFvs, with a c-myc tag and without the human Cκ tail. An individual colony was grown overnight and used to express antibody simultaneously in phage and soluble formats. This would eliminate any aggregation or steric hindrance caused by the Cκ detection tag. Antibodies produced in this manner are also expressed at dramatically lower concentrations than with the pIMS 147 vector, therefore, eliminating any problems that are concentration dependent, such as antibody precipitation. Even at such low concentrations and without the large human Cκ tag, scAbs still failed to behave in the 'model' fashion when presented with free THC (data not shown). Unfortunately at this stage, the lab supply of free THC became exhausted and the suppliers withdrew the drug from the European market. For this reason, no further ELISA analysis could be carried out on these clones.

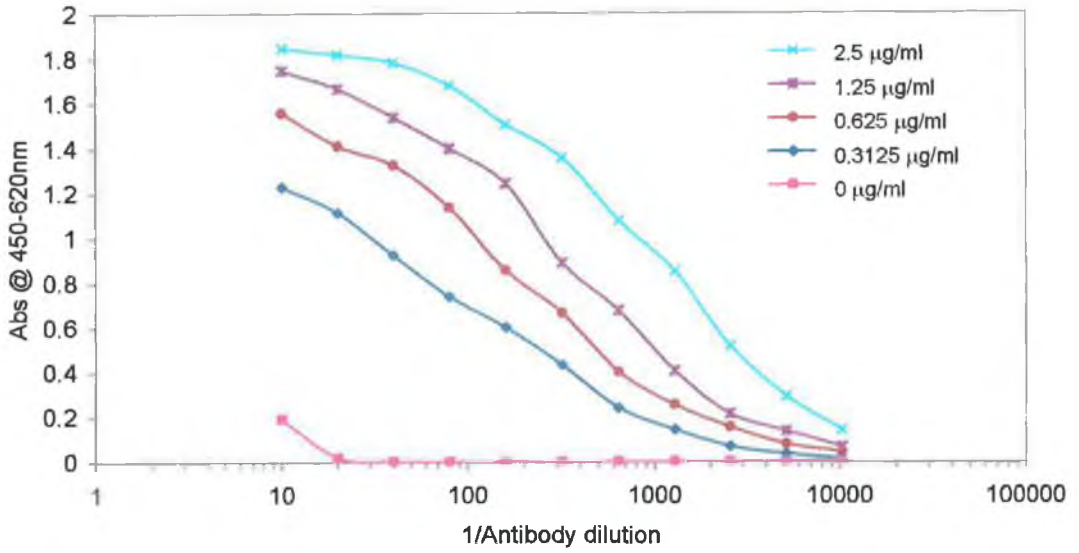


Figure 5.2.21: *Indirect checkerboard ELISA on scAb clone 2G6, for the determination of optimal conjugate coating concentration of THC-BTG, and optimal dilution of scAb for use in competition ELISA. THC-BTG was coated at 0, 0.3125, 0.625, 1.25 and 2.5 µg/ml and doubling dilutions of scAb from 1 in 10 to 1 in 10,000 were carried out.*

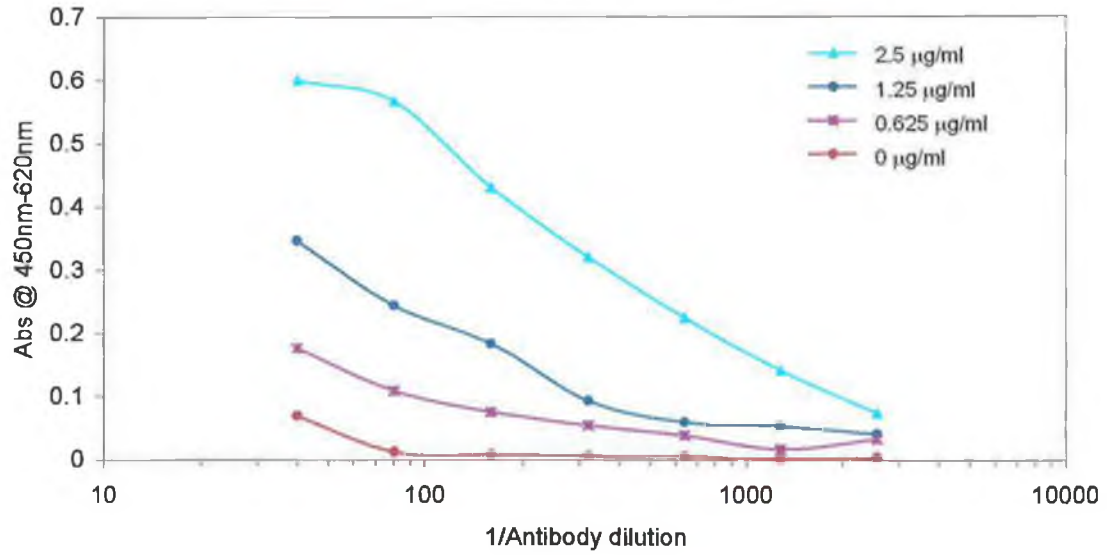


Figure 5.2.22: *Indirect checkerboard ELISA on scAb clone 4A5, for the determination of optimal conjugate coating concentration of THC-BTG, and optimal dilution of scAb for use in competition ELISA. THC-BTG was coated at 0, 0.625, 1.25 and 2.5 µg/ml and doubling dilutions of scAb from 1 in 10 to 1 in 2,500 were carried out.*

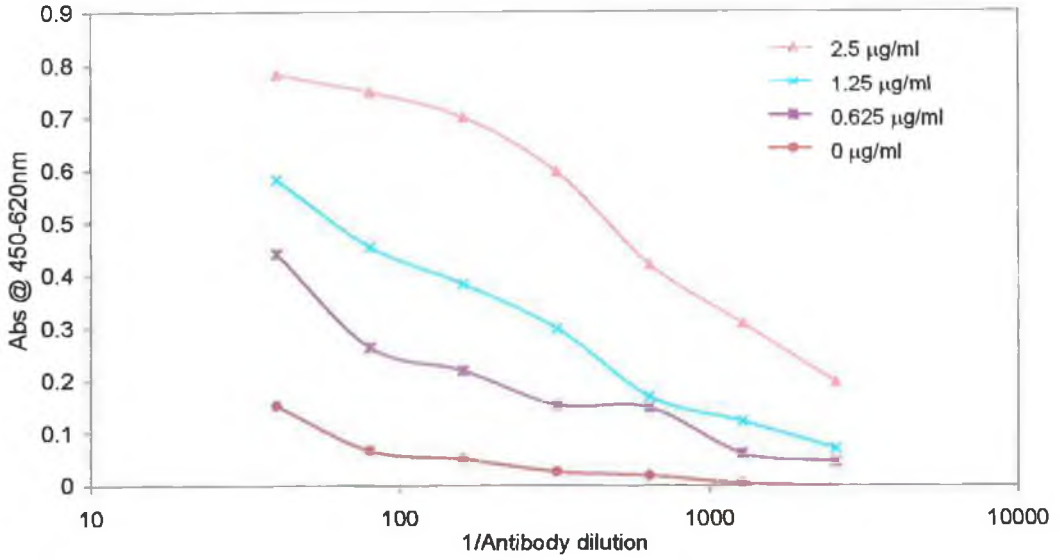


Figure 5.2.23: *Indirect checkerboard ELISA on scAb clone 5F12, for the determination of optimal conjugate coating concentration of THC-BTG, and optimal dilution of scAb for use in competition ELISA. THC-BTG was coated at 0, 0.625, 1.25 and 2.5 µg/ml and doubling dilutions of scAb from 1 in 10 to 1 in 2,500 were carried out.*

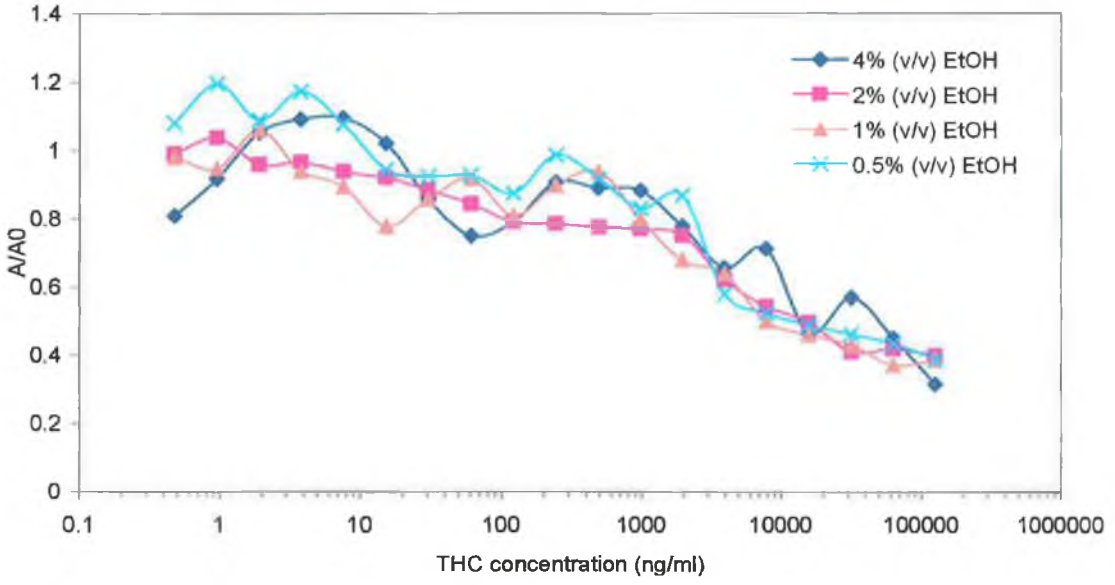


Figure 5.2.24: *Optimisation of ethanol concentration of THC standard for use in competitive ELISA with scab clone 2G6. A final concentration of 2% (v/v) ethanol was chosen as optimal as this gave the smoothest curve without affecting antibody activity.*

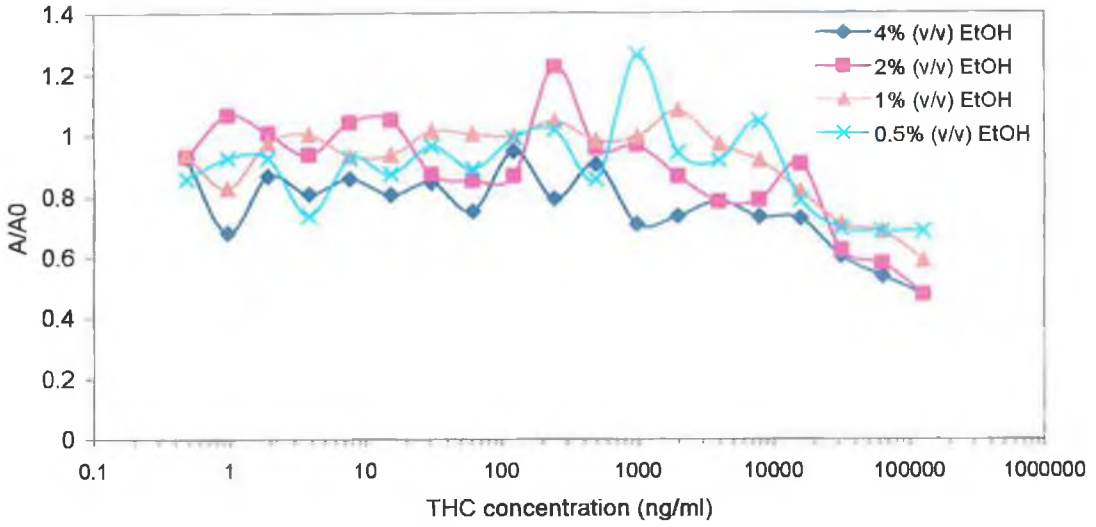


Figure 5.2.25: *Optimisation of ethanol concentration of THC standard for use in competitive ELISA with scAb clone 4A5. A final concentration of 4% (v/v) ethanol was chosen as optimal as this gave the smoothest curve without affecting antibody activity.*

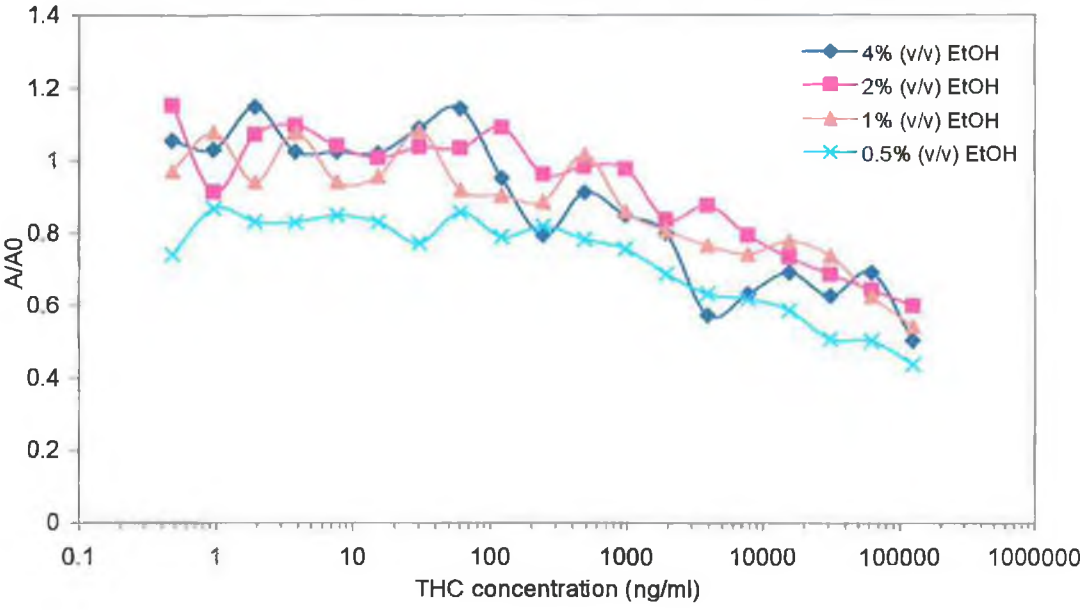


Figure 5.2.26: *Optimisation of ethanol concentration of THC standard for use in competitive ELISA with scAb clone 5F12. A final concentration of 0.5% (v/v) ethanol was chosen as optimal as this gave the smoothest curve without a ffecting antibody activity.*

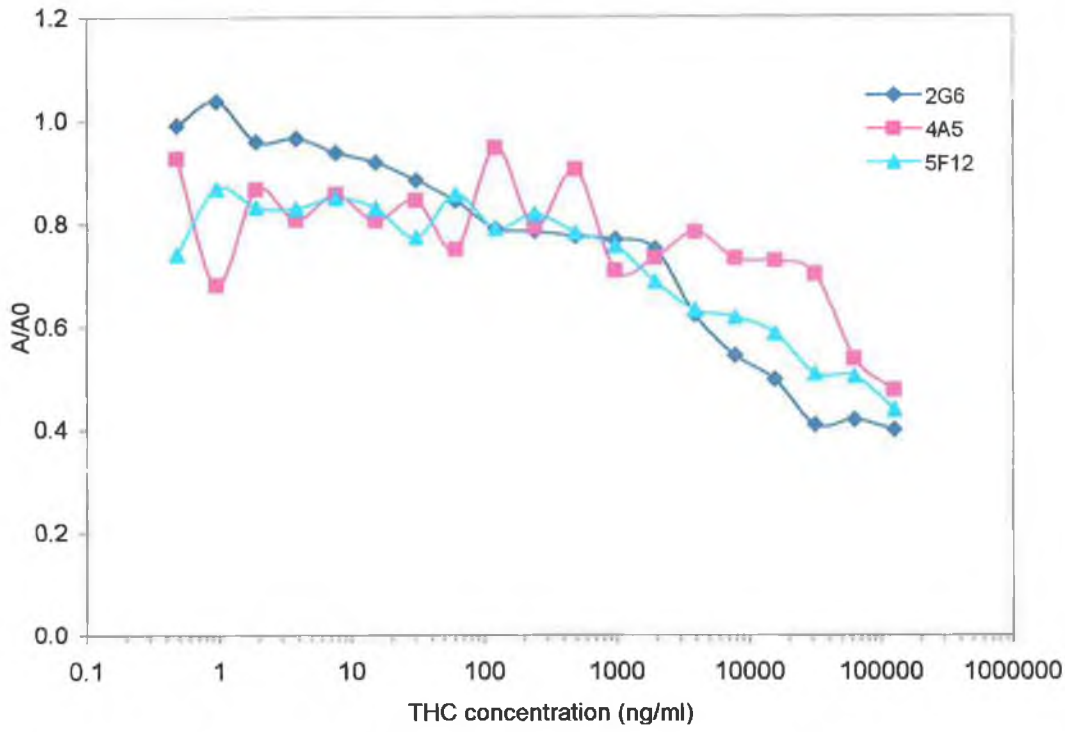


Figure 5.2.27: *Optimised competitive ELISA analysis using each scAb to detect free THC. THC concentrations from 0.1 to 200,000 ng/ml were analysed using optimised ELISA parameters detailed above. Assay curves deviated from the smooth sigmoidal curve expected from competitive antibody-antigen interactions. Clone 2G6, however, was deemed to be the most sensitive antibody.*

5.2.7 Determination of antibody aggregation by native gel electrophoresis

Native gel electrophoresis was carried out in order to access whether scAbs were spontaneously forming multimeric aggregates in solution. The occurrence of such multimers may explain the irreproducibility of ELISA results and a dramatic decrease in sensitivity between phage and soluble antibody formats. Native gel electrophoresis allows antibodies to be analysed in their native state as all denaturing agents such as SDS and mercaptoethanol are omitted from gel solutions. The omission of SDS from buffers, however, means that protein charge will also influence the rate of migration through the gel. For this reason a direct comparison between proteins and molecular weight markers cannot be made. The gel will however give a valuable insight into the native structure of the anti-THC scAbs when compared to a well-characterised positive control, clone CSBD9. All anti-THC antibodies appear to exist in a multimeric state, as can be seen in Figure 5.2.28. The molecular weights of the antibodies are more comparable to whole human IgG (150 kDa) than that of scAb (40 kDa).

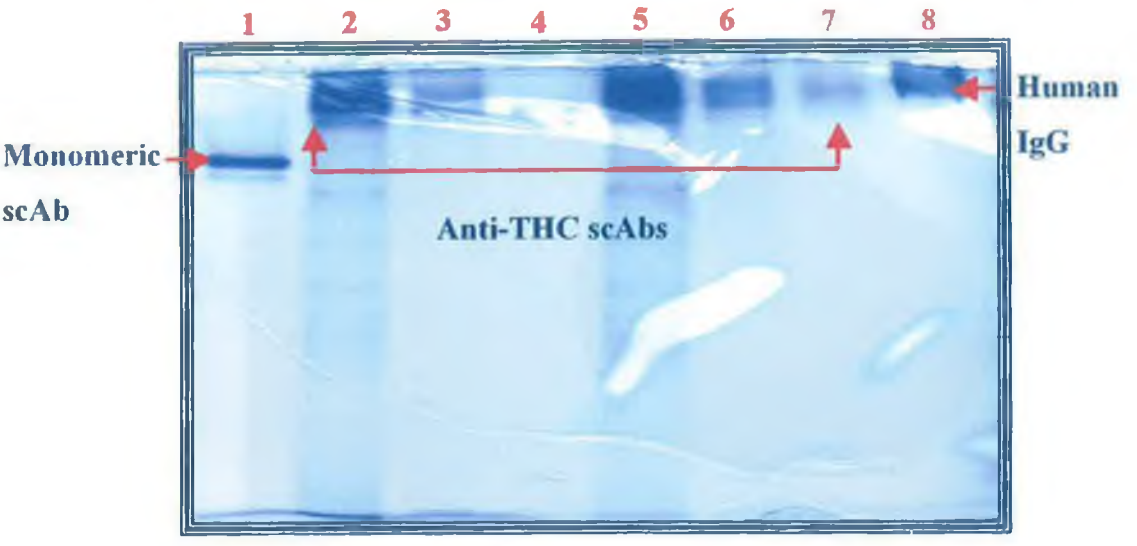


Figure 5.2.28: *Native gel electrophoresis on purified anti-THC scAbs to determine the degree of aggregation. Lane 1: Positive control scAb (clone CSBD9 against microcystin 40 kDa) at 300 µg/ml; Lane 2: Purified scAb clone 2G6, Neat; Lane 3: Purified scAb clone 2G6, 1 in 5 dilution; Lane 4: Purified scAb clone 4A5, Neat; Lane 5: Purified scAb clone 5F12, Neat; Lane 6: Purified scAb clone 5F12, 1 in 5 dilution; Lane 7: Purified scAb clone 5F12 at a 1 in 10 dilution; Lane 8: Standard human IgG (150 kDa) at 520 µg/ml.*

5.2.8 Re-evaluation of phage-displayed scFvs for the detection of THC in saliva

Several attempts were made to alleviate the aggregation problem of soluble antibodies by changing assay parameters, such as buffer pH. The problem appeared to be an intrinsic characteristic of the antibodies' protein sequence. It was decided to re-evaluate the use of phage-displayed scFvs as an analytical tool for the detection of THC in saliva. Clone 2G6, proved the most sensitive scFv in earlier analysis and was therefore chosen as a model for THC analysis in saliva. A pre-requisite for the development of a sensitive immunoassay is the preliminary optimisation of conjugate coating concentration and dilution of antibody, as previously discussed. This was carried out by means of a checkerboard ELISA (Figure 5.2.29). Concentrations of THC-BTG of 5, 2, 1 and 0 μg/ml were used to coat an ELISA plate. Dilutions of phage-displaying antibody from 1 in 8 to 1 in 2,560, diluted in 2% (w/v) MPBS, were added to the plate. Bound phage were detected using a peroxidase-labelled anti-M13 bacteriophage antibody and absorbance monitored at 450nm. Optimum conditions for the competitive ELISA were chosen to include a conjugate coating of 2 μg/ml THC-BTG, with an antibody dilution of 1 in 100. As earlier determined, an ethanol concentration of 2% (v/v), proved optimal for ELISA analysis of THC.

Using these parameters, a model ELISA assay was developed using clone 2G6 to detect THC in PBS. Figure 5.2.30, illustrates an intra-day assay (n=3) for THC, capable of detecting approximately 122 ng/ml THC in PBS. With one exception, all coefficients of variation lay between the acceptable limits of 20%. Figure 5.2.31, illustrates an inter-day assay carried out using phage-displayed clone 2G6 to detect THC in spiked-saliva samples. Saliva was obtained from THC negative donors and used to prepare varying dilutions of THC. The only sample preparation involved a brief (30 secs) centrifugation step, prior to analysis. The phage-displayed antibody was dilution in 2% (w/v) MPBS, containing 2% (v/v) ethanol. The assay proved that phage-displayed scFvs could be successfully used to detect THC in saliva. The assay had an increased limit of detection of approximately 500 ng/ml for quantitative analysis and 3.9 μg/ml for qualitative analysis, when compared to PBS. However, the assay demonstrated that phage-displayed antibodies could be successfully employed in analysis of 'real' biological samples, without extensive sample pre-treatment.

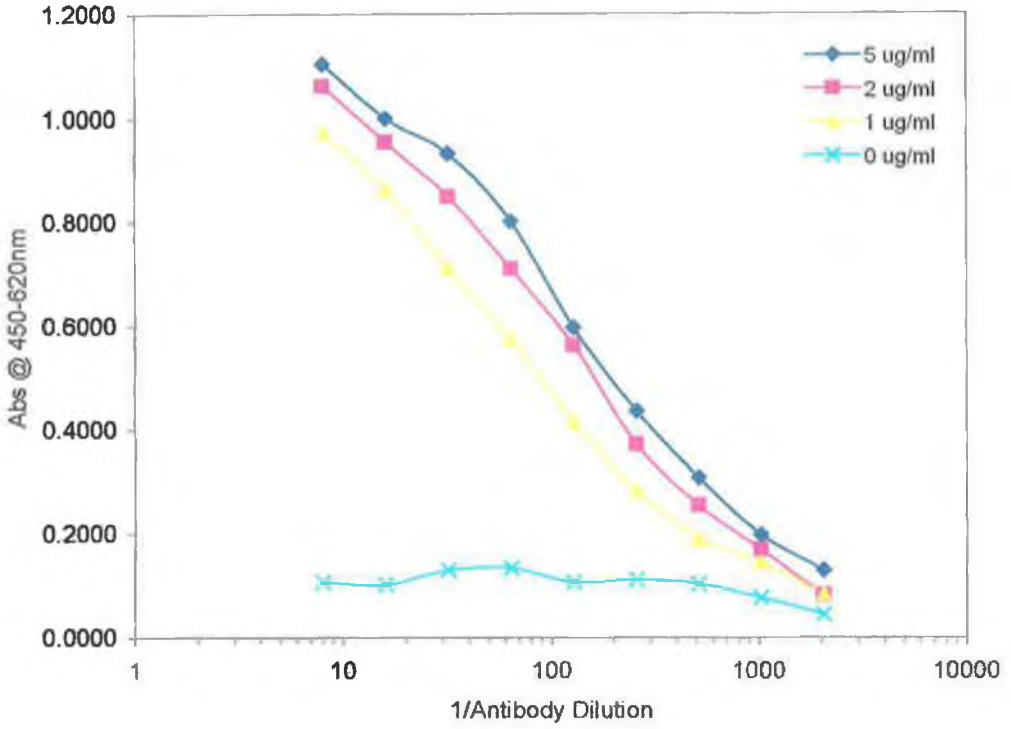
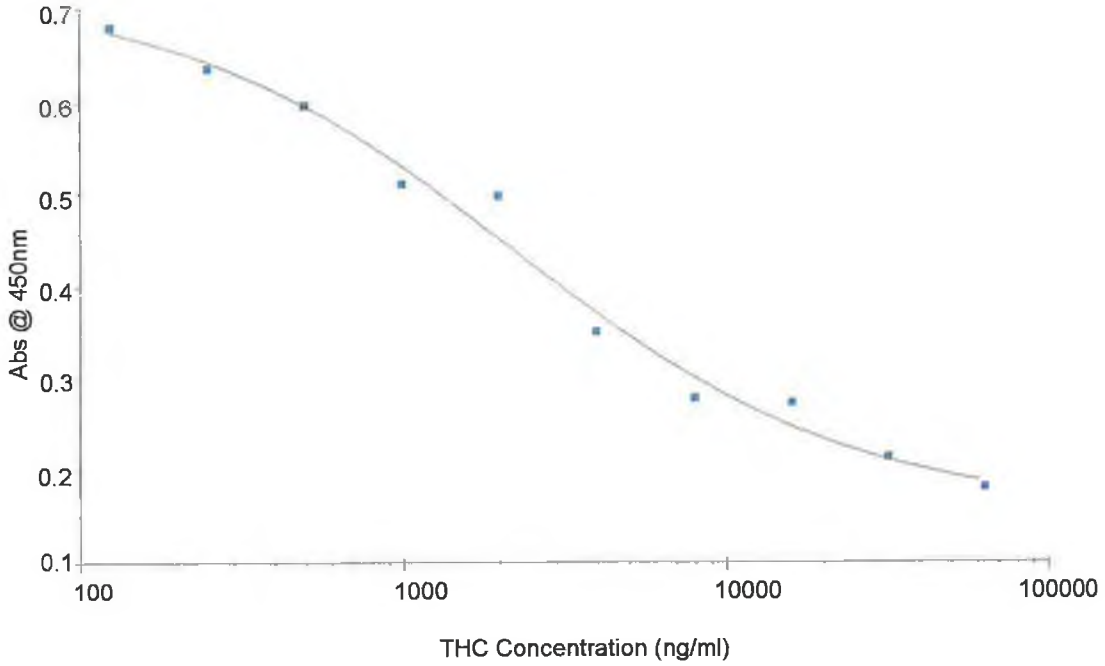
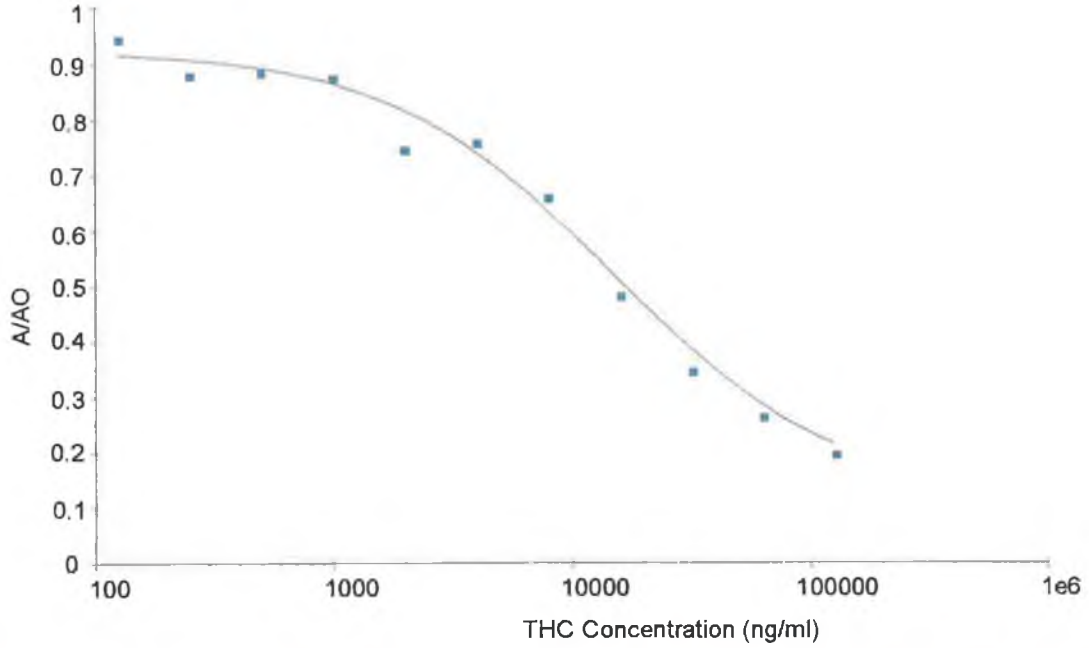


Figure 5.2.29: *Checkerboard ELISA to determine the optimal conjugate coating concentration of THC-BTG and optimal dilution of phage scFv for competitive ELISA. 2 μg/ml THC-BTG, with a 1 in 100 dilution of antibody-displayed phage were chosen as optimal.*



THC concentration (ng/ml)	Calculated mean Abs ± S.D.	Coefficient of variation (%)
62,500	0.181 ± 0.018	9.96%
31,250	0.215 ± 0.026	12.02%
15,625	0.273 ± 0.025	9.05%
7,812.5	0.278 ± 0.079	28.45%
3,906.25	0.350 ± 0.027	7.73%
1,953.13	0.497 ± 0.027	5.42%
976.56	0.544 ± 0.057	10.44%
488.28	0.595 ± 0.062	10.36%
244.14	0.636 ± 0.033	5.11%
122.07	0.681 ± 0.011	1.62%

Figure 5.2.30: *Intra-day assay using scFv, clone 2G6, displayed on the surface of phage, to detect THC in PBS. The results show an average of three sample replicates, carried out over one day.*



THC concentration (ng/ml)	Calculated mean A/A0 ± S.D.	Coefficient of variation (%)
125,000	0.148 ± 0.042	27.96%
62,500	0.261 ± 0.010	3.89%
31,250	0.351 ± 0.03	8.40%
15,625	0.470 ± 0.052	11.05%
7,812.5	0.718 ± 0.006	0.77%
3,906.25	0.782 ± 0.084	10.68%
1,953.13	0.782 ± 0.115	14.76%
976.56	0.891 ± 0.010	1.12%
488.28	0.921 ± 0.033	3.61%
244.14	0.891 ± 0.113	12.70%
122.07	0.971 ± 0.070	7.18%

Figure 5.2.31: *Inter-day assay for THC in spiked saliva samples, using scFv antibody derived from clone 2G6, expressed on the surface of phage. The inter-day assay, involved carrying out three sample replicates, over three separate days.*

5.3 Discussion

This chapter describes the production of genetically-derived scFvs against tetrahydrocannabinol. For this purpose a pre-immunised murine library was generated. A mouse was immunised with THC-BSA over a period of 12 weeks. Once a sufficient antibody titre (1 in 100,000) was obtained, the spleen was removed and RNA extracted. Variable heavy and light chain antibody genes were amplified by PCR, employing a mix of primers in a multiplex PCR. The primers detailed by Krebber *et al.* (1997), encompass all possible variations of murine heavy and light chain genes collected in the Kabat database (Kabat *et al.*, 1991), combined with an extended primer set described by Kettleborough *et al.* (1993). Heavy and light chain genes were annealed in the orientation V_L-$(Gly_4Ser)_4$-V_H, using a splice by overlap extension PCR. *Sfi 1* recognises the sequence GGCCNNNNNGGCC, where N can be any nucleotide. This recognition is introduced by the V_H for and scback primers and is very rare in antibody sequences. The phagemid vector pAK 100 has also been engineered to contain two *Sfi 1* sites, one complimentary to the light chain N nucleotides and one complimentary to the heavy chain N nucleotides. This allows directional cloning of the scFv gene in the correct orientation. The *Sfi 1* enzyme always cuts two sites at once, eliminating any intermediate digestion products, and generating two different palindromic ends with 3 bp overhangs, eliminating the possibility of self-ligation. The SOE product was digested with *Sfi 1* and ligated into the phagemid vector pAK 100.

This was transformed into XL-1 blue cells for phage production. Various methods of competent cell production and transformation were investigated, however, transformation efficiency proved poor, yielding a library of 3 x 10^4 cells. Commercially available supercompetant cells were also used but failed to produce a larger repertoire. It was hoped that the initial RNA would be highly specific to THC, eliminating the requirement for a large repertoire as the library was generated from pre-immunised spleen cells. Phage were rescued and enriched for positive binders by biopanning. Phage from each round were analysed by monoclonal phage ELISA. However, no positive binders were isolated, following four rounds of panning. At this stage it was decided to try alternative panning strategies. One such strategy involved

passive elution using male *E. coli* cells, followed by an alkaline shock as described by Wind *et al.*, (1997). Although a general enrichment was seen in phage towards THC-BTG in a polyclonal ELISA format, no positive monoclonal binders specific to THC were identified. Two reasons were identified as possibilities as to why the library failed to generate specific antibodies. The first possibility involved the type DNA polymerase used, as T*aq* polymerase does not have any proof reading ability. The quality of the primer set employed was also questionable. Primers synthesised by MWG were supplied at very low concentrations, which can be an indication of random single-base deletions. These would be amplified in the final scFv product, decreasing the functionality of the library.

At this stage, a natural human scFv repertoire was investigated as an alternative to the murine library created to circumvent some of the problems associated with the quality of molecular reagents available. This library described by Vaughan *et al.*, (1996) had a diversity of 1.2×10^{10} clones. The library was subjected to alternative biopanning strategy; rounds 2-4 were performed as before, alternating between BSA and BTG forms of conjugate. This should eliminate any binders to the carrier protein. In the fifth round of panning, phage recognising free THC were eluted using solutions with an increasing THC concentration. Higher affinity binders to the free antigen should be isolated with lower THC concentrations. After the fifth round, phage were analysed in a monoclonal format. Several positive binders were isolated and these were analysed in a competitive ELISA format, however, no recognition of free antigen was seen. A naïve human library represents a very uncontrolled diversity, limited in diversity by the IgM repertoire, which is frequently somatically mutated, the unknown history of B cell donors and the bias due to unequal expression of V-gene families (Hoogenboom, 1997).

The Griffin.1 Library is a semi-synthetic naïve human antibody library, constructed by recloning synthetic heavy and light chain variable regions from the lox library vector into the phagemid pHEN 2 vector (Griffiths *et al.*, 1994). The library was constructed *in vitro* using naturally occurring V genes as building blocks, with CDR 3 loops that have been partially randomised to mimic natural diversity generated through V-J gene recombination that occurs *in vivo*. The library represents a controlled and defined set of V-genes, representing antibodies, which may be

considered more natural than those, derived from natural IgM repertoires (Hoogenboom, 1997). The Griffin.1 library has been used successfully to isolate a variety of scFvs against haptens (Griffiths *et al.*, 1994; Strachan *et al.*, 2002; Brichta *et al.*, 2003). The library was subjected to five rounds of biopanning and monoclonal phage were analysed by monoclonal phage ELISA. Panning was performed by alternating between THC conjugates in the preliminary rounds, followed by free antigen elution, in latter rounds, to remove any interface binders. The phage population was enriched for specific THC binders as seen in Table 5.2.6. However, upon sequencing of positive binders, it could be seen that the same antibody sequences were being amplified and enriched by the panning strategy. This meant that multiple copies of each clone was present in the latter rounds of panning and, therefore, a higher percentage of phage population showed a positive binding interaction with both of the THC conjugates.

Sequence analysis of 7 positive THC revealed that 3 clones varied in protein sequence, especially in the critical CDR H3 region. Figures 5.2.16 and 5.2.17 illustrate homology modelling of clone 2G6, where the typical 'open conformation' structure observed in anti-hapten antibodies can be seen. A deep antigen-binding cavity, in which the hapten embeds itself, is clearly visible. This is formed by long CDR L1 and H3 loops, forming an antigen-binding pocket, increasing the antibody's surface area for antigen interaction. Three clones, 2G6, 4A5 and 5F12 were subcloned into the vector pIMS 147 for enhanced soluble expression as single chain Antibodies (scAb). The vector encodes the skp periplasmic chaperone gene, the human kappa constant light chain domain (Cκ) and a hexahistidine tag. The skp chaperone has been found to increase total and functional yield of antibody and alleviate protein aggregation in the bacterial periplasm (Strachan *et al.*, 1999, Hayhurst *et al.*, 2003). The human Cκ tail acts as an ideal antibody detection agent and the hexahistidine tag facilitates rapid purification via immobilised metal affinity chelate chromatography (IMAC). Single chain antibodies were expressed in *E. coli* XL-1 Blue cells under the control of the lac promoter. Soluble antibody was extracted from the periplasm of bacterial cells and purified by IMAC. Purity was accessed by SDS-PAGE analysis (Figure 5.2.19). Clone 2G6 appeared to behave anomalously when analysed by SDS-PAGE, yielding a protein band at a slightly lower apparent molecular weight than expected. This may be due to the very basic nature of the scAb (pI=8.99). Hames

(1990) has reported irregular migration of proteins in SDS-PAGE as a function of their inherent charge, which due to size or shape and conformation of the polypeptide, can contribute to overall charge. Antibody concentration could not be determined directly by capture ELISA (see Section 5.2.6) due to aggregation therefore, total protein concentration was used as an indication of antibody expression. Clone 2G6 was shown to be the best expressed of the three antibodies yielding nearly 19 mg of antibody per litre of culture. This also proved to be the most avid scAb, being used at a concentration of 11.35 nM in a competitive ELISA format. Clone 4A5 and 5F12 were both employed at higher concentrations, 66.7 nM and 68.58 nM, respectively. All scAbs were applied to a competitive ELISA format but results proved irreproducible.

Immunoassays developed for THC to date have been hindered by the highly lipophilic nature of THC. Immunoassays, by design, are carried out in aqueous media. In such a system, THC will adhere preferentially to glass or plastic in preference to dissolving in aqueous solution. Initially, an optimisation step was carried out to determine the ideal concentration of ethanol to be used in the preparation of standards. Competition ELISAs were carried out using these parameters and initially the limits of detection achieved were favourable. Upon reproducibility analysis however, it was noted that the assay lacked robustness. Ethanol concentrations were further increased until antibody activity was affected, but this failed to improve assay robustness. The scFv genes were re-sequenced to ensure that no mutations had been introduced during subcloning of the into the pIMS vector. Interference from the human Cκ tail (15 kDa), as reported by Hayhurst *et al.* (2003), was eliminated as a source of interference in the assay, by expressing the antibodies in a soluble format directly from the pHEN 2 vector. This vector encodes a detection label in the form of a c-terminal myc peptide, adjacent to a hexhistidine tag for purification. An individual clone of each antibody was used simultaneously to produce soluble and phage-displayed scFvs. Soluble antibody fragments produced in this manner were at significant lower concentrations than when expressed as scAb, with the aid of the skp chaperone. This eliminated any concentration dependant factors as the cause of irreproducibility. Antibodies expressed as a gene III fusion protein on the surface of phage behaved in a manner consistent with a 'model' competitive ELISA, displaying a reproducible sigmoidal shaped curve. Antibodies expressed as soluble fragments (scFv), however,

failed to exhibit any reproducible recognition of free drug. Hayhurst *et al.*, (2003), observed a decrease in sensitivity in phage antibodies when expressed in scAb format. They reasoned that the amplification in signal by the phage gVIII protein gave rise to a 100-fold increase in sensitivity. This may explain the decrease in assay sensitivity but fails to justify the irreproducibility associated with this assay for THC. As the problem persisted when antibodies were expressed in an scFv format, it suggested that antibodies were aggregating when expressed without the gene VIII protein. The formation of antibody aggregates was analysed by native polyacrylamide gel electrophoresis. Although a direct molecular weight determination cannot be made from this gel, it was apparent from that the scAbs were all forming aggregates, yielding a multimer, with molecular weight similar to that of a whole antibody. This increase in valency could significantly affect specificity of the scAbs. In the format of a competitive assay format, the amount of antibody detected is proportional to the amount scAb molecules remaining immobilised to THC-BTG surface. In contrast, dimeric scAbs contain two antigen binding sites, one available for binding to free antigen, while the other remains capable of binding THC-BTG conjugate. A dimeric scAb will therefore only be displaced by higher concentrations of competing antigen. This theory would explain the decrease in sensitivity observed between phage and soluble antibodies. A similar increase in scFv avidity for immobilised antigen has been seen between monmeric and dimeric scFvs (Brennan *et al.*, 2003). It appears that aggregation, in this case is spontaneous and varied in nature, giving rise to a heterogeneous mixture of multimers that displace at different rates in ELISA. This would explain the difficulty in achieving reproducible sensitivities. It was unclear why all antibodies, irrespective of protein sequence, spontaneously aggregated to form these multimers. Perhaps the nature of the primary protein sequence of scFvs required to bind such a lipophilic target molecule as THC, render them unsuitable for expression in a soluble format, without fusion to the gene VIII phage coat protein. To my knowledge this phenomenon has not previously been observed with the Griffin.1 library.

The possibility of using phage-displayed scFvs in a competitive ELISA for THC was re-evaluated. Assay conditions were optimised by means of an indirect checkerboard ELISA. A coating concentration of 2 μg/ml THC-BTG, with a 1 in 100 dilution of

phage was found to be optimal (Figure 5.2.29). A competitive immunoassay was carried out to detect THC in PBS. Figure 5.2.30, illustrates that the phage-displayed scFv can be used to specifically detect THC in PBS. All but one coefficient of variation was between the required 20% range for successful assay development. The phage-scFv was capable of detecting almost 100 ng/ml free THC. The possibility of applying this assay format to saliva sample analysis was also investigated. Saliva samples were obtained from THC negative donors. Samples were subjected to a brief centrifugation step, prior to analysis. Standards of varying THC concentration were prepared in saliva. Dilutions of phage were prepared in 2% (w/v) MPBS, % 2 (v/v) ethanol. The analysis was carried out on three separate days to calculate inter-day assay variability. Figure 5.2.31 illustrates that phage antibodies can be successfully applied to sample analysis in biological matrices, such as saliva. The phage could specifically detect THC, without suffering any significant effects from the complex, diverse nature of the sample matrix. The assay could quantitatively detect THC above a concentration of approximately 500 ng/ml in saliva. For qualitative analysis, a higher limit of detection than the LDD was chosen due to the high variability in the curve. The linear region of the curve from 3.9 μg/ml to 62.5 μg/ml proved suitable for qualitative. Within this region the coefficients of variation remain below 12%. Although %CV's below 10% are preferable, the acceptable levels for an optimised immunoassay should remain below 20% (Findlay *et al.*, 2000). This limit of detection is slightly higher than that reported with standard polyclonal antibodies for THC detection in saliva samples (Fanning, 2002). The relative change in absorbance observed per unit change in concentration is much greater for the phage assay, making it more sensitive over the dynamic range, i.e. slope of curve or $\Delta A/AO$ per Δ [THC]. Coefficients of variations for THC analysis are normally high, due to the amount of variability, associated analyte itself. In fact the C.V.'s reported here are below those reported for CAP FUDT (College of American Pathologists Forensic Urine Drug Testing) proficiency test surveys 1990-1991, which ranged from 14.2-37.1% (Joern, 1992). One specimen from the survey had a target value of 100 ng/ml, verified by quality control, but the average value determined by participants was found to be only 21 ng/ml. The successful development of an assay for THC using phage-displayed scFvs offers real potential in the search for a sensitive and specific antibody for THC analysis.

In summary, single chain antibodies directed against THC proved very difficult to generate. Initially, the natural immune response to foreign bodies was exploited to produce high affinity antibodies to THC. A recombinant antibody library was produced using RNA isolated from the spleen of a mouse immunised with THC-BSA. This however, failed to yield any antibodies specific to THC, either due to the unpredictability of the immune system when presented with obscure haptens or the quality of molecular biology reagents available. The natural diversity of a naïve human library created by Vaughan *et al.*, (1996) was also harnessed in pursuit of antibodies specific to THC. This led to the generation of antibodies specific for conjugated THC however, no antibodies specific to the free form of the molecule were identified. In a final effort to isolate recombinant antibody fragments to this problematic target antigen, the Griffin.1 library was subjected to 5 rounds of biopanning with various THC conjugates. The library was substantially enriched for antibodies to specific to free THC. Sequence analysis revealed that three clones isolated were genetically different. Antibodies were subcloned for expression in a scAb format. Protein expression yields proved high and the antibodies were successfully purified by IMAC. Upon assay development however, it was found that antibodies were spontaneously forming a heterogeneous population of multimers. The increase in avidity for immobilised conjugate led to a dramatic decrease in sensitivity for THC in solution. A variety of strategies were employed to alleviate the problem. However, antibodies continued to behave as aggregates and in a heterogeneous manner. It was concluded that a phage ELISA assay format proved the most suitable for THC detection. The ease of phage production and large amplification in reporter signal also make phage-displayed antibodies an ideal tool for analysis. Phage-displayed scFvs were used to develop a competitive immunoassay for THC. The antibodies could also be successfully applied to 'real-sample' analysis, in a biological matrix, such as saliva. To my knowledge, this is the first anti-THC single chain antibody, reported in the literature.

Chapter 6: Overall Conclusions

6.1 Overall Conclusions

The research discussed in this thesis describes the production of novel genetically-derived antibody fragments and their application in the detection of illicit drug residues in saliva.

Chapter 3 focused on the production and characterisation of three genetically derived scFv antibody fragments specific to M3G. The gene encoding a M3G-specific scFv was isolated and ligated into two expression vectors for the production of dimeric and alkaline phosphatase-labelled scFv. Optimal expression conditions for each of the scFvs were determined. Various purification strategies were investigated for each antibody. IMAC was found to be the most successful for large scale purification of monomeric and dimeric scFvs. The alkaline phosphatase-labelled scFv could not be successfully purified due to the poor quality of a commercially available anti-flag-sepharose resin. For applications where a high degree of purity is critical, affinity chromatography was found to be a superior alternative to IMAC for the purification of monomeric scFv. The three scFvs were applied to the development of competitive ELISAs for the detection of M3G. The optimised competitive ELISAs were extremely sensitive with the dimeric and alkaline phosphatase-labelled scFvs offering increased assay detection ranges. The monomeric and dimeric scFvs displayed minimal cross reactivity towards structurally related opiates. Although the alkaline phosphatase-labelled scFv offered decreased analysis time, it exhibited a high level of cross reactivity with 6-MAM, heroin and morphine.

Chapter 4 describes some possible applications of the recombinant scFvs produced. Affinity measurements of each antibody were determined using two different techniques, the classic ELISA-based method, described by Friguet *et al.* (1985), and a solution-phase determination using BIAcore. The equilibrium dissociation constants determined by the classic ELISA method were found to better reflect 'true' affinity, as the BIAcore system relied on active protein being equal to total protein concentration and global fitting parameters of the BIAevaluation models represented a completely monovalent binding interaction. The monomeric scFv was determined to have the best potential for analysis of morphine in saliva samples. A competitive

immunoassay for morphine, the main metabolite of heroin found in saliva, was successfully developed. The 'spiked' saliva samples were used as a reference calibration curve for the detection of morphine in the saliva of drug users. The monomeric scFv was then applied to the development of a BIAcore inhibition immunoassay for the detection of morphine in saliva. The inhibition assay offered improved sensitivity over the corresponding ELISA. A rapid colloidal carbon-based lateral flow immunoassay was also developed for the detection of M3G using monomeric and dimeric scFvs. This assay format proved to be extremely useful, with favourable detection limits, achieved in an assay time of a period of minutes. Detection limits for each assay were well below the 40 ng/ml cut-off level recommended by SAMHSA.

The production of good quality antibodies to the cannabis metabolite, tetrahydrocannabinol, has always proved problematic. Several different recombinant libraries were investigated for this purpose. A pre-immunised murine library was generated and two commercial libraries were panned in an effort to isolate antibody fragments specific to this analyte. Several phage-displayed antibody specific to THC was isolated from the Griffin.1 semi-synthetic human library. The gene encoding each of the antibodies was re-cloned into an enhanced expression vector for the production of soluble antibodies. Antibodies were purified by IMAC. However, upon further characterisation it was discovered that each of them was spontaneously forming a heterogeneous aggregate population. For this reason ELISA analysis suffered from irreproducibility. No solution to this problem could be found although several strategies were investigated. It appeared to be an intrinsic feature of the antibody sequences themselves. The application of phage-displayed antibodies to saliva sample analysis was then investigated. A competitive ELISA was developed using the most sensitive anti-THC scFv, expressed as a phage fusion protein. The assay range had a cut-off level of 250 ng/ml for quantitative analysis, which compares favourably with cut-off concentrations for commercial assay devices.

Several attempts were made to produce recombinant antibodies against amphetamine. The genes encoding an amphetamine specific-hybridoma were isolated and used to create a recombinant antibody library by the Krebber system of phage display (Krebber *et al.*, 1997). After several rounds of panning, no specific antibodies were

identified. The Griffin.1 library was also biopanned in an attempt to isolate anti-amphetamine antibodies. An amphetamine-BSA conjugate and biotinylated amphetamine, bound to streptavidin beads, were used alternatively for each round of panning. A high proportion of antibodies isolated simultaneously recognised amphetamine in conjugated and biotinylated forms. However, upon further analysis, none of the antibodies could recognise free amphetamine in solution. The small size of the amphetamine molecule (M.W. 135 Da) and simple molecular structure pushed the limitations of phage display beyond the scope of murine and human antibody libraries.

In summary, this work demonstrates the potential of recombinant antibodies for the detection of illicit drug residues. It highlights some of the main methods of recombinant antibody generation and derivatisation. This research also shows the applicability of recombinant antibody fragments to established immunoassay-based technologies.

Future work could entail the application of the recombinant antibody-based LFIA for the detection of morphine in saliva. This would facilitate 'on-site' monitoring for drugs of abuse in 'real sample' situations, within a matter of minutes. Affinity maturation strategies, like site-directed mutagenesis, for example, could be employed in an effort to express scFvs in a soluble form against THC. These antibodies could then be used in saliva sample analysis for illicit drug residues, either by ELISA, BIAcore or LFIA. Sheep antibody libraries have proved efficient for the production of recombinant antibodies to lower molecular weight haptens (Charlton *et. al.*, 2001). This technology could be investigated for the production of scFvs specific for amphetamine. The production of recombinant antibodies to cocaine would facilitate the development of an immunoassay system capable of detecting the major drugs of abuse. The utilisation of these antibodies in a multi-analyte detecting lateral flow immunoassay would allow sensitive, specific, rapid and simultaneous detection of all the major drug classes.

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Appendix 1A

Study on the analysis of saliva for drugs of abuse
Subject Information Sheet and Informed Consent Form

Collection of saliva

You are asked to take part in a study that will test your saliva for drugs of abuse. At your routine clinic visit, when you give a urine sample, you will also be asked to give a saliva sample. To collect the saliva you will chew on a piece of material like cotton wool given to you by a nurse. These are special saliva collecting devices and there are no known risks associated with using them. After a couple of minutes of chewing when the material has adsorbed the saliva, it will be place in a container and the saliva taken for testing

Confidentiality

Labelling the saliva sample with your initials and a number ensures your confidentiality. Your name will not appear on the container. The results will be compared to those of your urine test. Your name will not appear on any associated paperwork or publications.

AGREEMENT TO CONSENT

The research project has been fully explained to me. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that my decision not to take participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of record concerning my involvement in this project will be maintained in an appropriate manner. When required the records of this research will be reviewed by the sponsors of the research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the National Drug Treatment Centre. I have received a copy of

this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor at the clinic.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

_____

Client's name (Block capitals)

_____ _____

Client's signature **Date (to be dated by the patient)**

_____

Investigator name (Block capitals)

_____ _____

Investigators signature **Date (to be dated by the investigator)**

If applicable:

_____

Signature of Parent or Guardian

_____

Date

Appendix 1B

Glossary of terms and definitions commonly employed in Bioanalytical validation procedures

The terms listed below were referred to for validation purposes of certain procedures carried out in this thesis. The criteria under which they are defined defined under have been reviewed elsewhere (Hennion and Barcelo 1998; Dankwardt, 2000 and Findlay *et al.*, 2000).

Accuracy

Describes the aggreement between a measured test result and its theoretical true value.

Calibration curve

The dose response relationship between analyte concentration of samples and the binding response observed. The typical dose response is a sigmoidal curve.

Coefficient of Variation (% CV)

A quantitative measurement of precision expressed relative to the mean result. Coefficients of variation should be between 10 and 20% for an optimised assay (Dankwardt, 2000).

% CV = [Standard deviation/mean] x 100

Cross Reactivity

The degree to which an antibody recognises structurally similar compounds.

IC_{50}

The concentration of analyte that causes 50% binding inhibition.

IC_{90}

The concentration of analyte that causes 10% binding inhibition.

Least Detectable Dose (LDD)

The smallest concentration of analyte that produces a signal, which can be significantly distinguished from zero for a given sample matrix with a degree of confidence. Usually a dose that inhibits 10% of total antibody binding is selected, or 90% A/A0 (Hennion and Barcelo, 1998 and Dankwardt, 2000).

Mean

The average of replicates measurements (i.e. $n_1+n_2+n_3+.....n_x/x$), where n is value recorded for one particular measurement and x is the total numer of measurements made

Matrix Effects

The degree to which compounds present in a sample can interfere in the assay

Nominal Concentration

A stated or theoretical concentration that may or may not differ from the true concentration

Normalised Absorbance Values

Absorbance recorded at a particular antigen concentration (A) divided by absorbance observed in the absence of antigen (A0).

$$\text{Normalised Absorbance (A/A0)} = \frac{\text{Absorbance measured at analyte concentration}_x}{\text{Absorbance measured in the absence of analyte}}$$

Normalised Response Units

Response Units observed at a particular analyte concentration divided by response units observed in the absence of analyte.

$$\text{Normalised Response (R/R0)} = \frac{\text{Response at analyte concentration}_x}{\text{Response in the presence of zero analyte}}$$

Precision

A quantitative measure used to define the extent to which replicate analyses of a sample agree with each other.

Recovery

A quantitative measure of the closeness of an observed result to its theoretical value, expressed as a percentage of the theoretical concentration.

% Recovery = (Observed/Nominal) x 100

Repeatability

Precision of repeated measurements within the same analytical run. Repeatability is also termed intra-assay precision

Reproducibility

Precision of repeated measurements made in different assay runs. This can also be termed inter-assay precision.

Robustness

The precision is assay measurements carried out under different conditions by different operators.