Rapid methods for the detection of anti-parasitic drugs

A thesis submitted for the degree of Ph.D.
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Declaration

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Dedicated to My Family

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First and foremost I would like to extend a sincere thank you to Prof. Richard O'Kennedy for his supervision, advice and endless patience. I would also like to thank him for the IBO opportunities which brought me to two amazing countries I would never have visited on a post-grad wage!

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To the girls; Wen, Nat and Lu for thinking I made cow antibodies for 4 years! Thanks girls for everything over the years -pre and post PhD.

To Mum, Dad and Leanne, thank you for being my family and putting up with me! Thank you for the trips away, the dinners and the endless care packages. I am so lucky and grateful to have such a wonderful and caring family who showed me such love and support even if you didn't really know what you were supporting! And finally, to Shane, I am perpetually grateful for your words of encouragement, positive outlook, and for making me laugh throughout this very long journey, I wouldn't have made it without you! You have shared with me the highs and the lows while showing me nothing but love and support for which I am forever indebted to you.

Abbreviations

ABZ	Albendazole
ADI	Average Daily Intake
AP	Alkaline Phosphatase
APS	Ammonium Per Sulfate
BW	Body Weight
BSA	Bovine Serum Albumin
BTG	Bovine Thyroglobulin
BZD	Benzimidazole
BZT	Benzimidazole carbamates
CAM	Cambendazole
CHO	Chinese Hamster Ovary
CCα	Decision Limit
CCβ	Detection Capability
CM5	Carboxymethylated Dextran
DMSO	Di-methyl sulphoxide
DNA	Deoxyribonucleic acid
dSPE	Dispersive Solid Phase Extraction
EDC	1-Ethyl-3-(3-dimethylaminopropyl)Carbodiimide
ELISA	Enzyme-Linked Immuno Sorbent Assay
EMEA	European Agency for the Evaluation of Medicinal Products
EtOH	Ethanol
EU	European Union
Fab	Antibody Binding Fragment
FBZ	Fenbendazole
GC	Gas Chromatography
GFP	Green Fluorescent Protein
GI	Gastro Intestinal
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
L_1	Larval stage one of the parasitic life- cycle of a nematode
L_2	Larval stage two of the parasitic life- cycle of a nematode
L_3	Larval stage three of the parasitic life- cycle of a nematode
L_4	Larval stage four of the parasitic life-cycle of a nematode
LC	Liquid Chromatography
LFIA	Lateral Flow Immunoassay
LLOQ	Lower Limit of Quantification

LOB	Limit of Blank
LOD	Limit of Detection
mAb	Monoclonal Antibody
MeOH	Methanol
MBZ	Mebendazole
MPO	Myloperoxidase
MRL	Maximum Residue Limits
MS	Mass Spectrophotometry
NHS	N-hydroxy succinimide
NOEL	No Observable Effects Level
NTB	Netobimin
O.D	Optical Density
pAb	Polyclonal Antibody
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween
PCR	Polymerase Chain Reaction
PSA	Primary Amine Sorbent
QuEChERs	Quick, Easy, Cheap, Effective, Rugged and Safe
mRNA	Messenger Ribonucleic acid
R.T.	Room Temperature
RU	Response Units
SB	Super Broth
SHE	Syrian Hamster Embryo
scFv	Short Chain Variable Fragment
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis
SOC	Super Optimal Catabolite
SOE	Splice by Overlap Extension Polymerase Chain Reaction
TB	Terrific Broth
TBZ	Thiabendazole
TCB	Triclabendazole
TCB-SO	Triclabendazole Sulphate
TCB-SO_2	Triclabendazole Sulphone
TCEP	Tris [2-carboxyethyl]phosphine hydrochloride
TEMED	Tetramethylethylenediamine
TLC	Thin Layer Chromatography
TMB	3, 3´, 5, 5'-tetra-methylbenzidine
UV	Ultra Violet

V_H	Variable heavy chain of an antibody
V_L	Variable light chain of an antibody
VME	Veterinary Medicines Evaluation
VMP	Veterinary Medicinal Products

Units

µg	Micro-gram
µL	Micro-litre
g	Grams
(k) Da	(Kilo) Dalton
L	Litre
M	Molar
mg	Milli-gram
mL	Milli-litre
mM	Milli-molar
ng	Nano-gram
nm	Nano-meter
^{o}C	Degree Celsius
ppb	Parts per billion
RPM	Revolutions per minute
U	Unit
V	Volt
v/v	Volume per volume
w/v	Weight per volume
pg	Pico-gram
x g	x gravity

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Academic Outputs

Book chapters:

Darcy, E., Leonard, P., Fitzgerald, J., O'Kennedy, R., (2010). Purification of antibodies using affinity chromatography. *Protein Chromatography*, chapter in the *Methods in Molecular Biology* Book series, Cold Spring Harbour Press, Cold Spring Harbour, New York.

Fitzgerald, J., Leonard, P**., Darcy, E.,** and O'Kennedy, R., (2010). Immunoaffinity chromatography. *Protein Chromatography*, chapter in the *Methods in Molecular Biology* Book series. Cold Spring Harbour Press, Cold Spring Harbour, New York**.**

Publications:

Darcy, E., Keegan, J., Fitzgerald, J., O'Kennedy, R., (2010). Generation of a recombinant Fab antibody and assay development for the detection of thiabendazole. For submission to *Talanta* in June 2012.

Fitzgerald, J., Leonard, P., **Darcy, E.,** Danaher, M., O'Kennedy, R. (2011) Light Chain Antibody Shuffling from an Immune Sensitised Phage Pool allows 185-fold Improvement of an Anti-Halofuginone ScFv, *Analytical Biochemistry* ,410 (1), 27-33.

Presentations:

Darcy, E., Sheehan, F., Leonard, P., Keegan, J., Danaher, M., Brandon, D., and O' Kennedy, R. Generation of recombinant antibodies for monitoring small drug residues in food. Oral presentation at IXth International Conference on Agri-Food Antibodies (ICAFA), Oslo, Norway, September 9th, 2007.

Posters:

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Abstract:

The benzimidazoles (BZD), thiabendazole (TBZ), triclabendazole (TCB) and albendazole (ABZ), are veterinary agents employed for the eradication of parasitic infections in ruminant animals. Treatment with these drugs commonly results in trace residues remaining in the food product, thus requiring the development of tests capable of detecting below maximum acceptable residue levels (MRLs) as determined by European legislation. This research describes the development of novel, rapid, antibody-based methods for benzimidazole residue detection below the required MRL.

A recombinant antibody fragment (Fab) generated from a hybridoma secreting an anti-TBZ monoclonal antibody was utilised for the development of an ELISA for TBZ with a limit of detection (LOD) of 2.5 ng/mL. Thee sensitivity of this Fab was improved two fold by generation of a modified plasmid vector which converts the Fab from a mono-valent to a bi-valent antibody.

An avian polyclonal antibody (pAb) was raised against TCB and ABZ. This antibody was incorporated into competitive ELISA and Biacore-based assay with LODs for TCB in spiked pasteurised organic milk samples of 609 and 203 pg/mL, respectively. A short chain fragment (scFv) antibody library of 5.9×10^{11} was also constructed, bio-panned and screened for clones specific for TCB and ABZ.

Finally, the anti-TCB IgY was incorporated into a lateral flow immunoassay (LFIA) and a novel TCB-green fluorescent protein (GFP) LFIA assay with a detection threshold of 75-100 ng/ml of TCB in milk, which is at or below the required MRL.

Chapter one

Introduction

1.0 Introduction

The main aim of this work was the production of recombinant antibodies to the benzimidazole (BZD) family of veterinary drugs for their detection in different matrices such as liver, milk and fat. This introduction discusses the relevant aspects of the parasites that the drugs target, and their life-cycles. It also reviews the history of BZD's, their structures, current detection methods of the target residues, public health concerns and EU legislation. In addition, antibody production and phage display techniques are discussed.

1.1 Parasites

There are many definitions in the literature that describe a parasite. Chappell (Chappell, 1980) refers to parasites as protozoans, platyhelminths and nematodes that inhabit, at some point in their life-cycle, the body of a larger animal. Wilson (Wilson, 1979) suggested that parasitism is just one, among many types of associations, between two organisms and there is no single feature which can be used to label an animal undoubtedly a parasite. He states that nutritionally a parasite obtains food at the expense of its host by consuming either host tissues and fluids, or the contents of the host intestine. According to this definition the parasite may be recognised as a foreign invader causing the host harm and to which it can generate an immune response. There are three classes of parasitic worms that infect ruminants examples of these are shown in figure 1.1:

(1) Cestodes: The cestodes or tapeworms are flat worms chiefly of the alimentary tract of vertebrates. They have a complete lack of digestive tract in all stages of development (Chandler, 1961). They have a complex life-cycle with one or more intermediate hosts and almost invariably gain entry to the vertebrate final host via its food (Wilson, 1979). The cestode family include the orders *Tetraphyllidea, Trypanorhyncha, Proteocephal, Pseudophyllidea* and *Cyclophyllidea.* The latter two orders contain species that infect man and ruminants.

(2) Nematodes: The nematodes, more commonly known as parasitic roundworms are found in marine, fresh water and terrestrial habitats in addition to parasitizing plants and animals. The classification of nematodes is complicated as each of the worms has a remarkable similarity in body plan making precise identification difficult, particularly at larval stages. Several groups of nematodes have evolved and are important from a veterinary view point.

Such groups include the orders *Strongylida (Trichostrongyles), Ascardia, Spirurida and Trichenellida* (Wilson, 1979).

(3) Trematodes: The trematode species are more commonly referred to as flukes. The flukes are a class within the phylum *Plathyhelminthes* that contain two main groups monogenetic and diagenetic. The former have no asexual generations and are primarily external or semi external parasites of aquatic animals. The latter have two or more asexual generations and an alternation of hosts which can be internal parasites of all kinds of vertebrae (Chandler and Read, 1969). The sub-class, *Dignea,* which affects ruminant animals, include the orders *Strigeatoidea, Echinostomida, Reicolida, Opisthorchiida* and *Plagiorchiida.*

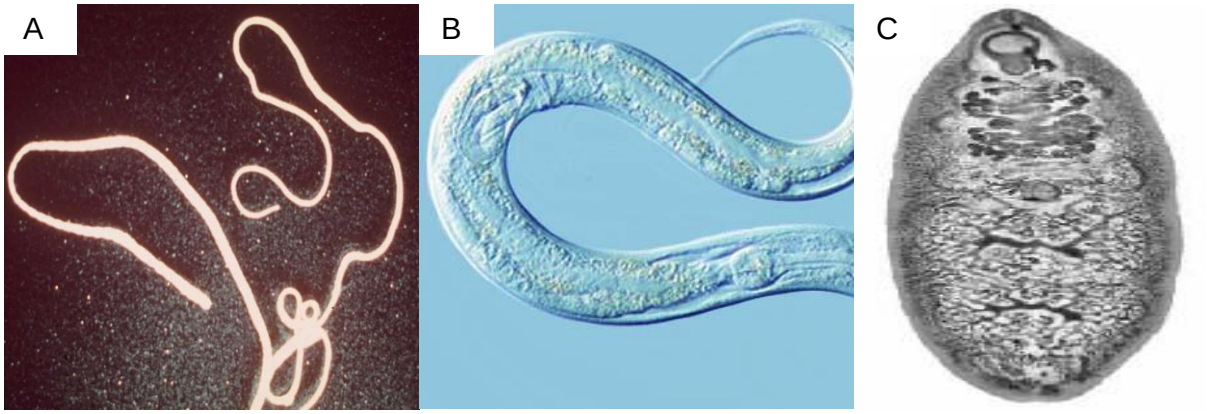


Figure 1.1: Examples of the three classes of parasitic worms; (1) cestodes (flatworm); (2) nematodes (round worm); and (3) trematodes (fluke).

1.2 Parasitic life-cycles

To understand the parasites and to fully comprehend the benzimidazoles mode of action for their eradication, an in-depth look at their life-cycles is required.

1.2.1 Direct life-cycle

A direct life-cycle is one that requires only one host for completion. One of the most important and widely prevalent groups of nematodes that undergo this type of cycle is the *Trichostrongyle* group. This group includes *Haemonchus, Ostertagia, Trichostrongylus, Mecistocirrus, Cooperia* and *Nematodirus* and the groups *Oesophagostomum* and *Bunostomum.*

The adult nematodes inhabit the gastro-intestinal tract of the ruminant animal. The eggs, produced by the female, are passed out in the faeces where they embryonate and hatch into first-stage larvae (L_1). These in turn moult into second-stage larvae (L_2) by shedding their protective cuticle. The L_2 larvae then moult into a third-stage larvae (L_3), but still retain the cuticle from the previous moult. These double-cuticled L_3 are the infective-stage larvae. These L_3 larvae migrate into herbage, are ingested by grazing ruminants, and pass into the abomasums or intestine. The cuticle of the larvae is shed during this infection process (Parkins and Holmes, 1989)

In the Trichostrongyle group, L_3 penetrates the mucous membrane (in the case of *Haemonchus* and *Trichostrongylus)* or enter the gastric glands *(Ostertagia).* After a number of days the L_3 moult and develop into larval stage four (L_4) which remains in the mucous membrane (or in the gastric glands) for about ten to fourteen days. They then emerge and moult into a young adult stage (L_5). The majority of *Trichostrongyles* mature and start egg production about three weeks after infection (Parkins and Holmes, 1989).

The parasitic part of the life-cycle of the *Oesophagostomum* class requires approximately 6 weeks for completion. The infective L_3 penetrate the intestinal wall, and the host responds by surrounding the L_3 in fibrous nodules. After two weeks the larvae move into the lumen of the intestine where they mature for a further four weeks.

The L_3 larvae of *Bunostomum* can infect ruminants by ingestion or penetration of the host's skin. Following skin penetration, the larvae are carried in the venous blood through the heart to the lungs, where they penetrate the alveoli, are coughed up and then swallowed, and thus pass to the small intestine. Here they moult and mature eight-nine weeks after infection causing damage to the host (Olsen, 1986).

1.2.2 Indirect cycle

Most parasites have a direct life-cycle. However, the flukes or trematodes undergo an indirect life-cycle with two distinct stages within two different hosts. The adult fluke lays its eggs in the liver of the host ruminant. The eggs pass through the bile duct into the intestine where they are excreted in the faeces. While on the ground the egg develops further and releases a miracidium which invades a suitable snail intermediate host within twenty four hours. The miracidium continues its development from sporocysts to rediae to cercariae in the snail host. The resulting cercariae are released from the snail as metacercariae where they can survive

for approximately 10 months on aquatic vegetation or other surfaces. Mammals acquire the infection by eating the vegetation. After ingestion, the metacercariae encyst in the duodenum and migrate through the intestinal wall, the peritoneal cavity and the liver parenchyma into the biliary ducts, where they develop into adults. The migration causes severe damage to the liver, particularly when large numbers of fluke are involved. It takes ten to twelve weeks from time of ingestion to maturation of the flukes (Zajac, 2006)

1.3 Economic impacts of parasitic infections

Infections caused by the parasites described in section 1.2 play a huge role in farming production losses as they affect feed ingestion and digestibility. These infections alter the value of animals and their products at slaughter as they reduce their weight gain, yield and quality of products. Some infections may also affect the animal's capacity for work, decrease the production of dung for fuel and fertiliser and more serious infections may cause premature death (Perry, and Randolph, 1999). The environment also plays a key role in supporting development stages of larvae and must be therefore considered in the eradication of parasites. However, the complexity of the environmental influences, severely reduce the prospect of complete obliteration. Hence, the use of anthelmintics or anti-parasitic drugs such as the benzimidazoles (BZD) is essential for, if not the eradication, but the control of these disease-causing parasites.

1.4 Benzimidazole (BZD) structure

The BZD structure is a bi-cyclic ring system in which benzene is fused to the 4 and 5 positions of the heterocyclic (imidazole) ring (Figure 1.4.1). BZD compounds are white crystalline powders, with high melting points and are insoluble, or minimally soluble, in water (Lanusse and Prichard, 1993). The synthetic pathways of most BZDs proceed through two steps. The first step is the formation of a benzene ring containing the desired substituent's and a 1,2-diamine grouping, followed by a closure of the 1,2- diaminobenzene derivative to construct the imidazole ring. Different modifications at the 2 and 5 position of the BZD ring have provided a range of anthelminthics that exhibit high efficacy against parasitic worms. For example, the sulphur-containing derivatives of BZD, such as albendazole (ABZ) and fenbendazole (FBZ) possess a high efficacy against lungworms and inhibit larval stages of most GI nematodes (Townsend and Wise, 1990).

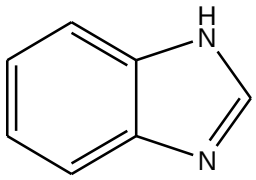
The preparation of ABZ (Fig 1.4.1) is a five step process, where the required propylthio group is attached to the benzene ring prior to the closure of the structure (Lanusse and

Prichard, 1993). A study carried out by (Lacey, 1990) showed that a carbamate substitution at the 2 position on the benzene ring was crucial for inhibition of microtubule formation in parasites. This work also suggested that both the chemical substitution at the 5 position and its conformational arrangement are relevant for access of the drugs to the active site and anti-parasitic activity.

Since their discovery, the BZD's have proven to be the least toxic of anthelmintic drugs, a major factor in their worldwide use over the last fifty years (Lanusse and Prichard, 1993). BZD's were originally introduced for the control of GI infections in livestock, however, they were later used for the treatment of GI infections in domestic animals. They offer many advantages in relation to spectrum (broad), efficacy against mature and immature stages of fluke, safety for the host and ovicidal activity. However, BZD's have a disadvantage as they have poor GI absorption and low water solubility therefore reducing their flexibilty for drug formulation in some drug preparations (Alvarez *et al.*, 2009). There are a number of BZD's available in the veterinary market today. These can be classified into four groups based on their structure and addition of methyl and thiol groups:

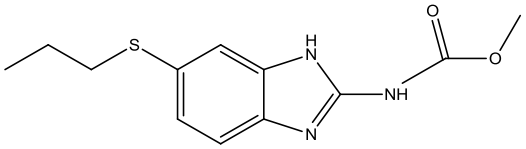
1. Benzimidazole thiazolyls including thiabendazole (TBZ) (Fig 1.4.1)
2. Benzimidazole methylcarbamates including mebenazole (MBZ), albendzole (ABZ) (Fig 1.4.1), and Fenbendazole (FBZ).
3. Halogenated benzimidazole thiols such as triclabendazole (TCB) (Fig 1.4.1).
4. Pro-benzimidazoles including fenbetal (FBT) and netobitim (NTB).

(A)

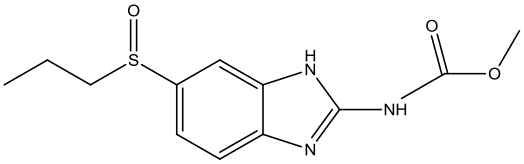


Benzimidazole*:* General structural form.

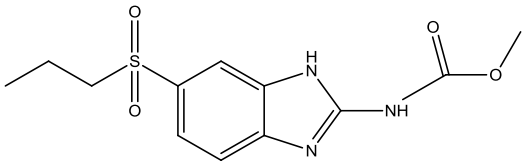
(B)



Albendazole: Methyl -[6-(propylthio)-1*H*-benzoimidazol-2-yl]carbamate

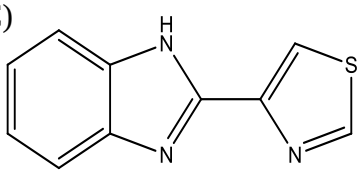


Albendazole Sulphone

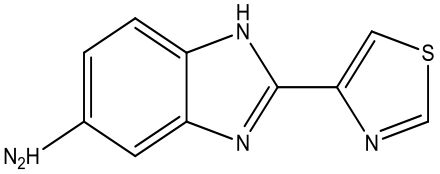


Albendazole Sulphoxide

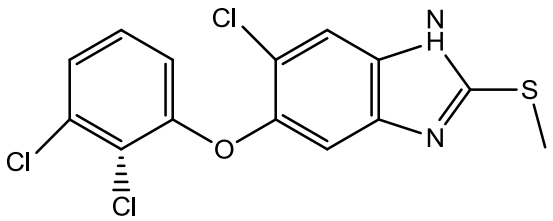
(C)



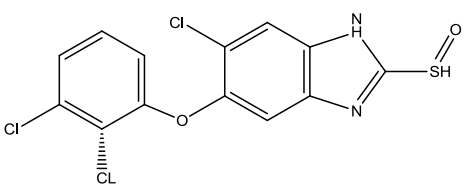
Thiabendazole: 4-(1*H*-1,3-benzodiazol-2-yl)-1,3-thiazole**.**



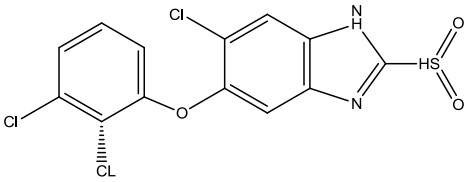
(D)



Triclabendazole: 5-chloro-6-(2,3-dichlorophenoxy)-2-(methylthio)-1-benzimidazole.



Triclabendazole Sulphone



Triclabendazole Sulphoxide

Figure 1.4.1: Chemical structures of benzimidazoles and their metabolites targeted within this research. These include (A) General benzimidazol structure; (B) albendazole, albendazole sulphone and albendazole sulphoxide; (C) thiabendazole and its metabolite thiabendazole NH_2 , and (D) Triclabendazole and its metabolites triclabendazole sulphone and sulphoxide.

1.5 Benzimidazole discovery and history.

The BZDs utilised for the prevention and treatment of the parasitic infections, discussed previously in section 1.3, was discovered in the 1944. Wayne Wooley, a leading expert in anti-metabolics at the time, noted that there was a similarity in structure of BZD to adenine and hypothesized that the former may act as an anti-metabolite. This research further demonstrated that the BZD molecule could inhibit the growth of bacteria and fungi thus proving its pesticide capabilities (Woolley, 1946). In 1953 research at the University of Michigan showed that subcutaneous injections of BZD in mice infected with poliomyelitis had a lowered mortality rate (Gillespie *et al.*, 1954) thus encouraging Merck researchers to synthesise BZD as a potential anti-viral drug. During routine screening of this drug for chemotherapeutic activity it was discovered that 2-phenylbenzamidazole had anti-helmintic properties. After examining hundreds of BZD analogues, it became apparent that thiabendazole (TBZ) was one of the most potent chemotherapeutic agents ever discovered. It was found to prevent the development of ascaris eggs *in-vitro* (Brown *et al.*, 1961), and

became the drug of choice for the treatment of strongyloidiasis which is a human/ animal parasitic disease caused by the nematode *Strongloides stercoralis* (Sneader, 2005).

Merck researchers screened analogues of TBZ that could resist metabolic inactivation thus allowing for improved penetration of the larvae. In order to achieve this they prevention of the enzymatic hydroxylation at the 5 position of the ring structure using aryl, alkyl, and halo substitutions was required. However, these approaches were unsuccessful due to metabolic conversion at the 5-position of the ring structure (Sneader, 2005). To overcome this, carbamates were produced, which are a group of organic compounds sharing a common functional group. From the carbamates synthesised, the compound Cambendazole was generated and was shown to be a highly potent, broad spectrum anti-helmintic (Hoff *et al.*, 1970). Following this discovery, the drug mebendazole which exhibited potent efficacy characteristics and broad spectrum activity was synthesized. Research continues on the production of anti-parasitic drugs resulting in a number of compounds which are safe for veterinary use and have broad spectrum activity against all stages of liver fluke and gastro intestinal parasites (Sneader, 2005).

1.6 Modes of action of benzimidazoles

In this section the mechanism by which the benzimidazoles eradicate parasitic infections is briefly discussed. The primary mode of action of these drug molecules involves their interaction with the eukaryotic cytoskeleton protein, tubulin. Tubulin provides the structural skeleton to the cell, and aids in the movement of chromosomes during cell division (Bell 1998). Tubulin (the microtubulin sub unit) is a dimeric protein consisting of α and β subunits of approximately 50 kDa each (Lacey, 1990).

Microtubules exist in balance with tubulin, and the ratio of dimeric tubulin to microtubulin is controlled by a number of regulatory proteins and co-factors known as micro-tubule inhibitors. These inhibitors bind to tubulin to prevent the self-association of the α and β subunits onto the microtubules (Lacey, 1990). This process is known as capping, where the inhibitor caps the microtubule at the associating end while the microtubule continues to dissociate from the opposite end resulting in loss of microtubule length. The benzimidazoles mebendazole and fenbendazole, were first discovered as inhibitors to induce the loss of microtubules of the tegumental and intestinal walls of cestodes and nematodes (Van den Bossche *et al*., 1982). This resulted in a loss of transport of secretory vesicles, a decrease uptake in glucose and an increased utilisation of stored glycogen. With the disruption of the

intestinal cell wall the parasite undergoes slow onset starvation and inhibition of egg production thus preventing further infection of the host animal (Martin *et al.*, 1997)

1.7 Benzimidazole residues and the EU directives

The employment of these veterinary drugs as preventatives for parasitic infections is essential for animal production. However, trace residues of benzimidazole may be found in the final meat or meat products. The control of these residues is important for public health, thus highlighting the requirement for rapid analytical methods for regulatory monitoring (Bergwerff and Van Knapen, 2006). By definition a residue is a trace of a substance, present in a matrix (meat, blood, urine etc) after some sort of administration (for example within the framework of veterinary practice and illegal use) to an animal. In all cases concentration levels in the ppb (µg/kg) range or even lower (ng/kg) have to be detected (Scarth *et al.*, 2009).

According to the EU directive 96/23/EC residues are divided into two major groups A and B. Group A includes illegal growth promoters that are abused for animal fattening, and the no maximum residue level substances. These can be sub-divided into four main groups; anabolic or anabolic steroids, thyreostats, beta-agonists and annex IV substances. Group B contains the veterinary drugs such as the benzimidazoles or veterinary medicinal products (VMPs) that include antibacterial substances, anthelmintics, coccidiostats, carbamates, pyrethroids, sedatives, non-steroidal anti-inflammatory drugs and other pharmacologically active substances.

The analytical requirements for both groups are different, for group A residues, the substances have to be identified in a large range of matrices at a low concentration level (zero tolerance principle). This requires a qualitative multi-residue method, closely followed by quantitative methods. For group B substances a less stringent approach is used whereby a qualitative method may be employed to confirm for the presence of such residues. A more detailed investigation of the screening methods available for the benzimidazole's detection is discussed in section 1.8.

The Veterinary Medicines Evaluation unit, as part of the European Agency for the Evaluation of Medicinal products (EMEA), compiles experimental data in order to determine maximum residue limits (MRLs) for veterinary drugs. These MRLs are established to prevent any contaminated food products reaching consumers (EMEA/CVMP/765/99-Rev.23). Included in

the section (1.7.1) is a summary of the key information reviewed from the EU directives regarding the targeted drugs in this research i.e. ABZ, TBZ and TCB. Some of the information reviewed here is not available from journals and are mainly based on the unpublished findings from the department of agriculture funded research projects.

1.7.1 Albendazole

EU directive number EMEA/MRL/865/03

A comprehensive series of developmental studies carried out in mice, rats, rabbits, and sheep showed albendazole to be teratogenic. The malformations included visceral, craniofacial and bone defects (including shortened limbs). The lowest no observable effects level (NOEL) for ABZ was 5 mg/kg of body weight (bw)/day. Netobimin (Prodrug of albendazole) and albendazole sulphoxide were also teratogenic with similar potency to albendazole (*EMEA/MRL/865/03, 2004)*

Reproductive effects of ABZ were investigated in multi-generation oral-dosing studies in rats. The drug reduced survival and growth of pups during the post-natal lactation period, with an NOEL for this effect of 5.8 mg/kg (*EMEA/MRL/865, 2005).*

Albendazole demonstrated negative results in bacterial mutation tests using numerous different strains of organisms. It produced no clastogenicity in an *in-vitro* metaphase analysis of Chinese hamster ovary (CHO) cells, and was negative in an *in-vitro* cell transformation assay in BALB/3T3 mouse cells. However, an *in-vivo* mouse bone marrow micronucleus test on ABZ, which had been isolated from a formulated product, gave a positive result. This result indicated that albendazole was an *in-vivo* somatic cell mutagen. In the absence of any tests on germ cells, it remains unclear whether or not ABZ can induce heritable mutations. Further studies showed that ABZ and NTB are *in-vivo* aneugens. A maximum/ minimum level of exposure to ABZ that presents a mutagenic risk to consumers was not identified.

ABZ was adequately tested in carcinogenicity bioassays, giving no evidence of neoplasia in either rats or mice, while albendazole sulphoxide was found to be non-irritant for the skin or eyes of rabbits (*EMEA/MRL/865/03 2004).*

ABZ may be administered to humans and domestic animals for the treatment of cestode parasitic infections. In human field trials of ABZ, 17 women in the first trimester of

pregnancy were given a single oral dose of 400 mg/person without any adverse effects on mother or child being apparent (*EMEA/MRL/865/03 2004).*

In cattle given 14C-labelled albendazole as a single dose of 15 mg/kg bw, the total residues in liver depleted from more than 20 mg/kg one day after treatment to 6 mg/kg four days after treatment and around 1.2 mg/kg 20 days after treatment. The liver has the highest residue levels and the kidney has the next highest and most persistent residue levels. Muscle and fat tissues have much lower residue levels and deplete rapidly (e.g. for muscle a level of 5 mg/kg one day after treatment reduced to 64 µg/kg four days after treatment and 20 µg/kg after 20 days). Ruminant species such as bovine, ovine and caprines share a similar gastro-intestinal physiology. The available pharmacokinetic and residue depletion data do not indicate any significant variability between cattle and sheep, therefore, the EMEA concluded that other ruminants were unlikely to show any significant differences in these parameters. Consequently, they recommended that the same MRL values would apply to all animal species tissues and organs. The same MRL for ABZ was also established for milk (Table 1.7.1). Based on the MRLs shown below it was calculated that the daily intake of extractible residues would amount to 310 µg/day i.e., 103 % of the ADI. It was considered that this would not constitute as a risk to consumers because at least 73 % of the residues in tissue were not bioavailable (*EMEA/MRL/865/03 2004).*

Table 1.7.1: Marker residue, target tissues and MRLs for Albendazole.

Pharmacologically active substances	**Marker residue**	**MRL'S**	**Tissue**
Albendazole	Sum of albendazole suphoxide, sulphone and 2-amino sulphone expressed as albendazole.	100 µg/kg 1,000 µg/kg 500 µg/kg 100 µg/kg 100 µg/kg	Muscle Fat Liver Kidney Milk

1.7.2 Thiabendazole

EU directive EMEA/ MRL/868

According to the EMEAs review on the toxic and mutagenic affects of TBZ, low acute toxicity is exhibited when TBZ is administered via the oral route. In mice oral LD50 values significantly varied from 2,400 to 3,810 mg/kg and 3,300 to 8,600 mg/kg in rats, and 3,850 mg/kg in rabbits. When administered by intraperitoneal injection and intravenously, LD50 values for mice were 430 and 130 mg/kg bw and for rats were 1,850 and 150 mg/kg bw, respectively. The signs of acute toxicity seen at high doses included weight loss, inanition, ataxia, narcosis and central nervous system depression, with subsequent death from respiratory failure (*EMEA/ MRL/868, 2002).*

Tada (1996) investigated the subchronic toxic effects of thiabendazole (TBZ) administered in the diet at levels of 0 (control), 0.8 and 1.6 % for 13 weeks to male and female ICR mice. Mean body weights of male mice fed 0.8 or 1.6 % TBZ showed a significant decrease compared with controls. Red blood cell parameters in male mice of treated groups were significantly lower than controls. Spleen or liver weights were significantly increased in male and female mice of treated groups. Relative kidney weights of treated mice tended to be increased in comparison with controls. In the liver, sinusoidal dilatation and enlargement of liver cells were found in treated mice. In the kidney, atrophy of tubules with peritubular fibrosis, cell infiltration and some tubular necrosis were found in treated mice. Slight hyperplasia was found in the urinary bladder of treated mice. The findings in the present study indicate that TBZ caused a slight anaemia and liver or kidney injury at both levels tested, under these conditions (Tada *et al.*, 1996).

Mice given TBZ in oral gavage doses of 250 or 500 mg/kg bw/day for up to seven days showed renal damage to the tubules and glomeruli. In pilot studies of three and six weeks duration, mice were fed diets which gave them doses of 0, 50, 150, 300, 600 and 900 mg/kg bw/day of TBZ with no effect on mortality or clinical appearance, but food intake and bodyweight gain were decreased at doses of 600 mg/kg bw/day or more. When treated with higher dosages over a thirteen week period both female and male mice showed decreases in red blood cell parameters (red blood cells, packed cell volume, MCHC, MCH). They also had an increased platelet count and a mild neutrophilia with concurrent lymphopaenia. Serum transaminases were raised in both male and female mice. The weights of the liver, spleen and

kidneys were increased in groups receiving high doses of TBZ. There was also centrilobular hepatocellular necrosis, bile duct proliferation, splenic haemosiderosis, necrosis, fibrosis and atrophy of renal tubules and urothelial hyperplasia (*EMEA/ MRL/868, 2002).*

Studies were carried out by (Lankas and Wise, 1993) that assessed the developmental toxicity of TBZ in New Zealand white rabbits and Sprague-Dawley rats. In rabbits, TBZ was administered orally at doses ranging from 24 to 600 mg/kg body weight/day in two separate studies. In rats, TBZ was administered at doses of 10, 40 or 80 mg/kg body weight/day. In all studies, TBZ was administered daily by gavage on gestation days 6 to 17. In the first rabbit study, weight loss occurred in the 600 mg/kg/day group and weight gain decreased for the rabbits administered with 120 mg/kg/day group. In addition, there were four complete litter resorptions and four abortions in the 120 and 600 mg/kg/day groups, respectively. This study was repeated and they found that there was a decreased maternal weight gain and decreased foetal weights at 600 mg TBZ/kg/day, but there was no evidence of selective developmental toxicity. During the rat study TBZ produced decreased maternal weight gain associated with slight decreases in foetal weights at doses of 30 and 80 mg/kg/day. No changes were found at 10 mg TBZ/kg/day and no evidence of selective developmental toxicity or teratogenicity was found at any dose. Based on these results they concluded that TBZ is not teratogenic or selectively foetotoxic in rats or rabbits (Lankas and Wise, 1993).

Mudry de Pargament (1987) reported the chromosomal damage produced by TBZ that was evaluated by "*in-vivo*" and "*in-vitro*" cytogenetic tests. Doses of 50, 100 and 200 mg/kg body weight were assayed in adult mice by the sister-chromatid exchange (SCE) and micronucleus tests. These doses were selected as they were within the range of those used in human antihelminthic treatments. SCE frequency was increased only in the last dose. A significant increase of micronucleated cells was shown in the 3 doses assayed. A notable increase in abnormal anaphase-telophase cells was only detected with the two highest concentrations assayed (0.60–0.24 μg/mL) $p < 0.01$ and $p < 0.05$ respectively. They state that the genotoxic effects of this residue indicate that TBZ itself is a mutagenic agent (Mudry de Pargament *et al.,* 1987). According to studies carried out by the EMEA their data indicates that TBZ does not produce gene mutations in bacterial tests (no mammalian cell gene mutation tests available), structural damage to chromosomes *in-vitro* (metaphase analyses), nor in Syrian hamster embryo (SHE) cell-transformation assays. The results of tests in fungal and mammalian cell systems (including *in-vitro* metaphase analyses, *in-vitro*

micronucleus/kinetochore assays, induction of mitoses, and yeast aneuploidy assays), however, provide consistent evidence of aneugenicity (mutations which cause the loss or gain of whole chromosomes) *in-vitro* (*EMEA/ MRL/868, 2002).*

They further concluded that the *in-vitro* aneugenicity of TBZ was shown to be a consequence of it binding to tubulin, which results in dysfunction of the spindle mechanism at cell division. All of the validated oral route *in vivo* mutagenicity assays in either, somatic cells (bone marrow metaphase analyses and bone marrow micronucleus tests) or germ cells (dominant lethal assays), were interpreted as being negative (*EMEA/ MRL/868, 2002).*
A study was carried out by (Ogata *et al.,* 1984) where TBZ was suspended in olive oil and tested for teratogenicity using pregnant Jcl:ICR mice at different stages of organogenesis. All foetuses were removed from the uterus on day 18 of gestation, and were examined for external and skeletal anomalies. In mice given 700, 1,300 or 2,400 mg TBZ/kg body weight/day on days 7–15 of gestation, dose-dependent external and skeletal anomalies, especially cleft palate and fusion of vertebrae, were observed. In mice given a single dose of TBZ (2,400 mg/kg) on any one of days 6–13, they observed an increased number of malformations. Various types of malformation occurred, especially in the mice treated on day 9. Reduction deformity of limbs was found in mice given TBZ on days 9–12. They also determined a dose-response relationship, whereby they administered groups of mice with one of 17 doses of TBZ (30–2,400 mg/kg) on day 9 of gestation. The number of litters having foetuses with reduction deformity of limbs and of those having foetuses with skeletal fusion increased in proportion to the dose of TBZ, thus proving that high doses of TBZ causes foetal abnormalities and mutations (Ogata *et al.,* 1984).

According to recent studies carried out by the EMEA the great weight of evidence indicates that thiabendazole does not cause any *in vivo* genotoxic effects when it was administered by the oral route, it was concluded that thiabendazole residues in food do not give rise to concern with respect to mutagenic hazards to consumers (*EMEA/ MRL/868, 2002).*

There is no significant variability between MRL's for different animal species. MRL's for milk were also established (Table 1.7.2). Based on these MRL's, the theoretical daily intake of total thiabendazole-related residues in milk and meat corresponds to around 7 % of the ADI.

Table 1.7.2: Marker residue, target tissues and MRLs of thiabendazole

Pharmacologically active substances	Marker residue	MRL'S	Target tissue
Thiabendazole	Sum of thiabendazole and 5-hydroxy-thiabendazole	100 µg/kg	Muscle
		100 µg/kg	Fat
		100 µg/kg	Liver
		100 µg/kg	Kidney
		100 µg/kg	Milk

1.7.3 Triclabendazole

EU directive EMEA/CVMP/320386

Triclabendazole has low acute toxicity when administered by the oral, intraperitoneal, dermal and inhalation routes. The sulphoxide and sulphone metabolites of triclabendazole also have low acute oral toxicity, produce minimal skin irritation, no eye irritation and had limited potential for skin sensitisation. TCB showed no teratogenicity in rats or chinchilla rabbits, although foetal development was retarded (low foetal body weight and low ossification) at doses which caused maternal toxicity (100 mg\kg bw in rats and 10mg/kg bw in rabbits). Oral administration to sheep up to 50 µg/kg bw had no adverse effects in reproductive parameters *(EMEA/CVMP/32038, 2004).*

TCB appeared negative in numerous *in-vitro* and *in vivo* mutagenicity tests. A carcinogenicity study was conducted in mice where the only pathological findings were increased serum levels of hepatic enzymes, increased liver-weight and benign hepatomas in females with a dietary supplement of 300 mg/kg of feed. Another study in rats also demonstrated no significantly adverse effects on tumour incidences at any dose *(EMEA/CVMP/320386, 2004).*

The bioavailability of the drug was assessed using the rat model and a number of different methods; these methods established that the rat's bioavailability from feed was approximately 70 %. The metabolism of rats is comparable to that of sheep, goats and cattle and it was therefore concluded that the residues of toxicological concern would be similar. The studies

also showed that the bioavailability of incurred residues was very low following administration of triclabendazole intravenously and orally, which allowed for an increase in previously established MRL's. Based on the MRL's below (Table 1.7.3), and taking into account the bioavailability of the residues in the tissues, the daily intake represents about 70 % of the ADI which is approximately 63 µg/day *(EMEA/CVMP/32038, 2004).*

Table 1.7.3: Marker residues, target tissues and MRL's of triclabendazole

Pharmacologically active substances	Marker residue	MRL'S	Target tissue
Triclabendazole	Sum of the extracted residues that may be oxidised to keto-triclabendazole	225 µg/kg	Muscle
		100 µg/kg	Fat
		250 µg/kg	Liver
		150 µg/kg	Kidney
		100 µg/kg	Milk

1.8 Screening methods for the detection of benzimidazoles.

Screening methods are defined according to commission decision 2002/657 as "methods that detect the presence of an analyte or class of analytes at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are specifically designed to avoid false compliant results". The level of interest referred to is the MRL.

1.8.1 Methods of detection for benzimidazoles.

A number of methods are available for the detection and quantification of benzimidazole residues and their metabolites. These include high performance liquid chromatography (HPLC), liquid chromatography (LC), gas chromatography (GC), mass spectrophotometry (MS) and thin layer chromatography (TLC). There are approximately 20 BZD residues that can be found in animal tissue (Danaher *et al.*, 2007).

Detection methods for BZD were developed soon after its discovery. Initial methods included direct spectrometric detection with fluorescence and colourmetric detection. However, these assays were only suitable for quantitation at high levels of BZD and a more selective chromatographic separation was required prior to spectrometric detection (Pease and Gardiner, 1969). TLC, which is a suitable technique for the semi-quantification of residues,

was further developed as a detection method. Norman *et al.* developed a TLC assay which was capable of detecting TBZ in citrus fruits (Norman *et al.,* 1972). Subsequently high performance TLC (HPTLC) was developed for the determination of benzimidazole residues in crops and the sensitivity was compared to that of HPLC which proved to be more sensitive a technique (Corti *et al.,* 1991). However, TLC methods had a higher limit of detection and were above EU regulations and standards as described in Tables 1.7.1-1.7.3.

The physical and chemical properties of BZD residues vary significantly thus making the advancement of chromatographic methods difficult. When developing these assays there are a number of things to take into account such as peak sharpness, resolution and run-time. A number of studies were carried out on LC-based systems investigating the use of columns with different bore sizes (Porter *et al.,* 1993) and concluded that using a narrow bore column reduced solvent usage, analysis time and pump water. Another study utilised different pH gradients and ion pair reagents to shorten retention time (Botsoglou *et al.,* 1994). Advances in separation techniques have led to the development of LC detection systems which are coupled with ultra-violet (UV), fluorescence and MS to determine BZD in different matrices such as milk, serum, tissue and crops. BZD possesses a strong UV chromophore and ABZ, TCB and TBZ possess naturally fluorescing chromphores which also make them suitable for detection by LC - fluorescence (Danaher *et al.,* 2007). Sorenson and Peterson developed a LC-UV method that allowed for the detection of eight benzimidazoles including ABZ, fenbendazole (FBZ) and ABZ-SO_2, a metabolite of ABZ in milk (Sorensen and Peterson, 1995) Kinabo and Bogan developed a LC-fluorescence method that had the ability to detect TCB and its metabolites TCB-SO and TCB-SO_2 at levels down to 40 µg/kg for both TCB and TCB-SO and 20 µg/kg for TCB-SO_2 (Kinabo and Bogan, 1988). Even though the detection methods described have well documented sensitivity, the technique of choice for the sensitive and selective detection of BZD's is predominantly LC-MS.

LC-MS facilitates the detection of residues in a range of matrices, and, when compared to LC-UV or fluorescence, can be used as a stand-alone method for the detection, confirmation and identity of benzimidazole residues. Takeba *et al*. described a confirmatory assay for TCB with a Limit Of Detection (LOD) range between 4 and 6 µg/kg in bovine milk (Takeba *et al.,* 2000). Improvement of the LC-MS system have allowed for the development of methods which can detect a number of residues simultaneously. Bushway *et al*. detected residues of TBZ and carbendazim (MBC) in fruit juices with a Limit of Quantification (LOQ) of 2 µg/kg

(Bushway *et al.*, 1994). Another study carried out by Balizs *et al*. used a triple-quadruple instrument to detect 15 different benzimidazole residues with the LOQ ranging between 5 and 30 µg/kg (Balizs and Erbach, 1999).

BZD determination can also be achieved by gas chromatography (GC). This method is a challenge for some BZD and its derivatives because of their low volatility. However, TCB and TBZ are sufficiently volatile to enable measurement in samples derived from crops and fruit juices with detection limits of approximately 10 µg/kg (Oishi *et al.*, 1994). The number of derivitisation steps required for GC may be time consuming and difficult. To eliminate long sample preparation and derivitisation steps a more rapid method can be employed for the detection and measurement of these residues such as enzyme-linked immunosorbent assay (ELISA) and biosensor assays

1.8.2 Enzyme-linked immunosorbent assay and Biosensor assays.

An ELISA is a quick, quantitative and robust system and may be used for the screening and determination of veterinary drug residues. It measures the biochemical interactions between specific antigens and antibodies for detection of residues in a sample. In 1992 a monoclonal antibody capable of detecting TBZ and its metabolites in bovine liver samples at levels down to 20 µg/kg was generated (Brandon *et al.*, 1992). This antibody was later used to detect TBZ in fruit, vegetable and fruit juices and a monoclonal (mAb) that had a cross reactivity with 11 of the benzimidazoles was also developed (Brandon *et al.*, 1994). A polyclonal antibody (pAb) that showed cross reactivity to 8 of the benzimidazole carbamates with limits of detection in serum of 2.6 µg/kg was also reported (Johnsson *et al.*, 2002). Increasing concern about the level of exposure of ruminants to veterinary drug residues necessitates the development of a rapid screening method for their determination in food. ELISA is a method that may be regularly used for confirmation and quantification of benzimidazole residues; however, the use of surface plasmon resonance (SPR) allows the development of an automated rapid screening assay for veterinary residues. SPR-biosensor assays employ label-free detection and have proven to be versatile, robust and capable of producing rapid and reliable results with minimum sample preparation (Baxter *et al.*, 2001). Keegan *et al.* developed an SPR-based assay, combined, with a well developed extraction procedure, for the determination of 11 benzimidazole residues in bovine milk with a LOD of 5 µg/kg (Keegan *et al.*, 2009). Brandon *et al*. (Brandon *et al.*, 2002) developed an inhibition biosensor assay to determine benzimidazole residues in serum and plasma using a monoclonal antibody

with a LOD of 5 µg/kg (Brandon *et al.,* 2002). There are few reports in the literature on the use of biosensors for the detection of BZD in food matrices.

In this research an ELISA assays were developed using a recombinant antibody binding fragment (Fab) which facilitated the detection of TBZ. ELISA, BIAcore™ and lateral flow immunoassays (LFIA) were also developed utilising an avian pAb for the detection of TCB in spiked milk samples. The use of recombinant antibodies has many advantages such as ease of production, immortalisation and most importantly the ability to modify properties through genetic modifications such as CDR walking, site directed mutagenesis and chain shuffling. The next sections describe the general structure of an antibody and its recombinant counterparts. It also describes the process of selection of highly specific and sensitive antibodies by the phage display technique.

1.9 Introduction to antibody structure and production.

1.9.1 Antibody structure

An antibody molecule is composed of four polypeptide chains linked by disulphide bonds consisting of 2 heavy chains and 2 light chains with molecular weights of 50 kDa and 25 kDa, respectively. The heavy chain is comprised of one variable (V_H) region which is involved in the antigen binding and three constant regions (C_{H1}, C_{H2}, C_{H3}) (Fig 1.9.1). The light chain is composed of one variable (V_L) region and is an integral part of the antigen binding site, and one constant (C_L) region (Feige *et al.,* 2009).

There are five classes of immunoglobulins (Ig). They are all distinguished by their heavy chains, these include, IgG, IgM, IgE, IgA and IgD. There are two types of light chain lambda (λ) and kappa (κ) which combine with heavy chains to form a complete Ig molecule. IgG is targeted for library construction as it is one of the major class of antibody which is produced during the immune response (Padoa and Crowther, 2006). The Fc portion of the antibody consists of the three constant heavy domains and these contribute to the effector functions of the antibody such as complement activation (Padoa and Crowther, 2006). The antigen binding region of the molecule consists of both the variable light and heavy domains that bring together the hyper variable loops known as the complementary determining regions (CDR). These regions usually have little difference in their sequence between classes; however, can exhibit a high level of sequence diversity (Conroy *et al.,* 2009).

1.9.2 Production of Antibodies

Antibodies have been used extensively as diagnostic and therapeutic applications and residual confirmatory tools (Borrebaeck, 2000). Initially immunoassays were dependent on the use of polyclonal antisera from immunised animals that provided a significant immunse response to the selected antigen (Borrebaeck, 2000). By definition, a polyclonal anti-serum contains many different antibody molecules varying in specificity and affinity. However, their supply is not limitless (Lee and Morgan, 1993). In 1975 the reporting of the discovery of monoclonal antibody (mAb) generation technology potentially led to the immortilisation of a single clone *in-vitro* and the availability of unlimited amounts of identical antibodies (Köhler and Millstein, 1975). Monoclonal antibodies are generated by the fusion of antibody-producing B cells to myelomas (malignant B cells). The fusion may be facilitated by the use of polyethylene glycol, a polywax that promotes cell adherence and the exchange of nuclei. To select for successful cell fusions, the cells are grown in a medium containing hypoxanthine aminopeterin and thymidine (HAT medium) in which only the hybrid cells can propagate. Following testing for specificity, the cultures are cloned by limiting dilution to obtain a homogenous cell clone secreting mAbs (Little *et al.,* 1988). Monoclonal antibodies offer several advantages over polyclonal antibodies as they have the ability to monitor precise conformational changes in biopolymers, or to target specific epitopes. However, a third recombinant method was developed for antibody production which have several advantages over mAb generation including, time necessary for production, the ability to locate epitopes that are inaccessible to larger antibody molecules: the ability to immobilise many more fragments on a small support such as a lateral flow or dipstick device (Lee and Morgan, 1993).

1.9.3 Recombinant antibody fragments

The development of an expression system by Pluckthun and Skerra (Skerra, A. 1988) allowing the production of a completely functional antigen binding fragment (Fab) in *E-coli* was a pivotal point in recombinant antibody engineering. This system showed that the assembly pathway for the Fv fragment is similar to that of the whole IgG molecule in eukaryotic cells (Skerra and Pluckton, 1988). The advent of this recombinant technology has allowed for the generation of smaller fragments that exhibit the stability and specificity of the larger IgG molecule (Conroy *et al.,* 2009). The smallest of the antibody fragments is the Fv portion which comprises of non-covalently associated V_H and $V_{L.}$ These have the same

affinity of the whole IgG molecule. High yields of this fragment can be produced in bacterial systems; however, the dissociation of the two variable domains may vary the stability (Bittar *et al.*, 1996). A more stable scFv fragment can be generated from this by incorporating a glycine serine flexible linker $(Gly_4Ser)_3$ (Verma *et al.*, 1998). The Fab fragment consists of the light chain and the Fd region, which is the V_H and C_{H1} region of the heavy chain with part of the hinge region. Since the Fab's antigen binding site is still intact, it binds in a similar fashion to the parent antibody molecule. It is composed of two chains: V_H-C_{H1} and V_L-C_L and mimics the binding of the parental IgG. Diabodies can be prepared by fusing a shorter polypeptide linker to two scFv fragments thus increasing the affinity of the fragment. Similar to this, a dimeric scFv can be produced by introducing a natural dimeric protein to attach both scFv fragments. Once these fragments are generated they (Welsh *et al.*, 1998) can then employed to select highly sensitive and specific antibodies from an extensive library (Bradbury and Marks, 2004).

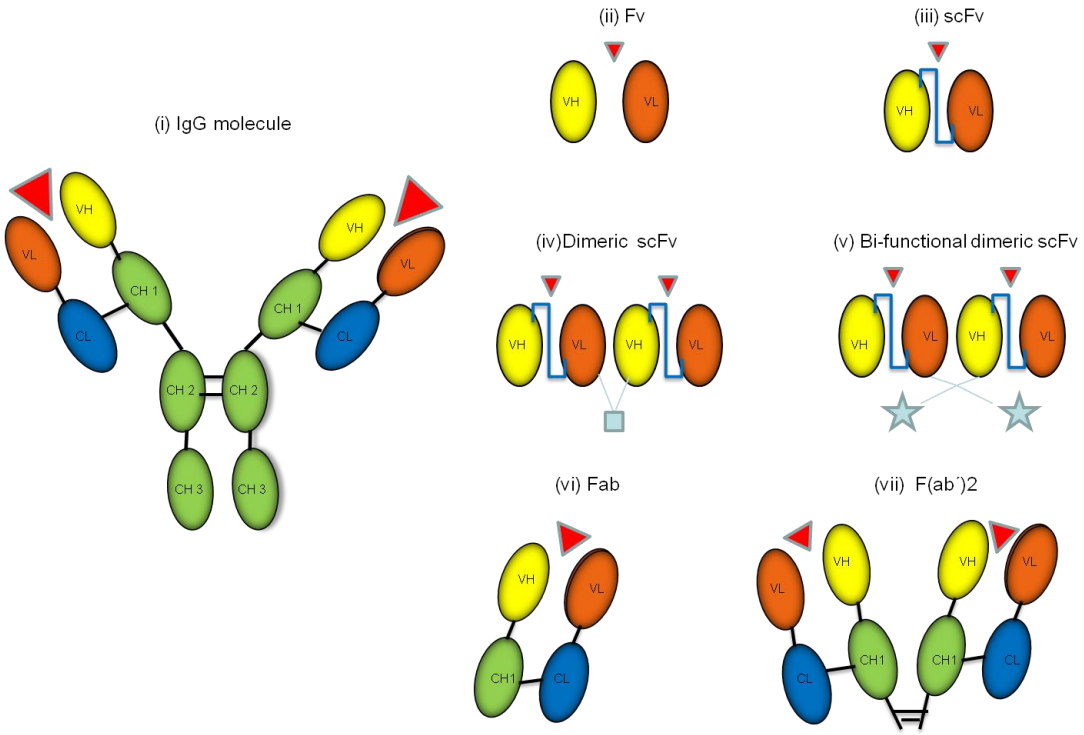


Figure 1.9.3.1: An illustration of a typical IgG molecule (i) (~150 kDa) and some of its recombinant antibody formats. The red triangles represent the antigen binding sites and the disulphide bonds are indicated as black lines. The Fv (ii) consists of the V_H (Yellow) and V_L (Orange) chains. The scFv (iii) consists of the Fv region with a flexible linker joining the terminal ends of the V_H and V_L. The dimeric scFv (iv) is a fusion of two scFv's via a naturally occurring protein such as a leucine zipper or a four helix bundle structure. The dimeric bi-valent scFv (v) incorporates an alkaline phosphatase label (star) to facilitate direct detection. The Fab fragment (vi) consists of fv fragments with both C_H (green) and C_L (blue) chain. The $F(ab')_2$ involves the linking of two Fab fragments with disulphide bonds. This can also be achieved by enzymatic cleavage of the full IgG molecule.

1.9.4 Phage display

Phage display was first introduced for the affinity selection of proteins fragments synthesized and expressed for cDNA fragments (Smith, 1985). This technique rapidly evolved to generate antibodies that recognised specific antigens with high affinity (Dennis and Lowman, 2004). Phage display can easily be described as the molecular link between phenotype and genotype. In general terms the protein is expressed on the surface of a phage particle as a fusion protein with a phage coat protein and the corresponding gene is inserted into the chromosome of the gene that is located within the phage particle (Jestin, 2008).

More specifically, the filamentous phage, M13, f1 and fd, from the genus Inovirus, are viruses that infect gram negative bacteria by using their pili as receptors (Zani and Moreau, 2010)(Figure 1.9.1). They are circular single stranded DNA that are packaged into a cylindrical shape known as a capsid. They are non lytic and do not assemble in the cytoplasm of the cell, instead they are continuously secreted across the bacterial membrane. The outer coat of the capsid is made up of five structural proteins. The more important of these 5 are the major coat proteins pVIII and pIII (Zani and Moreau, 2010). These proteins have both N-terminus region exposed to the exterior of the phage particle, and the sequences that are to be displayed on the surface are generally inserted at the N-terminus of the pVIII between the signal sequence and the beginning of the mature protein coding sequence. However, only small sequence may be inserted at this region as the sequence length prevents the correct phage assembly (Dennis and Lowman, 2004). The pIII protein located at the end of the phage is regularly utilised for most phage display functions as it allows long sequences to be inserted (Dennis and Lowman, 2004).

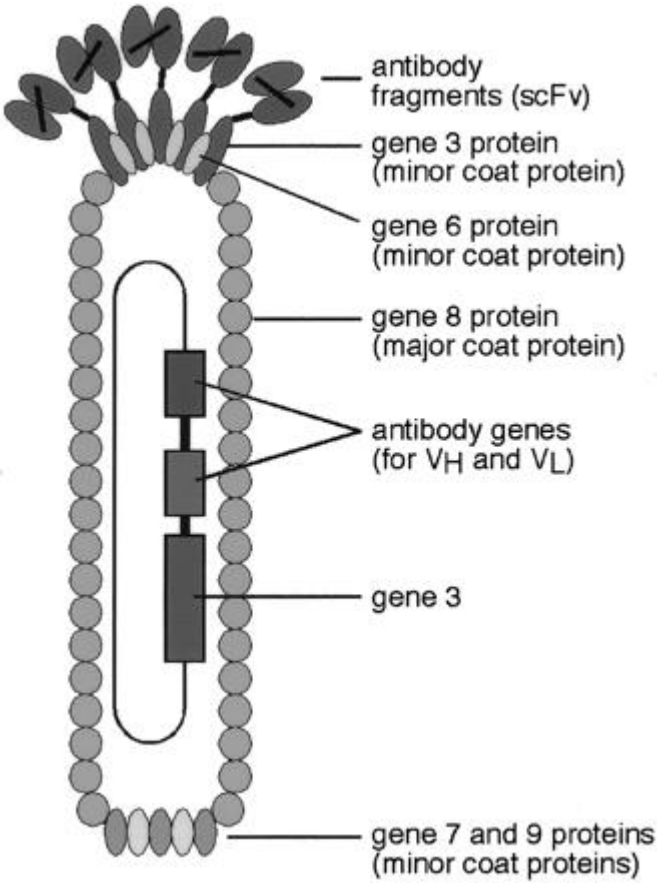


Figure 1.9.4.1: Structure of a filamentous phage displaying scFv fragments on its surface (Smith *et al*., 2005).

The protein of interest can also be displayed on the surface of the phage using a phagemid vector where the heterlogous sequence is inserted directly into the coding sequence of PIII or using a phage based phagemid vector that contains only the fusion protein gene (Zani and Moreau, 2010). Phagemid vectors are widely used because of their size and ease of cloning. The cDNA can be displayed by cloning into a small plasmid under the control of a weak promoter. Usually a phagemid vector carries both a plasmid origin of replication and a f1 origin of replication. The f1 ori allow the synthesis of a single stranded DNA and the formation of virions. These virions are produced from *E.coli* cells that possess these vectors by infecting the cells with helper phage. Infected cells then express wild type coat proteins encoded by the helper phage genome and a small amount of the fusion protein encoded by the phagemid vector. Finally, most virions contain the phagemid genome, as the helper phages genome has altered the packaging efficiency, therefore the linkage between genotype and phenotype is preserved (Zani and Moreau, 2010). Surface display of the antibodies then allows for affinity selection by antigen *in-vitro*, mimicking antigen selection in natural immunity (Petrenko and Vodyanoy, 2003). Affinity selection is achieved by passing the library over immobilised antigen molecules that are adsorbed to an immuno-sorbent material, binding clones are captured, while non binding clones are washed away. The specific phage particles can be eluted from antigen without losing infectivity and can then be propagated for another round of affinity selection (Petrenko and Vodyanoy, 2003).

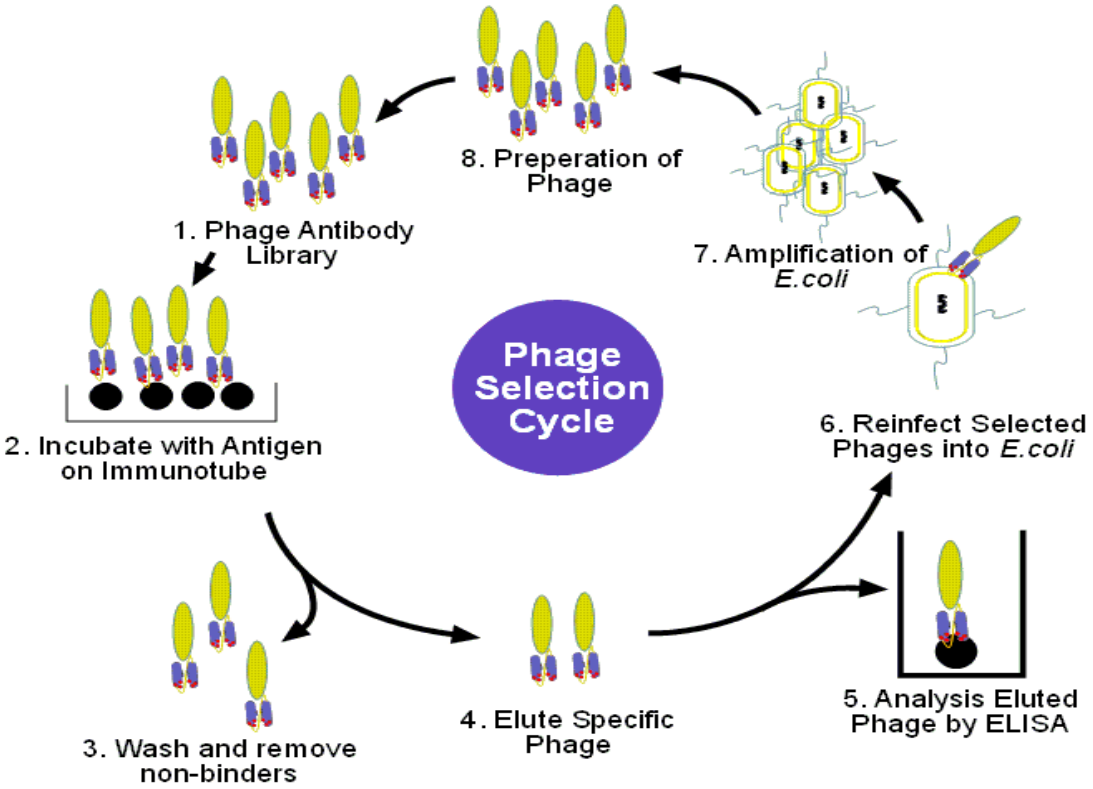


Figure 1.9.4.2: Diagrammatic representation of the typical steps involved in the panning of combinatorial phage-displayed libraries. (1) The phage-displayed antibody repertoire is packaged and isolated from *E.coli*. (2) Phage are subjected to selective pressure on immobilised antigen. (iii) Non-specific phage are removed by repetitive washing (4) Specific bound phage are eluted from immobilised antigen using specific conditions (i.e. acidic or basic solutions) (5) Eluted phage are analysed by ELISA. (6 and 7) Exponential growth phase *E.coli* containing F' plasmid are infected with eluted phage and allowed to amplify. Following infection monoclonal phage-antibodies can be obtained by isolating single infected colonies on selective agar (represented by red block arrow). (8) Panned phage amplified in *E. coli* are purified from bacteria and subjected to subsequent rounds of panning. Adapted from Christensen, 2001.

1.10 Thesis outline

The aim of the work presented in this thesis is the production and characterisation of recombinant antibody fragments for the detection of benzimidazoles in different matrices. Figure 1.10.1shows a schematic of the strategy employed during this research The production of a sensitive recombinant Fab fragment which was used in the development of an ELISA assay capable of detection of TBZ significantly below the MRL of 100 µg/kg is described. Recombinant antibody generation techniques included the production of phage-display Fab library from hybridoma cell lines secreting anti-TBZ monoclonal antibody. Twelve soluble Fab antibodies were isolated, expressed and purified from this library. These clones were then characterised by ELISA, SDS-PAGE and Western blotting. Subsequently, antibodies were evaluated by the development of inhibition immunoassays for the detection of TBZ in extraction buffer which contains 5 % (v/v) methanol.

The second section focuses on the improvement of the Fab's sensitivity by restructuring the pComb vector to contain a double helix gene which would create a bivalent Fab fragment. The dHLX gene was amplified by PCR from the pAK500 system. Positive clones were selected and characterised by ELISA, SDS-PAGE and Western blotting. Further confirmation of bivalency was carried out by HPLC. The development of the assay was initially carried out by ELISA. Working assays with desired performance characteristics (i.e. sensitivity and reproducibility) were again validated for the detection of TBZ in buffer.

Chapter four focuses on the generation of an avian polyclonal fromIgY from egg yolk and its subsequent implementation into an ELISA assay for the detection on TCB and ABZ in extraction buffer. This work outlines the optimisation of the assay, and the antibodies stability in a variety of buffers. Inter and intra assays were performed for sensitivity and reproducibility. Following this an avian scFv library was constructed and panned against the conjugates TCB-BTG and ABZ-BSA. Positive clones were selected and expression studies were carried out using a range of *E.coli* strains.

Chapter five describes the further development of an ELISA assay implementing the anti-TCB pAb for the detection of TCB extracted from spiked milk samples. Following this Biacore™ and lateral flow immunoassays were generated for the detection of TCB in spiked buffer and milk samples. The final section of this research details the conjugation of the TCB

hapten molecule to green flourescent protein (GFP) for the novel development of a rapid lateral flow immunoassay for the detection of TCB in buffer and milk samples.

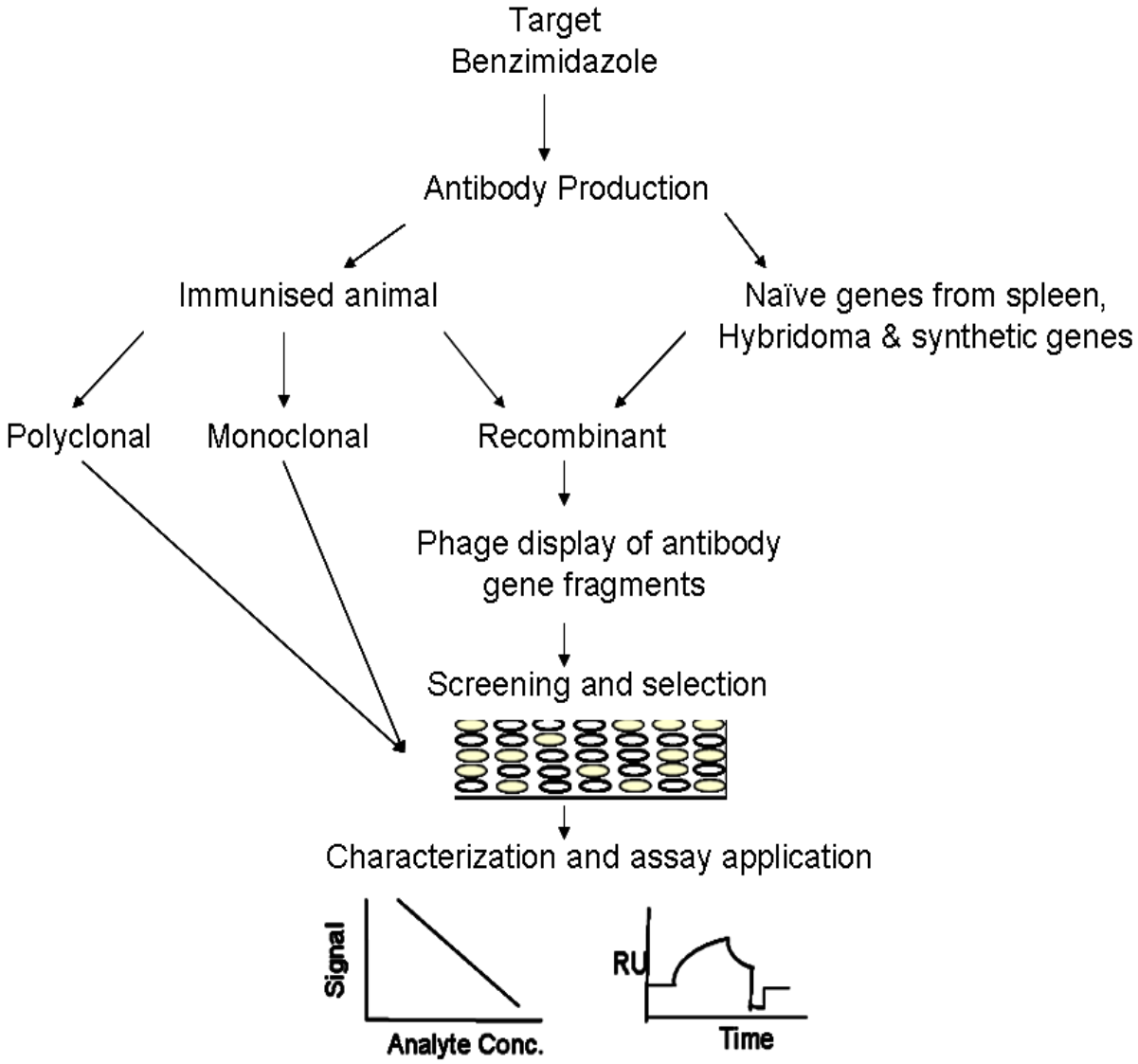


Figure 1.10.1: Diagram outlining the strategy employed for the generation of antibodies to the benzimidazoles and their application.

Chapter 2

Materials and Methods

2.0 Materials and methods

2.1 Equipment.

The following tables describe the equipment, media and buffer components, commercial antibodies and bacterial strains used though-out the course of this research.

Table 2.1.1: Equipment used over the course of this research and associated suppliers.

Equipment	Supplier
Biometra $T_{GRADIENT}$ PCR machine	LABREPCO, 101 Witmer Road, Suite 700, Horsham, PA 19044, USA.
Nanodrop™ ND-1,000	NanoDrop Technologies, Inc., 3411 Silverside Rd, 100BC, Wilmington, DE 19810-4803, USA.
Gene Pulser Xcell ™ electroporation System	Bio-rad Laboratories, Inc. 200 Alfred Nobel Drive, Hercules, California 94547, USA.
ORBI-safe TS netwise orbital shaking incubator	Sanyo Europe Ltd., 18 Colonial Way, Watford, WD24 4PT, United Kingdom

Equipment	Supplier
Trans-Blot® Semi-dry Transfer cell	Bio-Rad Laboratories, 2,000 Alfred Nobel Drive, Hercules, California 94547, USA.
Roller mixer SRT1	Sciencelab, Inc., 14025 Smith Rd. Houston, Texas 77396, USA.
Safire 2 plate reader	Tecan Group ltd Seestrasse103, CH-8708, Männedorf, Switzerland.
Biacore™ 1,000 and 3,000	GE Healthcare Bio-Sciences AB, SE-751 84, Uppsala, Sweden.
Vibra Cell™ sonicator	Sonics and Materials Inc., 53 Church Hill Road, Newtown, CT 06470-1614, USA.

Equipment	Supplier
Camag Linomat 5 (Lateral Flow Instrument)	Mason Technologies, Greenville Hall, 228 South Circular Road, Dublin 8, Ireland.
Typhoon 8600 variable mode flourescence imager.	Mason Technologies, Greenville Hall, 228 South Circular Road, Dublin 8, Ireland.
MiVac Quattro centrifugal concentrator	Mason Technologies, Greenville Hall, 228 South Circular Road, Dublin 8, Ireland.

2.2 Media compositions

Table 2.2.1: Media components used throughout the course of this research and their corresponding weights or volumes per litre. All media components were purchased from Cruinn Diagnostics, 5B/5C Hume centre, Parkwest Industrial estate, Nangor Road, Dublin 12, Ireland.

Media	Component	Weights/volumes per litre
2 x Ty	Tryptone	16.0 g
	Yeast extract	10.0 g
	NaCl	5.0 g
Super Broth (SB)	MOPs	10.0 g
	Tryptone	30.0 g
	Yeast extract	20.0 g
Super Optimal Catabolite (SOC)	Tryptone	20.0 g
	Yeast extract	5.0 g
	NaCl	0.5g
	1 M $MgCl_2$	10.0 mL
	1 M $MgSO_4$	10.0 mL
	1 M Glucose	16.0 mL
Terrific Broth (TB)	Tryptone	12.0 g
	Yeast extract	5.0 g
	Glycerol	4.0 mL
	KH_2PO_4	2.3 g
	K_2HP0_4	12.5 g

Table 2.2.2: Cell culture media components used throughout the course of this research and their corresponding volumes per litre. All cell culture media and components were purchased from Sigma Aldrich, 3050 Spruce StreetSt. Louis, MO 63103,USA.

Media	Components	Volume (mL) per litre
Supplemented RPM1 1640	100 mM Hepes	10
	100 mM Sodium pyruvate	10
	100 mM NEA	10
	Streptomycin	10
	15 % foetal calf serum	150
	10 % Briclone	100
	RPM1 1640	710

2.3 Bacterial cells and plasmids for cloning and expression

2.3.1: Bacteria strains used for gene cloning:

All bacterial cells used throughout the research were purchased from Statagene, Agilent Technologies, Unit 3, Euro House, Euro Business Park , Little Island, Cork, Ireland.

E. coli TOP10 F′ strain: {*lac*I^{q}, Tn*10*(Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G

E. coli XL1-Blue strain: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac *[F′* proAB lacI^{q}ZΔM15 *Tn*10 (*Tet*^{R})*]*.

E.Coli Rosetta™ 2: F^{-}*ompT hsdS*$_{B}$(r_{B}^{-} m_{B}^{-}) *gal dcm*(DE3) pRARE2 (Cam^{R})

2.3.2: Plasmid vectors

Pcomb 3Xss and Pcomb 3xTT were donated by Carlos Barbas III, The Scripps Research Institute, 10550 north Torrey pines road, IA Jolia, CA, 92037, USA.

PaK 500 vector was donated by Andreas Pluckthun, University of Zurich, Switzerland

2.4 Buffer components

2.4.1 ELISA Buffer Components

Table 2.4.1: Buffer components used throughout the course of this research and their corresponding weights or volumes, as appropriate, per litre. All buffer components were purchased from Sigma Aldrich, 3050 Spruce StreetSt. Louis, MO 63103, USA.

Buffer	Component	Weights or volumes per litre
Phosphate buffered saline (PBS), pH 7.5	150 mM NaCl 2.5 mM KCl 10 mM Na_2HPO_4 18 mM KH_2PO_4	8.0 g 0.2 g 1.4 g 0.2 g
PBSTween 20 (0.05 % (v/v) PBST), pH 7.5	150 mM NaCl 2.5 mM KCl 10 mM Na_2HPO_4 18 mM KH_2PO_4 0.05 % (v/v) Tween 20	8.0 g 0.2 g 1.4 g 0.2 g 0.5 mL
Milk-PBST (MPBST), pH 7.5	150 mM NaCl 2.5 mM KCl 10 mM Na_2HPO_4 18 mM KH_2PO_4 0.05 % (v/v) Tween 20 Specified % (w/v) milk marvel powder	8 .0 g 0.2 g 1.4 g 0.2 g 0.5 mL 0.5-5 % (w/v)

2.4.2 ELISA optimisation buffer components

Table 2.4.2: Buffers used for the optimisation of the coating concentrations for ELISA development and their weights or volumes, as appropriate, per 100 mL. All buffer components were purchased from Sigma Aldrich, 3050 Spruce StreetSt. Louis, MO 63103, USA

Buffer	Buffer Component	Weights or volumes per 100 mL
Citrate buffer, pH 4.7	Citric Acid	1.30 g
	Sodium citrate	1.10 g
Acetate buffer, pH 4.8	Sodium acetate	0.10 g
	Glacial acetic acid	0.06 mL
Carbonate buffer, pH 10.3	Sodium carbonate	0.07 g
	Sodium bi-carbonate	0.15 g
Tris buffer pH 7.4	Tris Base	0.30 g
	Sodium chloride	0.40 g
Tris Buffer pH 8.0	Tris Base	0.30 g
	Sodium chloride	0.40 g

2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting.

Table 2.5.1: Components used for the SDS-PAGE gel electrophoresis and their corresponding volumes for a 6 mL gel. All SDS-PAGE and Western Blot components were purchased from Sigma Aldrich, 3050 Spruce StreetSt. Louis, MO 63103, USA

12.5 % Separation gel	Volume per 6 mL gel
1 M Tris HCl, pH 8.8	1.5 mL
30 % (w/v) acrylamide	2.5 mL
2 % (w/v) methylamine bisacrylamide	1.0 mL
Distilled water	0.9 mL
10 % (w/v) sodium dodecyl sulfate (SDS)	0.04 mL
10 % (w/v) APS	0.04 mL
TEMED	0.01 mL

Table 2.5.2: Components used for SDS-PAGE gel electrophoresis and its corresponding volumes for a 2.6 mL gel.

4. 5 % Stacking Gel	Volume per 2.6 mL gel
1 M TrisHCl, pH 6.8	0.30 mL
30 % (w/v) acrylamide	0.37 mL
2 % (w/v) methylamine bisacrylamide	0.15 mL
Distilled water	1.74 mL
10 % (w/v) SDS	0.02 mL
10 % (w/v) APS	0.02 mL
TEMED	0.01 mL

Table 2.5.3: Components used for 10 x electophoresis buffer and its corresponding weights per litre.

10 x Electrophoresis buffer	Weight per litre
50 mM Tris, pH 8.3	30.0 g
196 mM Glycine	144.0 g
0.1 % (w/v) SDS	10.0 g
Distilled water to 1 L	

Table 2.5.4: Components used for the SDS-PAGE gel electrophoresis loading buffer and its corresponding volumes for a 10 mL solution.

SDS loading dye	Volume per 10 mL
Tris 0.5M, pH 6.8	2.5 mL
Glycerol	2.0 mL
2-mercaptoethanol	0.5 mL
20 % (w/v) SDS	2.5 mL
20 ppm Bromophenol blue	0.2 mg
Distilled water	2.5 mL

Table 2.5.5: Components used for the coomassie stain and its corresponing volumes and weights per litre

Coomassie Stain	Volume or weights per litre
Coomassie blue R-250	1.0 g
Methanol biotechnology grade, 99.93 %	450 mL
Distilled water	450 mL
Acetic acid *ReagentPlus*®, ≥ 99 %	100 mL

Table 2.5.6: Components used for the coomassie de-stain solution and its corresponding volumes per litre.

De-stain solution	Volume (mL) per litre
Acetic acid *ReagentPlus*®, ≥ 99 %	200 mL
Methanol biotechnology grade, 99.93 %	400 mL
Distilled water	600 mL

Table 2.5.7: Transfer buffer components for Western blotting and its volumes per litre

Transfer buffer	Volume (mL) per litre
1 x electrophoresis buffer (Table 2.5.4)	800 mL
Methanol biotechnology grade, 99.93 %	200 mL

2.6 Commercial kits

Table 2.6.1: Commercial kits used over the course of this research and their associated suppliers.

Kit	Supplier
Superscript III reverse transcriptase kit	Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008, USA.
Perfectprep Gel Cleanup kit	Eppendorf AG, Barkhausenweg 1, Hamburg 22339, Germany.
Wizard Plus SV Miniprep™ kit	Promega, 2800 Woods Hollow Road, Madison, WI 53711, USA.
QIAquick™ gel extraction kit	Qiagen, 28159 Avenue Stanford, Valencia, CA 91355, USA.
Pierce™ "Eggcellent" kit	Fisher Scientific, Suite 3, Plaza 212, Blanchardstown corporate park 2, Ballycoolin, Dublin 15,

	Ireland.
Invitrogen Superscript™ III reverse transcriptase kit	Invitrogen Corporation, 791 Van Allen Way, Carlsbad, CA 92008, USA .
BIAcore™ CM5 dextran chips and immobilisation solutions	GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden.

2.7: Lists of commercial antibodies, enzymes and chemicals used during this research.

2.7.1 Commercial antibodies

Table 2.7.1: Commercial antibodies used over the course of this research and the associated suppliers.

Antibodies	Supplier
Anti-M13 antibody (HRP-labelled)	GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden.
Mouse anti-HA (HRP-labelled) mAb	Roche Diagnostics, Grenzacherstrasse 124, Basel 4070, Switzerland
Anti-histidine (HRP-labelled) mAb	Sigma Aldrich, 3050 Spruce Street St. Louis, MO 63103, USA.
Anti-chicken (HRP-labelled) mAb	Sigma Aldrich 3050 Spruce Street St. Louis, MO 63103, USA.
Anti- chicken (AP-labelled) mAb	Sigma Aldrich 3050 Spruce Street, St. Louis, MO 63103, USA.
Anti- mouse (HRP-labelled)	Sigma Aldrich 3050 Spruce Street

	St. Louis, MO 63103, USA.
Anti-GFP mAb	Sigma Aldrich 3050 Spruce Street St. Louis, MO 63103, USA.

2.7.2 Commercial Enzymes

Table 2.7.2: Commercial enzymes used over the course of this research and the associated suppliers.

Enzymes	**Supplier**
Restriction enzymes *Sfi*1, *Bspe*1,*Bsiw*1	New England BioLabs, 75-77 Knowl Piece, Wilbury Way, Hitchin, SG4 OTY,
Antarctic phosphatase	United Kingdom. New England BioLabs, 75-77 Knowl Piece, Wilbury Way, Hitchin, SG4 OTY,
T4 Ligase	United Kingdom. New England BioLabs, 75-77 Knowl Piece, Wilbury Way, Hitchin, SG4 OTY, United Kingdom.

Dream Taq™	Fisher Scientific, Suite 3, Plaza 212, Blanchardstown corporate park 2, Ballycoolin, Dublin 15, Ireland.
Phusion Taq	New England BioLabs, 75-77 Knowl Piece, Wilbury Way, Hitchin, SG4 OTY, United Kingdom.
Green Taq Polymerase	Invitrogen Corporation, 791 Van Allen Way, Carlsbad, CA 92008, USA

2.7.3 Chemicals

able 2.7.3: Miscellaneous chemicals used over the course of this research and the associated suppliers. All chemicals were supplied by Sigma Aldrich unless otherwise stated.

Chemicals	Supplier
Rnase Zap™	Sigma Aldrich
Chlorform	3050 Spruce Street
Trizol™	St. Louis,
Isoproponal (2-proponal)	MO 63103,
Glycerol	SG4 OTY,
Polyethylene Glycol	USA.
Bovine Serum Albumin	
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	
$MgSO_4$	.
Imidazole	
Tween 20	
Freunds Adjuvant Complete	
Freunds Adjuvant Incomplete	
Protein G Fast flow	
Ammonium sulphate	
NaCl	
C18 sorbent	
Aceto Nitrile	
DMSO	
Ethanol	
Methanol	
EDTA	
Kanamycin	
Carbencillin	
Tris [2-carboxyethyl]phosphine hydrochloride and Sulfo GMBS	Fisher Scientific, Suite 3, Plaza 212, Blanchardstown corporate park 2, Ballycoolin, Dublin 15.

2.8 Generation and characterisation of mouse anti-TBZ Fab phage library

2.8.1 Recovery of anti-TBZ hybridoma cell line.

Anti-TBZ hybridoma (mouse/huma) cells donated by Dr. David Brandon of the United States Department of Agriculture (USDA) were recovered from liquid nitrogen frozen stocks and transferred to a T25 culture vessel containing supplemented RPM1 1640 (section 2.2). The flasks were incubated at 37ºC in a humidified 5 % (v/v) CO_2 incubator. Cells were allowed to grow until they reached 70 % confluency in T25 flasks, the cells were removed from the flask surface by vigorous pipetting and pelleted by centrifugation at 2,000 x g for 10 min (Hereus Chist, Labofuge 6000) at room temperature (R.T.) and re-suspended in 2 mL fresh media. The re-suspended cells were transferred to two T75 culture flasks containing 14 mL supplemented media. Flasks were incubated at 37ºC until greater than 70 % cell viability was reached i.e. until the flask surface was covered with more than 70 % of cells.

This method was adapted from Alfredo Sheehan, (2006).The production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the ß-lactam antibiotic, cephalexin, in milk. PhD thesis, DCU, Ireland.

2.8.2 Iso-typing of the anti-TBZ monoclonal supernatant.

Supernatant from the hybridoma cells was collected by continuously culturing cells in RPM1 1640 media. This was stored at 4°C with the addition of sodium azide (0.02 %, (w/v)). The sample was diluted 1:100 by adding 5 µL of supernatant to 495 µL of sample diluent provided. To each cassette 150 µL of the diluted sample was applied to each of the wells. A successful test will results in a red band at the "C" or control location and another band at one of the three iso-types location on each cassette. (Product insert instruction for Pierce Rapid Antibody Isotyping kit.)

2.8.3 Ammonium sulphate precipitation of anti-TBZ monoclonal antibody for protein-G purification.

Ammonium sulphate (99 % purity) was added to the monoclonal culture supernatant to a final concentration of 45 % (w/v). The mixture was stirred at 85 x g at R.T. for 30 min. The resulting precipitate was centrifuged at 4,500 x g for 20 min at 4°C and the pellet re-suspended with $1/5^{th}$ of the original volume of PBS containing 0.15 M NaCl, pH 7.4. Ammonium sulphate was added to a final concentration of 40 % (w/v), and stirred at 85 rpm until fully precipitated the mixture was centrifuged at 4,500 g at 4°C for 20 min. The resulting pellet was re-suspended with 1/2 of the original volume of PBS containing 0.15 M

NaCl, pH 7.4 and dialysed overnight with 5 L of PBS containing 0.15 M NaCl, pH 7.4. (Darcy *et al.*, 2010)

2.8.4: Purification of the anti-TBZ monoclonal antibody via protein G.

A 2 mL volume of Protein G Fast Flow sepharose in 20 % (v/v) ethanol was added into a clean column to settle, and any excess ethanol was drained off without letting the column run dry. The column was equilibrated with approximately 20 mL of sterile-filtered PBS containing 0.5 M NaCl, pH 7.4. The dialysed ammonium sulphate precipitated immunoglobulin (2 mL) was added to the column and the flow rate adjusted 1 mL/min. The flow-though was collected and applied to the column two more times. Wash buffer PBS (25 mL) containing 0.3 M NaCl, pH 7.4 though the column. The affinity captured antibody was eluted with 0.1 M glycine-HCl buffer (pH 2.5) and collected in 500 µl fractions. This was neutralised immediately with 150 µL of 2 M Tris/HCl, pH 8.7 (Darcy *et al.*, 2010)

2.8.5 Extraction and isolation of total RNA from anti-TBZ hybridoma cell line.

A Gelaire BSB 4 laminar unit was sprayed with both IMS and RNase™ ZAP. The cells secreting the TBZ monoclonal antibodies were pelleted from 2 x T75 flasks containing 15 mL of culture at 1,500 x g, at R.T. for 10 min (Hereus Chist, Labofuge 6000). The resulting pellet was re-suspended with 1 mL of Trizol™, transferred to 2 mL RNase-free Eppendorf tubes and incubated for 5 min at R.T. Chloroform (200 µL) was then added to the tube containing the supernatant, thoroughly mixed and incubated for 15 min at R.T. The addition of chloroform leads to the separation of the cells into an upper aqueous phase (containing RNA) and a lower organic phase (containing DNA and protein). The resultant mixture was centrifuged at 17,500 x g (Eppendorf centrifuge 5810R) for 15 min at 4°C. The centrifugation produced 3 layers, consisting of a lower red phenol/chloroform phase, a protein interphase and a colourless liquid upper phase containing the RNA. The upper aqueous phase was carefully transferred (ensuring no lower layer contamination) into a 2 mL tube and subjected to RNA precipitation by addition of 0.5 mL of isopropanol. The RNA was allowed to precipitate for 10 min at R.T. and then centrifuged at 17,500 x g (Eppendorf centrifuge 5810R) for 10 min at 4°C, resulting in a white RNA pellet. The isopropanol supernatant was carefully decanted and the pellet washed with 1 mL of 75 % (v/v) ethanol by centrifugation at 17,500 x g for 10 min at 4°C. Finally, the pellet was allowed to air dry in the laminar hood before re-suspension in 50-100 µL of molecular grade water. Method adapted from Alfredo Sheehan**,** (2006)**.** *The production of polyclonal, monoclonal and genetically-derived scFv*

*antibody fragments for the detection of the ß-lactam antibiotic, cephalexin, in milk***.** PhD thesis, DCU, Ireland**.**

2.8.6 Reverse transcription of total TBZ RNA to cDNA.

Following extraction the RNA was converted to cDNA using the commercial reverse transcription kit Superscript™ III. Method was taken from the product insert provided. This kit is fully optimized to synthesize first strand cDNA from total RNA.

Table 2.8.6.1: Components of mixture 1 for the reverse transcription of RNA to cDNA.

Mixture 1 Component	10 µL Volume	Conc. in 10 µL reaction
Total RNA	5.0 µL	5.0 µg
Oligo (dT) primer	1.0 µL	0.5 µg
dNTP mix	1.0 µL	1.0 mM
Molecular grade H_2O	3.0 µL	N/A

Table 2.8.6.2: Components of mixture 2 for the reverse transcription of RNA to cDNA.

Mixture 2 Component	10 µL Volume	Conc. in 10 µL reaction
10 X RT Buffer	2.0 µL	2 x
$MgCl_2$	4.0 µL	2.5 mM
DTT	2.0 µL	20.0 mM
RNase Out	1.0 µL	40 U
Superscript III enzyme	1.0 µL	200 U

A 20 x reaction for mixture 1 was set up on ice and then split up into 8 x 25 µL aliquots in sterile PCR tubes. The 8 PCR tubes were incubated at 65°C for 5 min and placed on ice, whilst a 20 x reaction for mixture 2 was set up. Next, 25 µL of the incubated mixture 2 was added to all 8 of the 25 µL aliquots of mixture 1 and left to incubate at 50°C for 50 min, followed by 85°C for 5 min. Finally, 5 µL of RNase ™ H was added to each of the 8 x 50 µL reactions and incubated at 37°C for 20 min. The addition of RNase H increases the sensitivity of this step by removing the RNA template from the cDNA: RNA hybrid by digestion.

2.9 Construction of the anti-TBZ Fab murine library

2.9.1 PCR primers and illustration of the amplification of mouse Fab (pComb series)

The primers listed below were obtained from Eurofins-MWG-Operon (318 Worple Road, Raynes Park, London, SW20 8QU) and are compatible with the primers set described by Barbas and co-workers (Barbas *et al.*, 2001) for the pComb vector system. All methods described from 2.9.1-2.9.13 were adapted from Barbas *et al.*, 2001.

Variable light chain 5´ Sense Primers

MSCVK-1

5´ GGG CCC AGG CGG CCG AGC TCG AYA TCC AGC TGA CTC AGC C 3´

MSCVK-2

5´ GGG CCC AGG CGG CCG AGC TCG AYA TTG TTC TCW CCC AGT C 3´

MSCVK-3

5´ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGM TMA CTC AGT C 3´

MSCVK-4

5´ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGY TRA CAC AGT C 3´

MSCVK-5

5´ GGG CCC AGG CGG CCG AGC TCG AYA TTG TRA TGA CMC AGT C 3´

MSCVK-6

5´ GGG CCC AGG CGG CCG AGC TCG AYA TTM AGA TRA MCC AGT C 3´

MSCVK-7

5´ GGG CCC AGG CGG CCG AGC TCG AYA TTC AGA TGA YDC AGT C 3´

MSCVK-8

5´ GGG CCC AGG CGG CCG AGC TCG AYA TYC AGA TGA CAC AGA C 3´

MSCVK-9

5´ GGG CCC AGG CGG CCG AGC TCG AYA TTG TTC TCA WCC AGT C 3´

MSCVK-10

5´ GGG CCC AGG CGG CCG AGC TCG AYA TTG WGC TSA CCC AAT C 3´

MSCVK-11

5´ GGG CCC AGG CGG CCG AGC TCG AYA TTS TRA TGA CCC ART C 3´

MSCVK-12

5´ GGG CCC AGG CGG CCG AGC TCG AYR TTK TGA TGA CCC ARA C 3´

MSCVK-13

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGA TGA CBC AGK C 3′

MSCVK-14

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGA TAA CYC AGG A 3′

MSCVK-15

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGA TGA CCC AGW T 3′

MSCVK-16

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGA TGA CAC AAC C 3′

MSCVK-17

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTT TGC TGA CTC AGT C 3′

Variable light chain 3′ Reverse Primers

MHybJK12-B

5′ AGA TGG TGC AGC CAC AGT TCG TTT KAT TTC CAG YTT GGT CCC 3′

MHybJK4-B

5′ AGA TGG TGC AGC CAC AGT TCG TTT TAT TTC CAA CTT TGT CCC 3′

MHybJK5-B

5′ AGA TGG TGC AGC CAC AGT TCG TTT CAG CTC CAG CTT GGT CCC 3′

Variable 5′ heavy chain sense primer

MHyVH1

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTR MAG CTT CAGGAGTC 3′

MHyVH2

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTB CAG CTB CAG CAGTC 3′

MHyVH3

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTG CAG CTG AAG SAS TC 3′

MHyVH4

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTC CAR CTG CAA CAR TC 3′

MHyVH5

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTY CAG CTB CAG CARTC 3′

MHyVH6

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTY CAR CTG CAG CAGTC 3′

MHyVH7

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTC CAC GTG AAG CAGTC 3′

MHyVH8

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTG AAS STG GTG GAA TC 3′

MHyVH9

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTG AWG YTG GTG GAG TC 3′

MHyVH10

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTG CAG SKG GTG GAG TC 3′

MHyVH11

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTG CAM CTG GTG GAG TC3′

MHyVH12

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTG AAG CTG ATG GAR TC 3′

MHyVH13

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTG CAR CTT GTT GAG TC 3′

MHyVH14

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTR AAG CTT CTC GAG TC 3′

MHyVH15

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTG AAR STT GAG GAG TC 3′

MHyVH16

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTT ACT CTR AAA GWG TST G 3′

MHyVH17

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTC CAA CTV CAG CAR CC 3′

MHyVH18

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTG AAC TTG GAA GTG TC 3′

MHyVH19

5′ GCT GCC CAA CCA GCC ATG GCC CTC GAG GTG AAG GTC ATC GAG TC 3′

V_H 3′ heavy chain reverse primers

MHyIgGCH1-B1

5′ CGA TGG GCC CTT GGT GGA GGC TGA GGA GAC GGT GAC CGT GGT 3′

MHyIgGCH1-B2

5′ CGA TGG GCC CTT GGT GGA GGC TGA GGA GAC TGT GAG AGT GGT 3′

MHyIgGCH1-B3

5′ CGA TGG GCC CTT GGT GGA GGC TGC AGA GAC AGT GAC CAG AGT 3′

MHyIgGCH1-B4

5′ CGA TGG GCC CTT GGT GGA GGC TGA GGA GAC GGT GAC TGA GGT 3′

Primers for the amplification of the human constant region and the pelB leader sequence from a cloned human Fab

HKC-F (sense)

5′ CGA ACT GTG GCT GCA CCA TCT GTC 3′

Lead-B (reverse)

5′ GGC CAT GGC TGG TTG GGC AGC 3′

Primers for amplification of the human CH1 chain from a cloned human Fab

HIgGCH1-F (sense)

5′ GCC TCC ACC AAG GGC CCA TCG GTC 3′

dpseq (reverse)

5′ AGA AGC GTA GTC CGG AAC GTC 3′

Primers for PCR assembly of mouse VL sequences with the human C. PCR product.

RSC-F (sense)

5′ GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC 3′

Lead-B (reverse)

(see above)

Primers for PCR Assembly of Mouse VH sequences with the Human CH1 PCR product.

leadVH (sense)

5′ GCT GCC CAA CCA GCC ATG GCC 3′

dpseq (reverse)

(see above)

Primers for PCR assembly of chimeric light-chain Fab sequences with chimeric heavy-chain (Fd) Sequences.

RSC-F (sense)

(see above)

dp-EX (reverse)

5′ GAG GAG GAG GAG GAG GAG AGA AGC GTA GTC CGG AAC GTC 3′

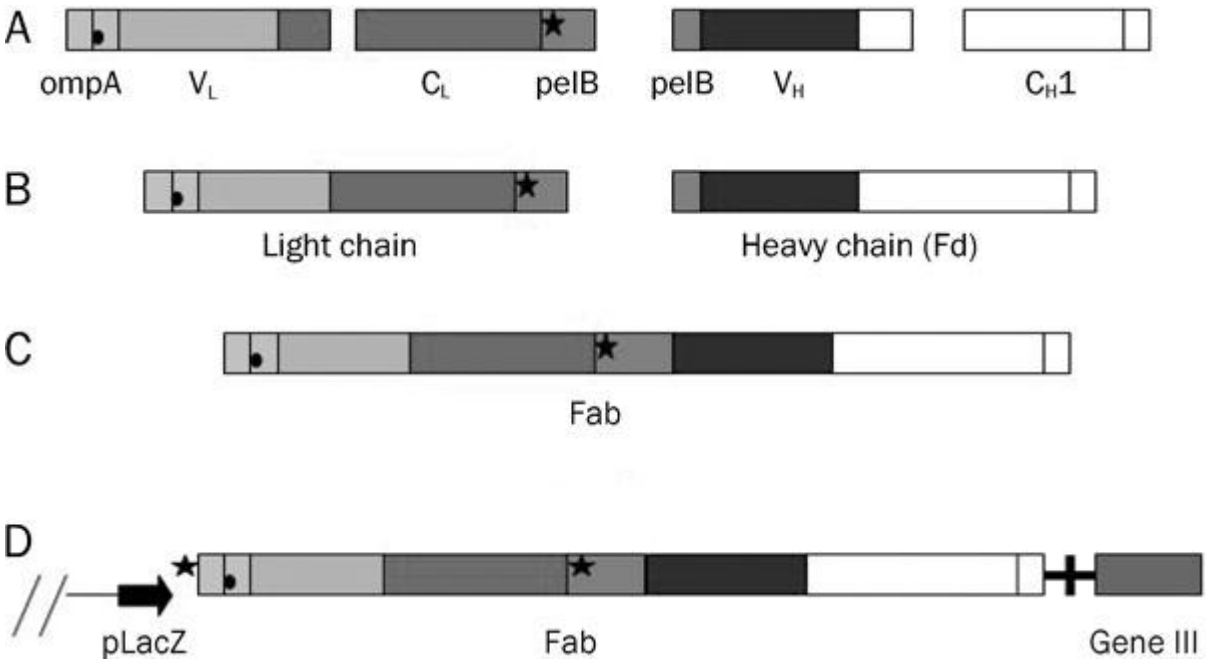


Figure 2.9.1: Schematic diagram illustrating the combination of genes by splice by overlap extension for the construction of a Fab fragment. The genes for the variable and constant regions are amplified separately. (B) Heavy-chain Fd and light chain DNA are assembled by variable regions and their constant counterpart respectively using over lap PCR. (C) Fd and light chain are fused to form Fab encoding sequences by over lap PCR. (D) Fab Genes are directionally cloned into Pcomb 3x phagemid vector where both the L chain fragment and Fd region are transported to the periplasmic space (Li *et a*l., 2010)

2.9.2 Amplification of antibody variable domain genes using the pComb primer series.

The following protocols describe the construction of a chimeric mouse/human Fab library by overlap extension PCR. It allows the expression and selection of chimeric Fabs that contain variable regions obtained from the mouse hybridoma, and light and heavy chains constant regions from a clone human Fab.

A 1 x reaction mix for amplification of the variable domains was formulated using the following components:

Table 2.9.1: Components of the PCR for the amplification of the murine variable domains and their subsequent volumes and concentrations in 50 µL.

Component	50 µL total volume	Conc in 50 µL reaction
5 x Buffer	10.0 µL	1 x
V_L/V_H Forward Primer	0.6 µL	60.0 pM
V_H/V_L Back Primer	0.6 µL	60.0 pM
cDNA	1.0 µL	1.0 µg
dNTP	2.0 µL	1.0 mM
$MgCl_2$	6.0 µL	3.0 mM
H_2O	28.8 µL	N/A
Green Taq ® Polymerase	1.0 µL	1U

The PCR for the amplification of the mouse antibody variable domain genes was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions:

Table 2.9.2: The PCR programme for the amplification of the murine variable domains.

Stage	Temp (^{o} C)	Time (seconds)
1 (1 cycle)	94	300
2 (30 cycle)	94	15
	56	30
	72	120
3 (1 cycle)	72	600
	4	600

2.9.3 Amplification of the constant kappa domain.

The constant kappa (C_κ) and pelB leader sequence was amplified from the pComb3xTT. The sense primer was specific for the 5' region of the human C_κ, the region used in the overlap extension PCR to create the chimeric light chain. The reverse primer was specific for the 3' end of the pelB leader sequence.

Table 2.9.3.1: Components of the PCR for the amplification of the human constant domains and their subsequent volumes and concentrations in 50 µL.

Component	50 µL total volume	Conc in 50 µL reaction
5 x Buffer	20.0 µL	2 x
HKC Forward Primer	0.6 µL	60.0 pM
Lead B Back Primer	0.6 µL	60.0 pM
pComb3xTT	1.0 µL	1.0 µg
dNTP	2.0 µL	1.0 mM
$MgCl_2$	10.0 µL	5.0 mM
H_2O	14.8 µL	N/A
Green Taq ® Polymerase	1.0 µL	1U

Table 2.9.3.2: The PCR programme for the amplification of the human constant domains.

Stage	Temp (^oC)	Time (seconds)
1 (1 cycle)	94	300
2 (30 cycle)	94	15
	56	30
	72	120
3 (1 cycle)	72	600
	4	600

2.9.4 Amplification of the human gamma chain.

The constant domain C_H1 was amplified from the pComb3xTT vector. The sense primer was specific for the 5' region of the C_H1 region that was used in the overlap extension PCR to create the heavy-chain (Fd) fragment. The reverse primer was specific for the decapeptide sequence that is located downstream of the C_H1 fragment and the 3´ *Sfi*1 site of pComb3xTT.

Table 2.9.4.1: Components of the PCR for the amplification of the human gamma chain and their subsequent volumes and concentrations in 50 µL.

Component	50 µL total volume	Conc in 50 µL reaction
5 x Buffer	10.0 µL	1 x
HIgGCH1-F Forward	0.6 µL	60.0 pM
dpseq Back	0.6 µL	60.0 pM
pComb3xTT	0.2 µL	1.0 µg
dNTP	2.0 µL	1.0 mM
$MgCl_2$	3.0 µL	1.5 mM
H_2O	32.6 µL	N/A
Green Taq ® Polymerase	1.0 µL	1U

Table 2.9.4.2: The PCR programme for the amplification of the human gamma chain.

Stage	Temp (^oC)	Time (seconds)
1 (1 cycle)	94	300
2 (30 cycle)	94	15
	56	30
	72	120
3 (1 cycle)	72	600
	4	600

2.9.5 Purification of VH and VL variable gene fragments using the Promega clean-up kit.

The V_H and V_L PCR products were resolved on a 1 % (w/v) agarose gel until single bands for both the variable heavy and variable light chains were observed. Both bands were excised using sterile scalpels and transferred into clean 1.5 mL micro-centrifuge tubes. Next, thee volumes of binding buffer was added for every one volume of gel slice and incubated at 50°C until the gel fragments had completely dissolved (approximately 10 min). The binding buffer contains guanidine isothiocyanate that allows for sufficient binding of the DNA to the silica membrane columns. One volume of isopropanol, equal to the original of the gel slice volume, was added and mixed to allow for precipitation of the DNA. The mixture was transferred to a binding column and collection tube and centrifuged at 17,500 x g (Eppendorf centrifuge 5810R) for one min to remove any residual buffer. The "flow-through" was discarded and the

column was washed first by centrifugation with 750 µL wash buffer and then with 250 µL wash buffer. The column was centrifuged at 17,500 x g (Eppendorf centrifuge 5810R) for 2 min to ensure all residual ethanol was removed and the DNA was eluted from the column using 50 µL molecular grade H_2O and was subsequently quantified using the Nanodrop™ ND-1000.

2.9.6 Splice by Overlap extension (SOE) PCR.

Gene Splicing by Overlap Extension or "gene SOEing" is a PCR-based method of recombining DNA sequences without reliance on restriction sites and of directly generating mutated DNA fragments in vitro. By modifying the sequences incorporated into the 5'-ends of the primers, any pair of polymerase chain reaction products can be made to share a common sequence at one end. Under polymerase chain reaction conditions, the common sequence allows strands from two different fragments to hybridize to one another, forming an overlap. Extension of this overlap by DNA polymerase yields a recombinant molecule (Horton *et al.*, 1990). The mouse V_L PCR products, that have a human constant sequence tail, were combined with the human constant–pelB products to create chimeric light-chain–pelB fragments. The sense extension primer (RSC-F) recognizes the sequence created by the first-round PCR V_L primers. The lead-B reverse primer recognizes the 3´ end of the pelB leader sequence that was amplified together with the human constant region from pComb3xTT. The 3´ end of the pelB sequence serves as the overlap region in the final overlap extension PCR.

The mouse V_H PCR products, that have a human C_H1 sequence tail, are combined with the human C_H1 PCR products to create the chimeric Fd fragment. The sense extension primer (leadV_H) recognizes the pelB leader sequence tail created by the first-round PCR V_H primers. The same reverse primer (dpseq) that was used for the generation of the C_H1 fragment was used in the overlap extension PCR of the heavy-chain fragment. The 3´ end of the pelB leader sequence served as the overlap region in the final overlap extension PCR.

Table 2.9.6.1: Components of the PCR for the overlap extension of the variable and constant light chains and their subsequent volumes and concentrations in 50 µL.

Component	50 µL total volume	Conc in 50 µL reaction
5 x Buffer	10.0 µL	1 x
Forward primer	0.6 µL	60.0 pM
Back Primer	0.6 µL	60.0 pM
V_L chain	1.5 µL	100 ng
C_L chain	2.5 µL	100 ng
dNTP	2.0 µL	1.0 mM
$MgCl_2$	3.0 µL	1.5 mM
H_2O	28.8 µL	N/A
Green Taq ® Polymerase	1.0 µL	1U

Table 2.9.6.2: Components of the PCR for the overlap extension of the variable and constant heavy chains and their subsequent volumes and concentrations in 50 µL.

Component	50 µL total volume	Conc in 50 µL reaction
5 x Buffer	10.0 µL	1 x
Forward primer	0.6 µL	60.0 pM
Back Primer	0.6 µL	60.0 pM
V_H chain	2.4 µL	100 ng
C_H chain	0.4 µL	100 ng
dNTP	1.0 µL	1.0 mM
$MgCl_2$	10.0 µL	5.0 mM
H_2O	23.0 µL	N/A
DMSO	1.0 µL	N/A
Green Taq ® Polymerase	1.0 µL	1U

Table 2.9.6.3: The PCR programme for the splice by overlap extension of the variable and constant genes.

Stage	Temp (^{o}C)	Time (seconds)
1 (1 cycle)	94	300
2 (20 cycle)	94	15
	56	30
	72	120
3 (1 cycle)	72	600
	4	600

2.9.7 Final splice by overlap extension (SOE).

The final overlap extension PCR combined the chimeric light chain–pelB fragment and the Fd fragment for the construction of the final mouse chimeric Fab library. The 3´end of the pelB leader sequence served as the overlap region for the two PCR products. The sense extension primer (RSC-F) used in this round of PCR was also used in the overlap extension amplification of the light chain and the reverse extension primer recognizes the decapeptide sequence downstream of the C_H1 region.

Table 2.9.7.1: Components of the PCR for the final overlap extension of the variable and constant heavy chains and their subsequent volume and concentration in 50 µL.

Component	50 µL total volume	Conc in 50 µL reaction
5 x Buffer	10.0 µL	1 x
Forward primer	0.6 µL	60.0 pM
Back Primer	0.6 µL	60.0 pM
V_{L+H} chain	1.5 µL	100 ng
C_{L+H} chain	2.5 µL	100 ng
dNTP	2.0 µL	1.0 mM
$MgCl_2$	10.0 µL	5.0 mM
H_2O	20.3 µL	N/A
DMSO	1.5 µL	N/A
Phusion Taq® Polymerase	1.0 µL	1U

Table 2.9.7.2: The PCR programme for the final splice by overlap extension.

Stage	Temp (^{o}C)	Time (seconds)
1 (1 cycle)	94	300
2 (15 cycle)	94	15
	56	30
	72	120
3 (1 cycle)	72	600
	4	600

2.9.8 SfiI restriction digests of the purified SOE-PCR fragment and ligation into the pComb3xSS vector.

The Fab fragment and pComb3xSS vector used for phage selection were digested using *Sfi*I restriction enzyme. The *Sfi*I enzyme allows for the unidirectional cloning of Fab fragment into the pComb phage display vector. The enzyme recognises 8 bases which are interrupted by 5 non-recognised nucleotides (5′ggccnnnnnggcc3′), thus virtually eliminating internal digestion in antibody sequences. Both digestions (outlined below) were carried out for 5 h at 50°C.

Table 2.9.8.1: Components for the restriction digest of the Fab PCR product and pComb vector.

SOE-PCR product Sfi*I *digest		***pComb vector Sfi*I *digest***	
Component	**150 µL volume**	**Component**	**50 µL volume**
SOE product	90.0 µL	pComb vector	18.9 µL
10 x Buffer 2	15.0 µL	10 x Buffer 2	5.0 µL
100 x BSA	1.5 µL	100 x BSA	0.5 µL
H_20	31.5 µL	H_20	7.6 µL
*Sfi*I	12.0 µL	*Sfi*I	18.0 µL

Following a 5 h incubation at 50°C, the digested pComb3xSS vector was treated with Antarctic phosphatase™ enzyme to prevent self-ligation. This enzyme catalyzes the removal of 5′ phosphate groups from DNA. The loss of the 5′ phosphoryl termini, therefore, prevents the ligase enzyme from working. To the digested vector 5 µL of Antarctic phosphatase buffer (10x) followed by 2 µL of antarctic phosphatase enzyme.

The mixture was incubated for 15 min at 37°C and then the enzyme was heat inactivated at 65°C for 5 min. Both digests were resolved via electrophoresis on a 1 % (w/v) agarose gel for the digested PCR product and a 0.6 % (w/v) gel for the digested vector. These were gel-purified, as described in section 2.9.6. The restricted Fab gene was then ligated into the pComb3xSS vector in a 1:2 (insert:vector) ratio under the conditions below and left at R.T. O/N.

Table 2.9.8.2: Components for the ligation of the Fab gene fragment into the pComb3xSS vector

Component	100 µL volume	Conc in 100 µL reaction
5 x ligase buffer	10.0 µL	1 x
Digested pComb3xSS	14.8 µL	1.4 µg
Digested Fab gene	37.8 µL	0.7 µg
H_2O	32.4 µL	N/A
T_4 DNA Ligase	5.0 µL	400 U

Following ligation the mix was ethanol precipitated using the following reagents.

Table 2.9.8.3: Components for the ethanol precipitation of the ligated gene fragment.

Component	Volume
Ligation mixture	100 µL
3M sodium acetate, pH 5.2	10 µL
100 % (v/v) ethanol	250 µL

The precipitation was incubated O/N at -20°C and the DNA was harvested by centrifugation at 17,500 x g (Eppendorf centrifuge 5810R) for 20 min at 4°C. The ethanol supernatant was decanted and the pellet was washed by addition of 1 mL of 70 % (v/v) ethanol and centrifuging at 17, 500 x g (Eppendorf centrifuge 5810R) for 20 min at 4°C. The pellet was allowed to air-dry briefly and dissolved in 20 µL of molecular grade H_2O.

2.9.9 Electro-transformation of Fab-containing plasmid into XL1-Blue E. coli cells.

Transformation of the ligated product was performed by electroporation into a commercially available strain of XL1-Blue *E. coli* cells (Stratagene). The apparatus used was a Gene Pulser Xcell ™ electroporation system with the parameters set to 25 µF, 1.25 kV and gene pulse controller at 200 Ω. After thawing on ice, 50 µL of the *E. coli* cells were added to the 5 µL of ligation product, mixed and left on ice for a 1 min incubation. The mixture was transferred to a chilled 0.2 cm electroporation cuvette, and placed into the 'Shockpod'. A pulse was passed though the cuvette, and immediately flushed with 1 mL of pre-warmed SOC medium using a sterile tip. The 1 mL of electroporated cells were transferred into a sterile 50 mL Falcon tube containing a further 2 mL of pre-warmed SOC medium. The 3 mL of transformant suspension was incubated for 1 h at 250 x g at 37°C and plated out on SB agar plates supplemented with 100 µg/mL carbenicillin. A negative control was also incorporated into the experiment by plating the XL1-Blue cells containing no plasmid onto the agar plates. All plates were incubated O/N at 37°C and scraped into SB medium containing 20 % (v/v) glycerol for long term storage at -80°C.

2.9.10 Phage rescue and precipitation of TBZ Fab library.

A conical flask containing 600 mL of sterile SB medium (100 µg/mL carbenicillin) was inoculated with 600 µL of the transformed library glycerol stocks and incubated at 220 rpm and 37°C until the O.D. reached 0.4 at 600nm. Helper phage (2 x 10^{11} cfu) was added. The culture was left static at 37°C for 30 min before transferring it to a shaking incubator at 37°C at 220 rpm for 1.5 h. Kanamycin was added to a final concentration of 50 µg/mL, before leaving the culture O/N at 37°C and 220 rpm. The O/N culture was centrifuged at 18,500 x g (Eppendorf centrifuge 5810R) for 20 min at 4°C and the phage supernatant transferred into 3 sterile 250 mL Sorvals™ centrifuge tubes containing 8 g of PEG 8,000 and 6 g of sodium chloride. The Sorvals™ were agitated at 220 rpm until the PEG/NaCl went into solution. The phage was precipitated for 1 h at 4°C. The phage was harvested by centrifugation at 6,440 x g for 25 min at 4°C and re-suspended in 2 mL of 1 % (w/v) BSA-PBS solution. The re-suspended phage pellets were added to a clean 1.5 mL micro-centrifuge tube and centrifuged at 20,000 x g for 5 min at 4°C to remove any bacterial debris. The remaining supernatant was refrigerated until ready for use.

2.9.11 Panning of the TBZ phage library against immobilized conjugates.

An immuno-tube (Maxisorp™, Nunc) was coated O/N at 4°C with 500 µL of 50 µg/mL TBZ-BSA in PBS. The tube was blocked for 1 h at 37°C with 3 % (w/v) BSA in PBS. Following one wash with PBST, 500 µL of the rescued phage (input phage) (Section 2.9.10) was added to the immuno-tube and incubated on a roller for 2 h at R.T. The immuno-tube was washed with PBST (x3) and PBS (x3) to remove non-specific phage. Specific phage was eluted by incubation with 1 mL of 10 mg/mL of trypsin in PBS for 30 min at 37°C. The trypsin eluted phage was removed after harsh pipetting and 800 µL was added to 5 mL of mid-exponential phase XL1-Blue cells. This was allowed to infect for 15 min static at R.T. and 6 mL of pre-warmed SB media containing 1.6 µL of 100 mg/mL carbenicillin and 12 µL of 5 mg/mL tetracycline was added (at this point 100 µl sample was removed for output titre determination). This 12 mL culture was incubated at 220 rpm at 37°C for 1 h. Carbenicillin (2.4 µL of 100 mg/mL) was added and incubated at 220 rpm at 37°C for 1 h. To this 12 mL culture, 800 µL of commercial helper phage (New England Biolabs) was added, followed by 88 mL of SB media supplemented with carbenicillin and tetracycline (final concentrations of 50 µg/mL and 10 µg/mL, respectively) and incubated for 2 h at 37°C at 220 rpm. Kanamycin was added to a final concentration of 70 µg/mL and incubated O/N at 220 rpm and 37°C.
An output titre was performed by making a 1 in 10 and 1 in 100 dilution of the XL1-Blue infected cells in SB media. These dilutions were plated out on SB agar plates containing 100 µg/mL of carbenicillin and incubated O/N at 37°C.

An input titre was performed by making serial dilutions (10^{-1}-10^{-12}) of the PEG-precipitated phage in exponential growth phase XL1-Blue cells. Following a 15 min infection at 37°C, the 10^{8} - 10^{12} serial dilutions were plated out on SB agar plates containing 100 µg/mL carbenicillin and incubated O/N at 37°C. All of the subsequent rounds of panning were performed as described above.

Table 2.9.11.1: Parameters varied during the panning of murine anti-TBZ Fab library.

Variables	Pan 1	Pan 2	Pan 3
Culture Volume	600 mL	100 mL	100 mL
TBZ-BSA coating concentration	100 µg/mL	50 µg/mL	25 µg/mL
Washes	3 x PBST, 3 x PBS	3 x PBST, 3 x PBS	3 x PBST, 3 x PBS

2.9.12 Fab gene insert check via 'colony-pick' PCR.

Single colonies (10) were randomly selected from all thee rounds of panning. A colony-pick' PCR was performed to ensure the vector incorporated the Fab fragment. A sterile tip was used to pick a single colony into the mixture described in 2.9.14.1, which was placed in a Biometra $T_{GRADIENT}$ PCR machine. The amplified Fab fragments were analysed via gel electrophoresis on a 1 % (w/v) agarose gel.

Table 2.9.12.1: Components of the PCR for the amplification of the Fab gene insert from a single colony and their volumes and concentrations in a 50 µL reaction.

Component	**50 µL total volume**	**Conc in 50 µL reaction**
10 x Buffer HF	10.0 µL	1 x
Forward primer	0.6 µL	60.0 pM
Back Primer	0.6 µL	60.0 pM
dNTP	2.0 µL	1.0 mM
$MgCl_2$	10.0 µL	5.0 mM
H_2O	24.3 µL	N/A
DMSO	1.5 µL	N/A
Phusion Taq® Polymerase	1.0 µL	1U
Colony	1	N/A

Table 2.9.12.2: PCR programme for the Fab colony pick PCR.

Stage	Temp (^{o}C)	Time (seconds)
1 (1 cycle)	94	300
2 (30 cycle)	94	15
	56	30
	72	120
3 (1 cycle)	72	600
	4	Pause

2.9.13 Direct and inhibition ELISA of Fab fragments in XL1-Blue for all thee rounds

Individual colonies (288 in total) were selected and grown O/N at 200 x g and 37°C in single wells containing 100 µL SB media with 100 µg/mL carbenicillin (stock plates). The 3 x 96 well stock plates were then sub-cultured into fresh SB media (180 µL) containing 1 x 505 (0.5 % (v/v) glycerol, 0.05 % (v/v) glucose final concentration), 1mM $MgSO_4$ and 100 µg/mL carbenicillin. Glycerol was then added to the O/N stock plates to a final concentration of 20 % (v/v) and then transferred to a -80°C freezer for long-term storage. Meanwhile, the sub-cultured plates were incubated at 37°C at 220 rpm until the cell suspension reached an O.D.of ~0.6. Expression was then induced by adding IPTG to a final concentration of 1mM and incubating at 30°C (200 rpm) O/N.

Also, for each plate of clones, 2 x 96 well plates (Maxisorp™, Nunc) were coated with 1 µg/mL TBZ-BSA in PBS solution (100 µL per well) and left O/N at 4°C. The following day, the antigen-coated plate was then blocked with a 5 % (w/v) milk-PBS solution (200 µL per well) for 1 h at 37°C. Meanwhile, the O/N plates of expressed clones were removed from 30°C and subjected to a freeze-thaw protocol for cell lysis. In this protocol the plates are placed at -80°C until frozen and then thawed at 37°C (this step was repeated a total of 3 times). The plates were centrifuged at 3,220 x g (Eppendorf centrifuge 5810R) for 15 min. The supernatant was removed added (100 µL per well) to its corresponding coated/blocked well and incubated at 37°C for 1 h.

A competitive ELISA was also performed, whereby the lysate was added to the wells followed by several concentrations of the free TBZ). Following incubation, the plates were washed using PBST (x3) and PBS (x3) to remove any unbound Fab. The antigen–antibody

complex was detected with 100 µL per well of 1/2,000 dilution of a HRP-labelled anti-HA that detects proteins tagged with the haemagglutinin epitope YPYDVPDYA. After a 1 h 37°C incubation with the secondary mAb, TMB was added (100 µL per well) and left react for 15 min at R.T. The reaction was quenched by the addition of 50 µL per well of 10 % (v/v) HCl, after which the absorbance was read at 450 nm on a Safire 2 plate reader.

This method was adapted from Alfredo Sheehan**,** (2006)**.***The production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the ß-lactam antibiotic, cephalexin, in milk***.** PhD thesis, DCU, Ireland**.**

2.9.14 Large-scale protein expression of anti-TBZ Fab and extraction from bacterial cultures.

Approximately 5 mL of SB media, containing 100 µg/mL carbenicillin, was inoculated with 100 µL of stock transformant and grown O/N at 37°C. A 5 mL volume of this culture was then inoculated into 500 mL TB media containing 100 µg/mL carbenicillin, 1 mM $MgSO_4$, 0.5 % (v/v) glycerol and 0.05 % (w/v) glucose. The sub-cultured clone was incubated at 37°C at 250 rpm until the cell suspension reached ~0.6 (OD 600 nm). Expression was then induced by adding IPTG to a final concentration of 1mM and transferring to 30°C (250 rpm) O/N.

The O/N expressed culture was then transferred to 2 x 250 mL sorval tubes and centrifuged at 15,000 x g in a GSM rotor for 20 min ('brake on'), to pellet the bacterial cells. The media supernatant was discarded into a bucket of Virkon™ and the excess media removed by inversion of the sorval tube onto a paper towel. Each cell pellet was thoroughly re-suspended in 20 mL of sonication buffer (1 x PBS, pH 7.4, 0.5 M NaCl and 20 mM imidazole). Each aliquot was sonicated on ice for 45 seconds (3 second intervals) at an amplitude of 40, using a microtip Vibra Cell™ sonicator and the cell debris removed by centrifuging at 20,817 x g for 10 min at 4°C. The lysate supernatant was passed though a 0.2 µm filter to remove any residual cell debris.

This method was adapted from Alfredo Sheehan**,** (2006)**.** *The production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the ß-lactam antibiotic, cephalexin, in milk***.** PhD thesis, DCU, Ireland**.**

2.9.15 Purification of anti-TBZ Fab fragments using immobilised metal affinity chromatography (IMAC).

Immobilized metal affinity chromatography is based on the interaction of amino acids, particularly histidine, to metals. This technique allows histidine tagged proteins with an affinity for metal ions such as Ni^{+}-NTA to be retained in a column and eluted with either a

low pH or high imidazole buffer. A 3 mL aliquot of Ni^{+}-NTA agarose resin (QIAgen) was added to a 1.45 x 5.0 cm (final volume 10 mL) column and equilibrated with 20 mL of running buffer (1 x PBS, 0.5 M NaCl, 20 mM imidazole and 1 % (v/v) Tween-20). The filtered lysate was applied to the equilibrated column and the flow-though collected in a 50 mL tube. The column was washed with 20 mL of running buffer to remove any loosely bound non-specific proteins, again collecting the flow-though in a 50 mL tube. The Fab fragment was eluted using 100 mM NaAc, pH 4.4, and collected in 24 x 400 µL aliquots in 1.5 mL micro-centrifuge tubes containing 100 µL of filtered neutralisation buffer (50 µL 100 mM NaOH and 50 µL of 10 x PBS). The 12 mL of neutralised Fab was then thoroughly buffer exchanged against filtered PBS (1 x) using a 5 kDa cut-off Vivaspin™ 6 column (AGB, VS0611). The buffer-exchanged Fab was quantified using the Nanodrop™ ND-1,000 and aliquoted into clean PCR tubes and stored at -20°C.

This method was adapted from Alfredo Sheehan**,** (2006)**.***The production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the ß-lactam antibiotic, cephalexin, in milk***.** PhD thesis, DCU, Ireland**.**

2.9.16 Transfer of purified anti-TBZ Fab to nitrocellulose membrane and Western blot analysis.

Six sheets of Whatmann™ filter paper and one sheet of 3 mm nitrocellulose (per gel) were cut to the same dimensions of the SDS gel (see section 2.5). Each of the layers of filter paper, the nitrocellulose membrane and the SDS gel were soaked in transfer buffer for 30 min. The first 3 sheets of filter paper were positioned on the trans blot, followed by the SDS Gel, nitrocellulose membrane and finally the filter paper. All air bubbles were removed by rolling the gel/filter paper sandwich with a disposable 10 mL serological pipette. Proteins were transferred from the acrylamide gel to the nitrocellulose using a Trans-Blot® Semi-Dry Transfer cell (BioRad) at 15 V for 15 min. The nitrocellulose membrane was transferred into a large weighing boat containing 20 mL of 5 % (w/v) milk-PBS solution and blocked for 2 h at R.T. or at 4°C O/N with agitation. The membrane was washed twice with PBS to remove any excess blocking agent. Following blocking, the blot was washed 3 times in PBS buffer and incubated with primary antibody (at the appropriate dilution in dilution buffer i.e. PBST containing 0.5 % (w/v) skimmed milk powder) for 1.5 h at R.T. while rocking. The blot was washed 3 times in PBS and 3 times in PBST prior to incubation with enzyme-labelled secondary antibody (e.g. HRP-labelled anti-HA) diluted in PBST containing 5 % (w/v)

skimmed milk for 1.5 h at R.T. The blot was washed 3 times in PBST then PBS. Next the membrane was developed using appropriate substrate solution (i.e. 3,3′,5,5′-tetramethyl-benzidine (TMB) for HRP-labelled and 5-bromo-4-chloro-3'-indolyphosphate / nitro blue tetrazolium chloride (BCIP/NBT) for alkaline phosphatase-labelled secondary antibodies). The colour was developed at R.T. until the bands were clearly visible. The reaction was stopped by washing with distilled water or by rinsing with 100mM EDTA.

This method was adapted from Alfredo Sheehan**,** (2006)**.***The production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the ß-lactam antibiotic, cephalexin, in milk***.** PhD thesis, DCU, Ireland**.**

2.9.17 ELISA to determine the working concentration of the purified murine anti-TBZ Fab.

A 96 well plate (Maxisorp™, Nunc) was coated with 1 µg/mL TBZ-BSA conjugate solution (100 µL per well) and incubated O/N at 4°C. The following day, the antigen-coated plate was then blocked with 200 µL per well of PBS (containing 5 % (w/v) milk) and incubated for 1 h at 37°C. Any excess blocking solution was removed with a single wash with PBST. Serial dilutions were prepared from the purified stock of anti-TBZ in PBST (containing 0.5 % (w/v) milk). A 100 µL aliquot of each of the dilutions were added in triplicate to the coated/blocked wells and incubated for 1 h at 37°C. Following incubation, the plates were washed using PBST (x3) and PBS (x3) to remove any unbound Fab. The antibody–antigen complex was detected using a 100 µL per well of a 1/2,000 dilution of a HRP-labelled anti-HA secondary mAb. After a 1 h incubation at 37°C, 100 µL per well of TMB was added and incubated for 15 min at R.T. and the reaction quenched with 50 µL per well of HCl. The absorbance was read at 450 nm on a Safire 2 plate reader.

This method was adapted from Alfredo Sheehan**,** (2006)**.***The production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the ß-lactam antibiotic, cephalexin, in milk***.** PhD thesis, DCU, Ireland**.**

2.9.18 Competitive ELISA for the purified murine anti-TBZ Fab

A 96 well plate (Maxisorp™, Nunc) was coated with 1 µg/mL TBZ-BSA conjugate solution (100 µL per well) and incubated O/N at 4°C. The following day, the antigen-coated plate was blocked with 200 µL per well of PBS (containing 5 % (w/v) milk) and incubated for 1 h at 37°C. The wells were washed once with PBS and an aliquot of 50 µL/well primary Fab

antibody solution (1:40 in PBST 0.05 % (v/v) Tween 20 containing 1 % (w/v) powdered milk and 5 % (v/v) MeOH) was added to each well. TBZ standards were serially diluted in 200 µL volumes of dilution buffer (PBST 0.05 % (v/v) Tween 20 containing 1 % (w/v) powdered milk and 5 % (v/v) MeOH). Each dilution was added to the corresponding wells (50 µL/well) and incubated for 1 h at 37°C. The plate was washed 3 times with PBST and 3 times with PBS. After washing, 100 µL/well of peroxidase-labelled secondary antibody (1:2,000 in dilution buffer) was added and incubated for 1 h at 37°C. After a 1 h incubation at 37°C, 100 µL per well of TMB was added and incubated for 15 min at R.T. and the reaction quenched with 50 µL per well of HCl. The absorbance was then read at 450 nm on a Safire 2 plate reader.

This method was adapted from Alfredo Sheehan**,** (2006)**.***The production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the ß-lactam antibiotic, cephalexin, in milk***.** PhD thesis, DCU, Ireland**.**

2.10 Generation of the pComb double helix vector for the enhancement of the anti-TBZ Fab.

2.10.1 Plasmid preparation of anti TBZ-Fab and pAK500.

Plasmid DNA purification was performed using the Wizard® Plus SV miniprep DNA purification kit (Promega) and the method performed as detailed in the product insert.. Single E. coli colonies containing plasmid (e.g. E.coli XL1-Blue containing pAK500 phagemid vector and pComb3xTT containing anti-TBZ Fab) were picked from a stock agar streak plate and used to inoculate 2 x 5 mL of SB supplemented with 30 µg/mL of chloramphenicol (pAK500) and 100 µg/mL carbenicillin (pComb3xTT). The cultures were grown O/N at 37°C with vigorous shaking. The cells were then pelleted by centrifugation for 5 min at 3,500 x g (Eppendorf 5810R). The supernatant was carefully decanted and the pellets thoroughly re-suspended in 250 µL re-suspension buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 10 µg/mL RNase A) and transferred to sterile 1.5 mL micro-centrifuge tube. Cell lysis solution (250 µL of 0.2 M NaOH and 1 % (w/v) SDS) was added to the re-suspended cells and mixed by inverting 4 times. The resulting mixture was incubated at R.T. for 5 min prior to the addition of 10 µL of alkaline protease solution and further mixing by inverting the tube 4 times and incubation for 5 min. Alkaline protease was added in order to inactivate endonucleases and other proteins released during cell lysis that can adversely affect the quality of DNA. 350 µL of neutralisation solution (buffer containing 4.09 M guanidine

hydrochloride, 0.76 M potassium acetate and 2.12 M glacial acetic acid, pH 4.2) was added and again mixed by inverting the tube 4 times. The resulting mixture was centrifuged at 14,000 x g for 10 min at R.T. The cleared lysate (supernatant) was carefully decanted into a spin column in a fresh 1.5 mL tube and centrifuged at 14,000 x g for 1 min at R.T. The column 'flow-though' was discarded from the 1.5 mL tube and 750 µL of wash solution (162.8 mM potassium acetate, 22.6 mM Tris-HCl, pH 7.5, and 0.109 M EDTA) pre-diluted with 95 % (v/v) ethanol as per manufacturers' guidelines, was added. The spin column was re-inserted in the micro-centrifuge tube and centrifuged at 14,000 x g for 1 min at R.T. and the 'flow-though' discarded again. The wash procedure was repeated using 250 µL of wash solution and centrifuged at 14,000 x g for 2 min. The 'flow-though' was again discarded. The spin column was transferred to a fresh sterile 1.5 mL micro-centrifuge tube and the plasmid DNA finally eluted by adding 100 µL of ultra-pure molecular grade water to the column, followed by centrifugation at 14,000 x g for 1 min at R.T. The volume collected in the 1.5 mL tube was kept for further analysis and the spin column discarded. DNA was stored at -20ºC until required. This method was adapted from Alfredo Sheehan**,** (2006)**.***The production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the ß-lactam antibiotic, cephalexin, in milk***.** PhD thesis, DCU, Ireland**.**

2.10.2 Amplification of the Double Helix (dHLX) gene from pAK500.

To further enhance the anti-TBZ Fab a modified pComb vector system for the improvement of antibody fragments was constructed whereby the dHLX gene is inserted down-stream of the Fab gene insert and upstream of the amber stop codon. The double helix region was amplified from the pAK500 using the designed primers shown. The forward primer was designed to amplify the N-terminus of the dHLX domain from pAK500 for cloning into pComb3xSS via the *BsiWI* site. The back primer was designed to amplify the C-terminus of the dHLX domain including the 5 histidines from pAK500 and for cloning into pComb3xSS via the *BspEI* site in pComb3xSS (deleting the HA tag).

dHLX forward

ACCCGTACGGACCCAAACCTAGCACCCCCCCT

dHLX reverse

TAGTCCGGAACATGGTGATGATGGTGCGGGCGC

Table 2.10.2.1: The components of the PCR c for the amplification of the dHLX gene. and their volumes and concentrations in a 50µL volume.

Component	50 µL total volume	Conc. in 50 µL reaction
10 x Buffer	5.0 µL	1 x
dHLX Forward Primer	0.5 µL	60.0 pM
dHLX Back Primer	0.6 µL	60.0 pM
dNTP	2.0 µL	1.0 mM
H_2O	40.9 µL	N/A
Dream Taq polymerase	1.0 µL	1 U

The PCR for the amplification of the double helix gene was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions:

Table 2.10.2.2: PCR conditions for the amplification of the dHLX gene.

Stage	Temp(^oC)	Time (seconds)
1 (1 cycle)	96	300
2 (20 cycle)	96	15
	56	30
	72	30
3 (1 cycle)	72	600
	4	600

Five µg of the pComb3xTT vector and Fab E6 were digested with 180 units of *SfiI* (New England Biolabs). The digested Fab insert was ligated into the pComb3xTT vector using T4 ligase (New England Biolabs) and subsequently transformed into XL1-Blue cells. The dHLX gene fragment and the vector containing the Fab were sequentially digested with the restriction enzymes *Bspe* I and *Bsw* 1. These were ligated together with T4 ligase and transformed into electrocompetent XL1-Blue cells. Colonies were picked and checked for both the Fab and dHLX insert by PCR amplification.

Table 2.10.2.3: The components for the restriction digest of the Fab gene fragment and pComb vector.

Anti-TBZ Fab SfiI digest (5 µg)		***pComb3xTT Sfi*I *digest (20 µg)***	
Component	**50 µL total volume**	**Component**	**50 µL total volume**
Plasmid prep	10.0 µL	pComb vector	20.0 µL
10 x Buffer 2	5.0 µL	10 x Buffer 2	5.0 µL
100 x BSA	0.5 µL	100 x BSA	0.5 µL
H_20	25.5 µL	H_20	6.5 µL
*Sfi*I	9.0 µL	*Sfi*I	18.0 µL

Table 2.10.2.3: The components for the sequential restriction digest of the pComb vector and the double helix gene fragment.

Double helix gene digest (5 µg)		***pComb3xTT containing Fab digest (20 µg)***	
Component	**50 µL volume**	**Component**	**90 µL volume**
Plasmid prep	14.0 µL	pComb vector	67.0 µL
10 x Buffer 3	5.0 µL	10 x Buffer 3	9.0 µL
H_20	26.0 µL	H_20	8.9 µL
BspEI	5.0 µL	*Sfi*I	5.0 µL

The reactions were incubated at 37°C for 1 h. The enzyme was inactivated at 80^{o}C for 20 min. To each reaction 5 µL of *BsiWI* was added and incubated at 55^{o}C for 1 h and inactivated at 80^{o}C for 20 min.
Both components were ligated together as outlined in section 2.9.8.

Table 2.10.2.4: The components for the ligation of the pComb vector with the double helix gene fragment.

Component	**60 µL Volume**	**Conc. in 60 µL reaction**
Digested pComb vector	39.0 µL	1.4 µg
Digested dHLX gene	8.5 µL	0.7 µg
5 x Ligase buffer	6.0 µL	1 X
H_2O	3.5 µL	N/A
T_4 DNA Ligase	3.0 µL	400 U

Following ligation, the reaction was inactivated, ethanol precipitated and transformed as described in sections 2.9.10-2.9.13. A colony pick PCR was performed to determine the clones that contained the dHLX and Fab insert as described in section 2.9.15.

2.10.3 ELISA for the titration of the positive anti-TBZ Fab dHLX clones selected.

Positive clones were selected and O/N cultures were prepared in 10 mL of SB media containing 100 µg/mL of carbenicillin and were allowed to shake at 220 rpm at 37°C. Each clone was then sub-cultured into the same volume of media and grown until the O.D. reached 0.6 at 600 nm. These cultures were then induced with 1mM IPTG and continued to grow O/N at 30°C shaking at 220 rpm. The cultures were centrifuged at 3,500 x g for 10 min at 4°C. The pellet was re-suspended in 1 mL of PBS (pH 7.4) and subjected to cell lysis by 3 cycles of freeze-thawing. These cultures were then centrifuged at 3,500 x g at 4°C. The resulting supernatant was stored at 4°C. A nunc maxisorb plate (Maxisorp F96, Fisher Scientific Ireland) was coated with 100 µL/well of 1 µg/mL of the TBZ-BSA conjugate (donated by USDA) in PBS (pH 7.4) O/Nat 4°C. Subsequently, the plate was blocked with 200 µL/well of blocking solution (5 % (w/v) skimmed milk in PBS) for 1h at 37°C. The antibody supernatant was serially diluted in PBS (pH 7.4) and 50 µL of each dilution was transferred to the wells of the ELISA plate and incubated for 1 h at 37°C. The plate was washed 3 times with PBST and 3 times with PBS. After washing, 100 µL/well of peroxidase-labelled secondary antibody (1:2,000 in dilution buffer) was added and incubated for 1 h at 37°C. After the wells were washed 3 times with 300 µL/well of PBST and PBS, 100 µL/well of 3,3´,5,5´-tetra-methylbenzidine (TMB) was added and incubated for 15 min at R.T. The reaction was stopped by adding 50 µL/well of 1 M HCl and the absorbance measured at 450 nm.

These positive clones were purified by IMAC as described in section 2.9.15.

This method was adapted from Alfredo Sheehan**,** (2006)**.**The production of polyclonal, monoclonal and genetically-derived scFv antibody fragments for the detection of the ß-lactam antibiotic, cephalexin, in milk**.** PhD thesis, DCU, Ireland**.**

2.10.4 High performance liquid chomatography (HPLC) of the anti-TBZ Fab and anti-TBZ Fab DHLX.

The composition of the two IMAC purified antibody fragments (dHLX and Fab E6) were determined by size exclusion chomatography. Protein separations were performed on a Bio-Sep-SEC-S2000 stationary phase column (Phenominex 300 x 7.8 mm) with 1 x PBS pH 7.4 selected as a mobile phase. The samples (50 µL) were assayed at a flow rate of 0.5 mL/min using a UV detection 280 nm) and confirmation of the sample size was determined by

referencing against a range of protein standards namely, bovine thyroglobulin (670 kDa), immunoglobulin G (150 kDa), ovalbumin (44 kDa), myoglobin (17 kDa). This method was adapted from an in-house protocol entitled "HPLC-based analysis" written exclusively for the Applied Biochemistry group by Dr Barry Byrne.

2.11 Generation and characterization of chicken anti-TCB/ABZ antibodies

2.11.1 Immunisation of a Leghorn chicken with both TCB-BTG and ABZ-HRP conjugates and subsequent the antibody titre determination.

A white Leghorn chicken, aged 5-8 weeks, was immunised with the conjugates TCB-BTG and ABZ-HRP. On day 1, the chicken was immunised with 250 μg/mL of each conjugate in 600 μL of PBS, mixed in a 1:1 ratio with 600 μL of Freund's complete adjuvant (FCA). Freund's complete adjuvant is an emulsified mineral oil antigen solution used as an immunopotentiator (Bennett, B. 1992). The conjugate was mixed with the Freund's adjuvant and was vortexed until a stable emulsion was formed. This was passed twice though a 1 mL syringe with a 0.5 x 25 mm needle to disperse any air bubbles. On day 14, 35, 49 and 70, boosts were given to the chicken using 125 μg/mL of both conjugates in PBS, mixed in a 1:1 ratio with Freund's incomplete adjuvant (FICA). This solution was injected over four separate subcutaneous sites of each chicken for immunisation. A bleed was taken from the chickens 10 days after each injection and the antibody titre against TCB and ABZ determined. Once a high response from each hapten was obtained, the chicken was given a final boost, sacrificed 6 days later and the spleen and bone marrow removed. Eggs contain a high level of IgY comparable to that from the serum, therefore eggs were collected continuously though-out the immunisation period and from the yolk TCB/ABZ IgY was purified (section 2.8.3).

This method was adapted from Barry Mc Donnell (2010). *Development of novel antibody-based diagnostics for the early and rapid detection of cardiac markers*. PhD Thesis, DCU, Ireland.

2.11.2 Extraction and isolation of total RNA from the TCB/ABZ immunised chicken.

The inner surface of a Gelaire BSB 4 laminar unit was sprayed with both IMS and RNase ZAP to remove any RNases that may degrade RNA during the isolation procedure. The spleen and bone marrow were removed from the chicken and all excess fat was removed from the spleen before being transferred into a 50 mL 'RNase-free' tube containing 30 mL of Trizol ™ reagent. A homogenizer, previously autoclaved at 120°C for 15 min and baked O/N at 180°C, was used to homogenize the spleen/bone marrow. The mixture was incubated for 5

min at R.T. to allow for the total dissociation of nucleoprotein complexes while maintaining the integrity of the RNA. The homogenized spleen/bone marrow was centrifuged at 2,465 x g (Eppendorf centrifuge 5810R) for 10 min at 4°C to pellet any excess cell debris and the supernatant transferred into a 50 mL polypropylene Oakridge tube. Chloroform (6 mL) was added to the tube containing the supernatant, thoroughly mixed though and incubated for 15 min at R.T. The resultant mixture was centrifuged at 17,500 x g (Eppendorf centrifuge 5810R) for 15 min at 4°C. This centrifugation step produced 3 layers, a lower red phenol/chloroform phase, a protein inter-phase and a colourless liquid upper phase containing the RNA. The upper aqueous phase was carefully transferred (ensuring no lower layer contamination) into an 85 mL polycarbonate Oakridge tube and the RNA was precipitated by addition of 15 mL of isopropanol for 10 min at R.T.. It was centrifuged at 17,500 x g (Eppendorf centrifuge 5810R) for 25 min at 4°C resulting in a white RNA pellet. The isopropanol was carefully decanted and the pellet washed with 30 mL of 75 % (v/v) ethanol by centrifugation at 17,500 x g (Eppendorf centrifuge 5810R) for 10 min at 4°C. The pellets were air dried in the laminar hood and re-suspended in 500 µL of molecular grade water. The RNA preparation was quantified using the Nanodrop™ ND1000.

This method was adapted from Barry Mc Donnell (2010).*Development of novel antibody-based diagnostics for the early and rapid detection of cardiac markers*. PhD Thesis, DCU, Ireland.

2.11.3 Reverse transcription of total TCB/ABZ RNA to cDNA.

Following extraction the RNA was converted to cDNA using the commercial reverse transcription kit Superscript™ III. Method was taken from the product insert provided. This kit is fully optimized to synthesize first strand cDNA from total RNA.

Table 2.11.3.1: The components of mixture 1 for the reverse transcription of RNA to cDNA.

Mixture 1 Component	10 µL Volume	Conc. in 10 µL reaction
Total RNA	5.0 µL	5.0 µg
Oligo (dT) primer	1.0 µL	0.5 µg
dNTP mix	1.0 µL	1.0 mM
Molecular grade H_2O	3.0 µL	N/A

Table 2.11.3.2: The components of mixture 2 for the reverse transcription of RNA to cDNA.

Mixture 2 Component	10 µL Volume	Conc. in 10 µL reaction
10 X RT Buffer	2.0 µL	2 X
$MgCl_2$	4.0 µL	2.5 mM
DTT	2.0 µL	20.0 mM
RNase Out	1.0 µL	40 U
Superscript III enzyme	1.0 µL	200 U

2.11.4 PCR primers for amplification of the avian scFv library (pComb series).

The primers listed below were obtained from Eurofins-MWG-Operon (318 Worple Road, Raynes Park, London, SW20 8QU) and are compatible with the primers set described by Barbas and co-workers (Barbas, C.F. 2001) for the pComb vector system. All methods described from 2.11.4-2.11.10 were adapted from (Barbas *et al.*, 2001) unless otherwise stated.

Variable heavy chain:

CSCVHo-F (sense)

5´ GGT CAG TCC TCT AGA TCT TCC GCC GTG ACG TTG GAC GAG 3´

CSCG-B (reverse)

5´ CTG GCC GGC CTG GCC ACT AGT GGA GGA GAC GAT GAC TTC GGT CC 3´

Variable light chain:

CSCVK (sense)

5´ GTG GCC CAG GCG GCC CTG ACT CAG CCG TCC TCG GTG TC 3´

CKJo-B (reverse)

5´ GGA AGA TCT AGA GGA CTG ACC TAG GAC GGT CAG G 3´

Splice by overlap extension (SOE) primers

CSC-F (sense)

5´ GAG GAG GAG GAG GAG GAG GTG GCC CAG GCG GCC CTG ACT CAG 3´

CSC-B (reverse)

5´ GAG GAG GAG GAG GAG GAG GAG CTG GCC GGC CTG GCC ACT AGT GGA GG 3´

The following protocols describe the construction of an avian scFv gene construct by overlap extension PCR. It allows the expression and selection of scFv that contain variable regions obtained from the spleen and bone marrow of the immunised chicken.
A 1 x reaction mix for amplification of the variable domains was formulated using the following components.

Table 2.11.4.1: Components of the PCR for the amplification of the the avian variable domains and their subsequent volumes and concentrations in 50 µL.

Component	50 µL total volume	Conc in 50 µL reaction
10 x Buffer	5.0 µL	1 x
V_L/V_H Forward Primer	0.6 µL	60.0 pM
V_H/V_L Back Primer	0.6 µL	60.0 pM
cDNA	1.0 µL	1.0 µg
dNTP	2.0 µL	1.0 mM
$MgCl_2$	4.0 µL	2.0 mM
H_2O	36.6 µL	N/A
Dream Taq ® Polymerase	0.2 µL	1U

The PCR for the amplification of the avian antibody variable domain genes was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions:

Table 2.11.4.2: The PCR programme for the amplification of the variable heavy and light scFv chains.

Stage	Temp(^oC)	Time (seconds)
1 (1 cycle)	94	300
2 (30 cycle)	94	15
	56	30
	72	120
3 (1 cycle)	72	600
	4	600

2.11.5 Purification of V_H and V_L variable gene fragments using the Promega clean-up kit.

The V_H and V_L PCR products were resolved on a 1 % (w/v) agarose gel until single bands for both the variable heavy and variable light chains were observed. Both bands were excised

using sterile scalpels and transferred into clean 1.5 mL micro-centrifuge tubes. Next, thee volumes of binding buffer was added for every one volume of gel slice and incubated at 50°C until the gel fragments had completely dissolved (approximately 10 min). The binding buffer contains guanidine isothiocyanate hat allows for sufficient binding of the DNA to the silica membrane columns. One volume of isopropanol, equal to the original of the gel slice volume, was added and mixed to allow for precipitation of the DNA. The mixture was transferred to a binding column and collection tube and centrifuged at 17,500 x g (Eppendorf centrifuge 5810R) for one min to remove any residual buffer. The "flow-through" was discarded and the column was washed first by centrifugation with 750 µL wash buffer and then with 250 µL wash buffer. The column was centrifuged at 17,500 x g (Eppendorf centrifuge 5810R) for 2 min to ensure all residual ethanol was removed and the DNA was eluted from the column using 50 µL molecular grade H_2O and subsequently quantified using the Nanodrop™ ND-1000.

Table 2.11.5.1: The components for the PCR to combine the two variable regions of the scFv subsequent volume and concentration in 50 µL.

Component	50 µL total volume	Conc in 50 µL reaction
10 x Buffer	5.0 µL	1 x
CSC Forward Primer	0.6 µL	60.0 pM
CSC Back Primer	0.6 µL	60.0 pM
V_H purified product	0.5 µL	100 ng
V_L purified product	1.0 µL	100 ng
dNTP	2.0 µL	1.0 mM
DMSO	2.0 µL	N/A
$MgCl_2$	4.0 µL	2.0 mM
H_2O	34.1 µL	N/A
PlatinumTaq® Polymerase	0.2 µL	1U

Table 2.11.5.2: The PCR programme for the splice by overlap extension of the variable light and heavy chains.

Stage	Temp(^{o}C)	Time (seconds)
1 (1 cycle)	94	120
2 (25 cycle)	94	15
	56	15
	72	90
3 (1 cycle)	72	600
	4	600

2.11.6 SfiI restriction digests of the purified SOE-PCR fragment and ligation into the pComb3xSS vector.

The scFv fragment and pComb3xTT vector used for phage selection were digested using *Sfi*I restriction enzyme. The *Sfi*I enzyme allows for the unidirectional cloning of scFv fragment into the pComb phage display vector. The enzyme recognises 8 bases which are interrupted by 5 non-recognised nucleotides (5′ggccnnnnnggcc3′), thus virtually eliminating internal digestion in antibody sequences. Both digestions (outlined below) were carried out for 5 h at 50°C.

Table 2.11.6.1: Components for the restriction digest of the Fab PCR product and pComb vector.

Component	Vector digest 200µL volume	Spleen digest 200µL volume	Bone digest 200µL volume
Template	86	40	46
10 x buffer	20	20	20
100 x BSA	2	2	2
*Sfi*I	6	12.5	12.5
H_2O	86	125.5	119.5

Following a 5 h incubation at 50°C, the digested pComb3xSS vector was treated with Antarctic phosphatase™ enzyme to prevent self-ligation. This enzyme catalyzes the removal of 5′ phosphate groups from DNA. The loss of the 5′ phosphoryl termini, therefore, prevents

the ligase enzyme from working. To the digested vector 5 µL of Antarctic phosphatase buffer (10 x) followed by 2 µL of antarctic phosphatase enzyme.

The mixture was incubated for 15 min at 37°C and then the enzyme was heat inactivated at 65°C for 5 min. Both digests were resolved via electrophoresis on a 1 % (w/v) agarose gel for the digested PCR product and a 0.6 % (w/v) gel for the digested vector. These were gel-purified, as described in section 2.9.6. The restricted scFv gene was then ligated into the pComb3xTT vector in a 1:2 (insert:vector) ratio under the conditions below and left at R.T. O/N.

Table 2.11.6.2: Components of the ligation of the scFv gene fragment into the pComb vector.

Component	200 µL volume	Conc in 200 µL reaction
5 x ligase buffer	20 µL	1 x
Digested pComb3xTT	25 µL	1.4 µg
Digested scFv spleen gene	29 µL	0.7 µg
H_2O	116 µL	N/A
T_4 DNA Ligase	10 µL	800 U

Table 2.11.6.3: Components of the ligation of the scFv gene fragment into the pComb vector.

Component	200 µL volume	Conc in 100 µL reaction
5 x ligase buffer	20 µL	1 x
Digested pComb3xTT	25 µL	1.4 µg
Digested scFv bone gene	50 µL	0.7 µg
H_2O	95 µL	N/A
T_4 DNA Ligase	10 µL	800 U

Following ligation the mix was ethanol precipitated using the following reagents.

Table 2.11.6.4: Components for the ethanol precipitation of the ligated gene fragment.

Component	Volume
Ligation mixture	200 µL
3M sodium acetate, pH 5.2	20 µL
100 % (v/v) ethanol	500 µL

The precipitation was incubated O/N at -20°C and the DNA was harvested by centrifugation at 17,500 x g (Eppendorf centrifuge 5810R) for 20 min at 4°C. The ethanol supernatant was decanted and the pellet was washed by addition of 1 mL of 70 % (v/v) ethanol and centrifuging at 17, 500 x g (Eppendorf centrifuge 5810R) for 20 min at 4°C. The pellet was allowed to air-dry briefly and dissolved in 20 µL of molecular grade H_2O.

2.11.7 Electro-transformation of scFv-containing plasmid into XL1-Blue E. coli cells.

Transformation of the ligated products was performed by electroporation into a commercially available strain of XL1-Blue *E. coli* cells (Stratagene). The apparatus used was a Gene Pulser Xcell ™ electroporation system with the parameters set to 25 µF, 1.25 kV and gene pulse controller at 200 Ω. After thawing on ice, 50 µL of the *E. coli* cells were added to the 5 µL of ligation product, mixed and left on ice for a 1 min incubation. The mixture was transferred to a chilled 0.2 cm electroporation cuvette, and placed into the 'Shockpod'. A pulse was passed though the cuvette, and immediately flushed with 1 mL of pre-warmed SOC medium using a sterile tip. The 1 mL of electroporated cells were transferred into a sterile 50 mL Falcon tube containing a further 2 mL of pre-warmed SOC medium. The 3 mL of transformant suspension was incubated for 1 h at 250 x g at 37°C and plated out on SB agar plates supplemented with 100 µg/mL carbenicillin. A negative control was also incorporated into the experiment by plating the XL1-Blue cells containing no plasmid onto the agar plates. All plates were incubated O/N at 37°C and scraped into SB medium containing 20 % (v/v) glycerol for long term storage at -80°C.

2.11.8 Phage rescue and precipitation of TCB/ABZ scFv library

A conical flask containing 600 mL of sterile SB medium (100 µg/mL carbenicillin) was inoculated with 600 µL of the transformed library glycerol stocks (bone and spleen) and incubated at 220 rpm and 37°C until the O.D.reached 0.4 at 600nm. Helper phage (2×10^{11} cfu) was added. The culture was left static at 37°C for 30 min before transferring it to a shaking incubator at 37°C at 220 rpm for 1.5 h. Kanamycin was added to a final concentration of 50 µg/mL, before leaving the culture O/N at 37°C and 220 rpm. The O/N culture was centrifuged at 18,500 x g (Eppendorf centrifuge 5810R) for 20 min at 4°C and the phage supernatant transferred into 3 sterile 250 mL Sorvals™ centrifuge tubes containing 8 g of PEG 8,000 and 6 g of sodium chloride. The Sorvals™ were agitated at 220 rpm until the PEG/NaCl went into solution. The phage was precipitated for 1 h at 4°C. The phage was harvested by centrifugation at 6,440 x g for 25 min at 4°C and re-suspended in 2 mL of 1 %

(w/v) BSA-PBS solution. The re-suspended phage pellets were added to a clean 1.5 mL micro-centrifuge tube and centrifuged at 20,817 x g for 5 min at 4°C to remove any bacterial debris. The remaining supernatant was refrigerated until ready for use.

2.11.9 Panning of the TCB/ABZ phage library against immobilized conjugates.

An immuno-tube (Maxisorp™, Nunc) was coated O/N at 4°C with 500 µL of 100 µg/mL TCB-HRP and ABZ-HRP in PBS. The tube was blocked for 1 h at 37°C with 3 % (w/v) BSA in PBS. Following one wash with PBST, 500 µL of the rescued phage (input phage) (Section 2.9.10) was added to the immuno-tube and incubated on a roller for 2 h at R.T. The immuno-tube was washed with PBST (x3) and PBS (x3) to remove non-specific phage. Specific phage was eluted by incubation with 1 mL of free TCB/ABZ in PBS for 2 h at 37°C. The competitively eluted phage was removed after harsh pipetting and 800 µL was added to 5 mL of mid-exponential phase XL1-Blue cells. This was allowed to infect for 15 min static at R.T. and 6 mL of pre-warmed SB media containing 1.6 µL of 100 mg/mL carbenicillin and 12 µL of 5 mg/mL tetracycline was added (at this point 100 µl sample was removed for output titre determination). This 12 mL culture was incubated at 220 rpm at 37°C for 1 h. Carbenicillin (2.4 µL of 100 mg/mL) was added and incubated at 220 rpm at 37°C for 1 h. To this 12 mL culture, 800 µL of commercial helper phage (New England Biolabs) was added, followed by 88 mL of SB media supplemented with carbenicillin and tetracycline (final concentrations of 50 µg/mL and 10 µg/mL, respectively) and incubated for 2 h at 37°C at 220 rpm. Kanamycin was added to a final concentration of 70 µg/mL and incubated O/N at 220 rpm and 37°C.

An output titre was performed by making a 1 in 10 and 1 in 100 dilution of the XL1-Blue infected cells in SB media. These dilutions were plated out on SB agar plates containing 100 µg/mL of carbenicillin and incubated O/N at 37°C.

An input titre was performed by making serial dilutions (10^{-1}-10^{-12}) of the PEG-precipitated phage in exponential growth phase XL1-Blue cells. Following a 15 min infection at 37°C, the 10^{8} - 10^{12} serial dilutions were plated out on SB agar plates containing 100 µg/mL carbenicillin and incubated O/N at 37°C. All of the subsequent rounds of panning were performed as described above.

Table 2.11.9.1: Parameters varied during the panning of avian scFv TCB/ABZ library

Round	Free drug concentration µmol	Coating concentration µg/mL	Wash conditions
1	100	100	5 x PBST, 5 x PBS
2	50	50	5 x PBST, 5 x PBS
3	25	25	5 x PBST, 5 x PBS
4	12.5	12.5	5 x PBST, 5 x PBS
5	6.5	6.5	5 x PBST, 5 x PBS

2.11.10 ScFv gene insert check via 'colony-pick' PCR.

Single colonies were randomly selected from both the spleen and bone transformed stock plates. A 'colony-pick' PCR was performed to ensure the vector incorporated the scFv fragment. A sterile tip was used to pick a single colony into the mixture described in *2.9.14.1*, which was placed in a Biometra $T_{GRADIENT}$ PCR machine. The amplified scFv fragments were analysed via gel electrophoresis on a 1 % (w/v) agarose gel.

Table 2.11.10.1: PCR components for amplification of the scFv gene fragment from selected clones.

Component	50 µL total volume	Conc in 50 µL reaction
10 x Buffer	5.0 µL	1 x
Colony	1	N/A
CSC Forward Primer	0.6 µL	60.0 pM
CSC Back Primer	0.6 µL	60.0 pM
dNTP	2.0 µL	1.0 mM
DMSO	2.0 µL	N/A
$MgCl_2$	4.0 µL	2.0 mM
H_2O	35.6 µL	N/A
PlatinumTaq® Polymerase	0.2 µL	1U

Table 2.11.10.2: The PCR programme for amplification of the the scFv gene fragment from selected clones.

Stage	Temp(^{o}C)	Time (seconds)
1 (1 cycle)	94	120
2 (25 cycle)	94	15
	56	15
	72	90
3 (1 cycle)	72	600
	4	600

2.11.11 Direct and inhibition ELISA of scFv fragments in XL1-Blue for all thee rounds.

Individual colonies (288 in total) were selected and grown O/N at 200 x g and 37°C in single wells containing 100 µL SB media with 100 µg/mL carbenicillin (stock plates). The 3 x 96 well stock plates were then sub-cultured into fresh SB media (180 µL) containing 1 x 505 (0.5 % (v/v) glycerol, 0.05 % (v/v) glucose final concentration), 1mM $MgSO_4$ and 100 µg/mL carbenicillin. Glycerol was then added to the O/N stock plates to a final concentration of 20% (v/v) and then transferred to a -80°C freezer for long-term storage. Meanwhile, the sub-cultured plates were incubated at 37°C at 220 rpm until the cell suspension reached an O.D. of ~0.6. Expression was then induced by adding IPTG to a final concentration of 1mM

and incubating at 30°C (200 x g) O/N. Also, for each plate of clones, 2 x 96 well plates (Maxisorp™, Nunc) were coated with 1 µg/mL TBZ-BSA in PBS solution (100 µL per well) and left O/N at 4°C. The following day, the antigen-coated plate was then blocked with a 5 % (w/v) milk-PBS solution (200 µL per well) for 1 h at 37°C. Meanwhile, the O/N plates of expressed clones were removed from 30°C and subjected to a freeze-thaw protocol for cell lysis. In this protocol the plates are placed at -80°C until frozen and then thawed at 37°C (this step was repeated a total of 3 times). The plates were centrifuged at 3,220 rpm (Eppendorf centrifuge 5810R) for 15 min. The supernatant was removed added (100 µL per well) to its corresponding coated/blocked well and incubated at 37°C for 1 h. (A competitive ELISA was also performed, whereby the lysate was added to the wells followed by several concentrations of the free TBZ). Following incubation, the plates were washed using PBST (x3) and PBS (x3) to remove any unbound Fab. The antigen–antibody complex was detected with 100 µL per well of 1/2,000 dilution of a HRP-labelled anti-HA that detects proteins tagged with the haemagglutinin epitope YPYDVPDYA. After a 1 h 37°C incubation with the secondary mAb, TMB was added (100 µL per well) and left react for 15 min at R.T. The reaction was quenched by the addition of 50 µL per well of 10 % (v/v) HCl, after which the absorbance was read at 450 nm on a Safire 2 plate reader.

This method was adapted from Barry Mc Donnell (2010).*Development of novel antibody-based diagnostics for the early and rapid detection of cardiac markers.* PhD Thesis, DCU, Ireland

2.11.12 Expression studies of the TCB/ABZ avian scFv clones TCB E4 and ABZ D6.

Both clones were grown overnight in 5 mL of SB (section 2.2.1) containing 100 µg/mL of carbenicillin at 37°C at 220 rpm. These were sub-cultured into 4 x 200 ML (2 for E4 and 2 for D6) of SB containing 100 µg/mL of carbenicillin and grown at 37°C at 220 rpm until the O.D. reached 0.6. A 5 mL samples were removed from each flask, two were induced with 10 mM IPTG and incubated at 30°C overnight. The remaining two were un-induced. The remaining cultures were induced with 1 mM IPTG and incubated at 30°C and 37°C. Samples (5 mL) were taken every h for 6 h and the remaining cultures were incubated overnight at 30°C and 37°C at 220 rpm. Each sample was centrifuged at 3,500 x g (Eppendorf 5810 R) for 10 min at 4°C, and the pellet re-suspended in 5 mL of sonication buffer (1 x PBS, pH 7.4, 0.5 M NaCl and 20 mM imidazole) and split into 2 x 2.5 mL samples. Half of the samples were lysed via the freeze thaw method (freeze at -80°C for 15 min and thaw at 37°C for 15 min

repeating cycle thee times), and the other half by sonication. Each sample was analysed for activity by direct ELISA (section 2.9.13).

Expression studies were adapted from Barry Mc Donnell (2010). *Development of novel antibody-based diagnostics for the early and rapid detection of cardiac markers*. PhD Thesis, DCU, Ireland

2.12 Purification, characterisation and assay development for the antiTCB/ABZ polyclonal IgY from chicken egg.

2.12.1 Purification of anti-TCB/ABZ polyclonal IgY from chicken egg.

This purification was carried out using the Pierce™ chicken IgY "eggcellent" purification kit. The method was used as per the product insert. Thee egg yolks were separated from the egg whites. The yolk sacs were rinsed with de-ionised water, punctured and the egg yolks collected. Cold delipidation solution (provided in the kit) was added (5 times the combined original yolk volume) with continuous stirring to ensure the solution was entirely mixed. This was incubated for 6 h at 4°C, and centrifuged for 15 min at 3,500 x g (Eppendorf centrifuge 5810R) at 4^{o} C. The supernatant was decanted and an equal volume of IgY precipitation reagent was added. The suspension was incubated at 4°C O/N and centrifuged for 15 min at 3,500 x g (Eppendorf centrifuge 5810R) at 4°C. The supernatant was discarded and the retained pellet was re-suspended in a volume of 1 x PBS pH 7.5, equal to the original egg yolk volume. The purified IgY protein was quantified using a pre-programmed 'IgG' option on the NanoDrop™ ND-1000.

2.12.2 SDS-PAGE of the purified anti-TCB/ABZ polyclonal IgY.

Samples containing the purified anti-TCB/ABZ IgY were separated using 12.5 % (w/v) SDS-PAGE gels (20 µl /Lane) to analyse purity and to determine the apparent molecular weight. The gels were prepared, as described in section 2.5, and left to polymerise between two clean glass plates. After the stacking gel was poured, a comb was inserted to make wells in preparation for loading of the protein samples. The samples were prepared by adding loading buffer to a ratio of 1:5 volumes of sample. The gels were placed in an electrophoresis apparatus and submerged in electrophoresis buffer (section 2.5). A 20 µg quantity of each protein sample was added into each well in a total volume of 20 µL. A PAGE ruler ™ (Fermentas) was added to one well per gel to determine the molecular weight of each protein. The apparatus was attached to a power supply and a voltage of 200 V applied. The gels were allowed to run until the tracking dye had reached the bottom of the gel, taking approximately 45 min. The gels were removed from the apparatus and stained using Coomassie stain for 3 h.

Finally, the stained gels were de-stained O/N using Coomassie de-stain. The de-stain solution was changed 2-3 times (until the background non-specific staining was removed).

2.12.3 Selection of optimal coating buffers for use with TCB and ABZ in ELISA development.

A number of coating buffers were investigated to determine which buffer best facilitated optimum conjugate binding. A 96 well plate (Maxisorp™, Nunc) was coated with varying concentrations of TCB-BTG and ABZ-BSA in different buffers as outlined in section 2.5 and left O/N at 4°C. The following day, the antigen-coated plate was blocked with a 5 % (w/v) milk-PBS solution (200 µL per well) for 1 h at 37°C. A 1/ 100 dilution of the previously purified anti-TCB/ABZ was added to each well (100 µL per well) and incubated at 37°C for 1 h. Following incubation, the plates were washed using PBST (x3) and PBS (x3) to remove any unbound pAb. The antigen-pAb complex was detected using 100 µL per well of a 1/2,000 dilution of a HRP-labelled anti-chicken IgY secondary mAb produced in rabbit. After a 1 h incubation at 37°C the plate was washed again with PBST (x3) and PBS (x3) to remove any unbound detection antibody. TMB substrate was then added (100 µL per well) and left to react for 15 min at R.T.. The reaction was quenched by the addition of 50 µL per well of 10 % (v/v) HCl, after which the absorbance was determined at 450 nm on a Safire 2 plate reader.

2.12.4 Checkerboard ELISA of the purified anti-TCB/ABZ pAb antibody.

A checkerboard ELISA was performed to determine the optimum conjugate coating concentration and antibody dilution to be used though-out the assay development. A 96 well plate (Maxisorp™, Nunc) was coated with varying concentrations of TCB-BTG and another with ABZ-BSA in 1 x TBS (pH 8.0) and left O/N at 4°C. The following day, the antigen-coated plate was then blocked with a 5 % (w/v) milk-PBS solution (200 µL per well) for 1 h at 37°C. Serial dilutions of the purified pAb were made in 0.5 % (w/v) milk in PBST, added (100 µL per well) to the corresponding coated/blocked well and incubated at 37°C for 1 h. Following incubation, the plates were washed using PBST (x3) and PBS (x3) to remove any unbound pAb. The antigen-antibody complex was detected using 100 µL per well of a 1/2,000 dilution of a HRP-labelled anti-chicken IgY secondary mAb produced in rabbit (Sigma #A6154). After a 1 h incubation at 37°C the plate was washed again with PBST (x3) and PBS (x3) to remove any unbound detection antibody. TMB substrate was then added (100 µL per well) and left to react for 15 min at 37°C. The reaction was quenched by the addition of 50 µL per well of 10 % (v/v) HCl, after which the absorbance was determined at 450 nm on a Safire 2 plate reader.

2.12.5 A study of the affect of different solvents used during the extraction of TCB and ABZ residues on the pAb anti-TCB/ABZ.

Most benzimidazoles are not water soluble and are dissolved in solvents such as ethanol and methanol. Therefore, before assay development the antibody's tolerance to solvents such as methanol had to be examined. Methanol is generally used as an extraction solvent for ELISA because it has less effect on antigen-antibody interactions and HRP activity than other solvents. A 96 well plate (Maxisorp™, Nunc) was coated with varying concentrations of TCB-BTG and ABZ-BSA in 1 x TBS (pH 8.0) and left O/N at 4°C. The following day, the antigen-coated plate was blocked with a 5 % (w/v) milk-PBS solution (200 µL per well) for 1 h at 37°C. Different concentrations of methanol, ethanol and DMSO were prepared ranging from 1-100 %. A 1/1,000 dilution of the antibody was added to each concentration and 100 µL of the extraction buffer/antibody solution was added to the corresponding wells and incubated for 1 h at 37°C. Following incubation, the plates were washed using PBST (x3) and PBS (x3) to remove any unbound pAb/buffer The antigen-antibody complex was detected using 100 µL per well of a 1/2,000 dilution of an HRP-labelled anti-chicken IgY secondary mAb produced in rabbit. After a 1 h incubation at 37°C the plate was washed again with PBST (x3) and PBS (x3) to remove any unbound detection antibody. TMB substrate was added (100 µL per well) and left to react for 15 min at R.T. The reaction was quenched by the addition of 50 µL per well of 10 % (v/v) HCl, after which the absorbance was determined at 450 nm on a Safire 2 plate reader.

2.13 Development of an ELISA method for the detection of TCB in spiked milk

2.13.1 QuEChERs method

Milk samples (12g) were spiked with free TCB concentrations ranging from 12 µg/mL to 7 pg/mL. The TCB was extracted from the milk using a slurry containing acetonitrile:MgSO4:NaCl (12:4:1 v/w/w) by shaking vigorously by hand for 1 min. The samples were centrifuged at 3,500 x g for 10 min at 4^{o}C and the supernatants were transferred to tubes containing 0.5g of C18 sorbent and 1.5 g $MgSO_4$. Each tube was shaken for 1 min and centrifuged at 3,500 x g for 10 min at 4^{o}C. Two layers were formed and the upper acetonitrile layer (2 mL) was transferred to 2 mL eppendorfs and evaporated to dryness using a MiVac Quattro centrifugal concentrator (Mason technologies) at 50^{o}C. The extracts were reconstituted in 666 µL of DMSO by vortexing for 2 min followed by a brief sonication in a sonicator water bath (Mason Technologies) for 10 min. The reconstituted extracts were diluted in a 1:1 methanol:water ratio and vortexed for 1 min followed by filtration through a

0.22 μm filter. All samples were then further diluted in a ratio of 1: 4 with HBS buffer; pH 7.4. The anti-TCB pAb was mixed with the milk extracts in a ratio of 1:3 prior to ELISA and Biacore™ analysis. (ref)

2.13.2 Direct centrifugation method

A 2 mL spiked milk stock solution (10 μg/mL) was prepared and serial dilutions ranging from 12 μg/mL to 7 pg/mL were made in organic milk (Organic milk is milk without synthetic chemicals, hormones or antibiotics). Each sample was incubated for 30 mins at R.T followed by centrifugation at 14,000 rpm at R.T for 30 min. The aqueous phase was gently removed using a 200 μl pipette and mixed with a 1/2,560 of anti-TCB pAb in a ratio of 1:1.

2.13.3 Inhibition ELISA for the detection of TCB in spiked milk samples

A 96 well plate (Maxisorp™, Nunc) was coated with 1,500 ng/mL TCB-BTG conjugate solution (100 μL per well) and incubated O/N at 4°C. On the following day, the antigen-coated plate was blocked with 200 μL per well with PBS, pH 7.5, containing 5 % (w/v) milk marvel and incubated for 1 h at 37°C. The wells were washed once with PBS and 100 μL of the extracted TCB samples, pre-mixed with a 1/2,560 anti-TCB pAb, was added to each well and incubated for 1 h at 37°C. After incubation the plate was washed 3 times with PBST and 3 times with PBS. After washing, 100 μL/ well of peroxidase-labelled secondary antibody (1/2,000 in PBS containing 0.5 % w/v milk marvel) was added and incubated for 1 h at 37°C. Following this, 100 μL per well of TMB was added and incubated for 15 min at R.T. The reaction was quenched with 50 μL per well of HCl and the absorbance was then read at 450 nm on a Safire 2 Tecan plate reader.

2.14 Development of SPR-based immunoassay using a Biacore™ 3000TM biosensor

BIAcore™ assay development was carried out on a BIAcore 3000™ instrument using carboxymethylated dextran (CM5) sensor chips. The running buffer for all BIAcore™ experiments was HBS buffer, pH 7.4, this was was filtered (pore size of 0.45 μm) and degassed using a filtration apparatus (Millipore sintered glass filtration unit) immediately before use.

2.14.1 Pre- concentration studies

Pre-concentration studies were carried out to determine the optimal buffer conditions required for maximum binding of the ligand to the un-modified CM5 dextran chip surface. At low ionic strength and a pH lower than the iso-electric point of the conjugated-hapten, the protein is electrostatically attracted to the negatively charged surface matrix. The TCB-BTG conjugate was diluted in 10 mM sodium acetate solution at pHs of 4.0, 4.2, 4.4, 4.6, 4.8 and 5.0, and injected over a blank surface for 1 minute at a flow rate of 10 µL/min. The pH at which the highest binding was observed, was used for TCB-BTG immobilisation.

2.14.2 Immobilisation of TCB-BTG to the CM5 dextran chip surface

The CM5 dextran surface is a three dimensional surface containing carboxyl groups (COOH) attached to the gold surface layer. The surface was activated by injecting a 1:1 mixture of 400 mM EDC (N-ethyl-N-(dimethyl-aminoprsopyl) carbodiimide hydrochloride) and 100 mM NHS (N-hydroxysuccinimide) for 7 minutes at a flow rate of 10 µL/min. Later, TCB-BTG (50 µg/mL) conjugate was diluted in the appropriate pH buffer and injected over the activated surface for 30 minutes at 10 µL/min. Un-reactive NHS groups were capped by passing 1 M ethanolamine hydrochloride, pH 8.5, for 10 minutes at 10 µL/min and non-covalently bound proteins removed

2.14.3 Regeneration studies of the TCB-BTG immobilised CM5 chip

The stability of the immobilised TCB-BTG surface was measured by passing a known concentration of antibody over the chip surface, and by regenerating the surface with various concentrations of NaOH (10 mM, 20 mM, 50 mM, 100 mM and 200 mM) and HCl (5 mM, 10 mM and 20 mM). Glycine (10 mM) at different pH 2.0, 2.5 and 3.0, and NaOH with 0.5 % acetonitrile at concentrations of 10 mM, 50 mM, 100 mM and 200 mM were also studied for regeneration of the surface. The cycle of regeneration was repeated for approximately 50 cycles and the binding signal was measured at the end of each regeneration cycle to assess the stability and suitability of the immobilised surface for the development of a sensitive and reproducible assay.

2.14.4 Flow rate, contact time and antibody dilution optimisation.

The working antibody dilution, contact time and flow rate that was optimum for the development of a BIAcore™ inhibition assay was determined. Anti-TCB pAb dilutions were prepared (1/10, 1/50, 1/100, 1/125, 1/250, 1/500 and 1/1000) in HBS buffer, pH 7.4, and passed over the immobilised flow cell using a range of contact times (1 min, 3 min and 5 min) and flow rates (10, 20 and 40 µl/min). The combination of dilution, contact time and flow rate that yielded a Response Unit (RU) from 100-500 was chosen.

2.14.5 BIAcore™ inhibition buffer curve generation using a TCB-gluteraldehyde linked chip.

PBS containing 50 % (v/v) methanol was spiked with free TCB, concentrations ranging from 12 ug/mL to 7 pg/mL, and mixed 1:1 with 1/125 dilution of anti-TCB pAb prior to injection and placed in the BIAcore™ 3000 auto-sampler and randomly passed over the TCB immobilised chip surface (in triplicate) at a flow rate of 10 µl/min for 4 minutes. The surface was regenerated with three pulses of 200 mM NaOH at a flow rate of 10 µl/min for 1 min. The mean response (RU) was calculated and expressed as a ratio of RU/RU_0, where the 'spiked' sample response (RU) was divided by the response obtained from the sample containing no free TCB (RU_0). A decrease in binding response was observed with increasing free TCB concentrations.

2.14.6 BIAcore™ Inhibition milk assay generation using a TCB-gluteraldehyde linked chip.

Milk was spiked with varying concentrations of TCB ranging from 1 µg/mL to 7 pg/mL and samples were extracted and prepared as in section 2.13.1. A 1/125 anti-TCB pAb and milk extract were mixed in a ratio of 1:3 and incubated for 30 mins at R.T and passed randomly over the gluteraldyhyde linked TCB chip at a flow rate of 10 uL /min for 4 min (the gluteraldehyde linked chip was donated by Dr. Martin Danaher of Teagasc Ashtown). The surface was regenerated using 200 mM NaOH at a flow rate of 10 ul/min for 2 min. Inter-day studies were performed for 3 days and the co-efficient of variation and percentage accuracies were determined for each concentration from back-calculated concentration values obtained from the intra-assay calibration curve.

2.15 Development of lateral flow immunoassays for the rapid detection of TCB.

2.15.1 Optimisation of the anti-TCB IgY concentration for the lateral flow immunoassay development.

Strips of HiFlow Plus (Millipore, HF07504) nitrocellulose membrane was cut to dimensions of approximately 5 cm x 1 cm. TCB-BTG was diluted to a concentration of 1,500 ng/mL in spotting buffer and sprayed across the width of the strips approximately 1.5 cm from the bottom using the Linomat 5 system (CAMAG) at a flow rate of 400 nL/sec. Anti-MPO (control antibody) was diluted to a concentration of 72 µg/mL in spotting buffer and sprayed at 3.5 cm up the strip (control line). The membrane was incubated at 37^{o}C for 15 min to ensure adequate drying of both control and test lines. Following this, each strip was incubated for 1 h at 37^{o}C in a blocking solution and dried for 5 mins at R.T. Cellulose wicking material was attached to the top of each strip and allowed to stand vertically with the bottom of the strip touching the surface of a petri dish. Purified anti-TCB pAb was diluted 100, 50, 25 and 10 µg/mL in running buffer was applied to the strip. All strips were washed with a 200 µL of wash buffer. Following this, a 1/2,000 dilution of HRP-labelled anti-chicken antibody in running buffer was applied to the strips, and washed with 200 µL of wash buffer followed by the addition of 200 µL substrate TMB.

2.15.2 Optimisation of the control line for the lateral flow immunoassay development.

Strips of HiFlow Plus (Millipore, HF07504) nitrocellulose membrane was cut to dimensions of approximately 5 cm x 1 cm. Anti-MPO was diluted in spotting buffer to concentrations ranging from 2-600 µg/mL. Half the samples were sprayed across the width of the strips approximately 1.5 cm from the bottom, and the other half, 3.5 cm from the bottom of the strip using the Linomat 5 system at a flow rate of 400 nL/sec. All membranes were treated as described in section 2.15.1

2.15.3 Generation of the competitive lateral flow immunoassay for the detection of TCB in spiked PBS and milk samples.

A strip of HiFlow Plus (Millipore, HF07504) nitrocellulose membrane was cut to dimensions of approximately 5 cm x 1 cm. TCB-BTG was diluted to a concentration of 1,500 ng/mL in spotting buffer and sprayed across the width of the strips approximately 1.5 cm from the bottom using the Linomat 5 system (CAMAG) at a flow rate of 400 nL/sec. Anti-MPO (control antibody) was diluted to a concentration of 72 µg/mL in spotting buffer and sprayed

at 3.5 cm up the strip (control line). The membrane was incubated at 37^{o}C for 15 min to ensure adequate drying of both control and test lines. Following this, each strip was incubated for 1 h at 37^{o}C in a blocking solution and dried for 5 mins at R.T. Cellulose wicking material was attached to the top of each strip and allowed to stand vertically with the bottom of the strip touching the surface of a petri dish. Samples spiked with TCB (PBS and milk) were mixed (ratio 1:1) with 100 µg/mL of anti-TCB IgY. Samples (200 uL) were applied to the petri dish and allowed to travel up the length of the nitrocellulose strip via capillary action. The nitrocellulose strip was washed with 200 µL of 50 mM sodium phosphate pH 7.5, containing 0.05% (v/v) Tween. A 200 µL volume of a 1/2,000 dilution of HRP-labelled anti-chicken antibody in running buffer was applied to the strip, and washed with 200 µL of wash buffer followed by the addition of 200 µL substrate TMB. Blank sample buffer was used as a negative control

2.15.4 Reduction of the GFP using TCEP disulfide reducing gel

Tris [2-carboxyethyl]phosphine hydrochloride (TCEP)(Thermo scientific, 77712) was used for the reduction of the green fluorescent protein's (GFP) disulphide bonds to allow for the conjugation of the hapten to the protein via Sulfo-GMBS chemistry. TCEP is a highly effective agent for the reduction of disulfide bonds in proteins and peptides. The TCEP gel was washed 3 times with the addition of 300 µL 50 mM PBS, pH 7.5, and 5 mM EDTA. The gel matrix was centrifuged at 1,000 x g for 1 min and the supernatant removed. The GFP (150 µL) was added to the washed TCEP gel and vortexed for 1 min. The GFP and the TCEP were incubated for 1 h at R.T. The sample was centrifuged at 1,000 x g for 1 min. The supernatant containing the reduced GFP was removed and stored at 4^{o}C.

2.15.5 Conjugation of TCB to GFP using Sulfo GMBS

A 50 M excess of Sulfo-GMBS (0.372 mg) was added to 0.525 mg of the hapten TCB and 50 mM PBS, pH 7.5, was added to a final volume of 1 ml. This reaction was incubated at 4^{o}C for 4 h. Excess cross-linker was removed by dialysis with 5 L of 50 mM PBS, pH 7.5. Following the removal of excess cross-linker, the previously reduced GFP (150 µL) was combined with the TCB hapten and the reaction was incubated at 4^{o}C O/N.

2.15.6 GFP lateral flow assay:

A strip of HiFlow Plus (Millipore, HF07504) nitrocellulose membrane was cut to dimensions of approximately 5 cm x 1 cm. Purified anti-TCB pAb was diluted to 100 µg/mL in spotting buffer. The diluted anti-TCB pAb was sprayed across the width of the strip at approximately

at 1.5 cm up the strip using the Linomat 5 system at a flow-rate of 400 nL/sec. Anti-GFP mAb (control antibody);(Sigma Aldrich, G6539) was diluted to a concentration of 10 µg/mL in spotting buffer and sprayed at 3.5 cm up the strip (control line). TCB samples (500, 100, 50, 25 and 5 ng/mL) and the conjugated TCB-GFP (1/5) were initially prepared in running buffer and mixed in a ratio 1:1. Following this, milk was spiked with 500, 100, 50, 25 and 5 ng/mL of TCB and extracted via QuEChERs method as described in section 2.13.1. The conjugated TCB-GFP was diluted 1/5 in running buffer and mixed in a ratio of 1:3 with the extracted samples. Cellulose wicking material was attached to the top of each strip and allowed to stand vertically with the bottom of the strip touching the surface of a petri dish. A 200 uL of each sample to be tested was applied to the petri dish and the sample travelled up the length of the nitrocellulose strip via capillary action. The strips were then visualised using a typhoon 8600 variable mode flourescence imager at excitation and emmission wavelengths of 488 nm and 507 nm respectively.

Chapter 3

The generation of an anti-Thiabendazole Fab from a monoclonal antibody-secreting hybridoma.

3.0 Introduction: Murine antibody generation

Hybridoma technology was first introduced in 1975 by Köhler and Milstein whereby a method was described for producing cell lines that secrete a single species of antibody. A hybridoma is a cell hybrid that is produced *in-vitro* by the fusion of a lymphocyte (mouse spleenocyte) that produces a specific antibody and a myeloma cell. This hybrid proliferates into a clone that produces a continuous supply of monoclonal antibody (Köhler and Milstein, 1975). This technology has since been applied for the generation of monoclonal antibodies (mAb) against a wide variety of targets such as, viruses (Wrammer *et al.,* 2008), bacteria (Hearty *et al.,* 2006), and veterinary drug residues. These include the benzimidazoles, including albendazole, fenbendazole, oxibendazole and thiabendazole (Bushway *et al,*. 1995), as described in section 1.8.1.

In 1989, Orlandi *et al.* designed a set of oligonucleotide primers to amplify the cDNA of mouse immunoglobulin heavy and light chain variable domains by the Polymerase Chain Reaction (PCR). They applied this technique to amplify the variable domains of five hybridoma antibodies and to express a mouse-human chimeric antibody that bound to the human mammary carcinoma line, MCF-7. They hypothesized that this technique would lead to the cloning of antigen-binding specificities directly from immunoglobulin genes, thus bypassing hybridoma technology. Ward *et al*. found that by using PCR they could isolate V_H domains with good antigen-binding affinities. Diverse libraries of V_H genes were cloned from murine spleen genomic DNA and expressed and secreted from *E. coli* and it was suggested that the isolation of variable domains may offer an alternative to mAbs and serve as the key to building high-affinity human antibodies (Ward *et al.,* 1989).

Marks and co-workers subsequently reported the generation of antibodies via gene technology, where variable genes were amplified from hybridomas or B cells, by PCR and cloned into expression vectors. Soluble antibody fragments secreted from bacteria were screened for binding activities. They revolutionized this approach by successfully displaying antibody V domains on the surface of fd bacteriophage. The phage would bind specifically to antigen and therefore, could be detected and subsequently isolated (Marks *et al.,* 1990). Using this method, McCafferty *et al.* showed that they could mimic the features of immune selection to make human antibodies in bacteria by generating diverse libraries of V_H and V_L by PCR with subsequent expression on the phage surface. Specific antigen-binding phage was selected by panning against the target antigen. They established that a single large phage

display library could be used to isolate human antibodies against any antigen, thus eliminating the requirement of hybridoma technology and immunisation (McCafferty *et al.*, 1990). These advances in the generation of diverse libraries of variable heavy and light genes from genomic DNA and the subsequent expression of these genes on the surface of bacteriophage have resulted in an increase in the use of recombinant antibodies for both diagnostic and therapeutic purposes (Ward *et al.*, 1989; McCafferty *et al.*, 1990; Röthlisberger, Honegger and Pluckthun, 2005).

When generating recombinant antibodies there are many arguments for and against the decision to generate a Fab or a scFv library. Hoogenboom suggested that the smaller size of the scFv format makes these libraries genetically more stable than Fab libraries (Hoogenboom, 2002). However, many scFvs can form dimers and trimers, which may complicate selection and characterization. The Fab fragments lack this polymerisation tendency and can more easily be incorporated into assays and binding kinetic experiments (Hoogenboom, 2002). Studies carried out by Reiter *et al*. and Röthlisberger *et al*.; indicate that some scFv fragments are likely to be less stable than Fab fragments, partly due to the lack of intra-molecular disulfide bonds and constant domains and the altered stability of the variable domains involved in the antibody construct (Reiter *et al.*, 1994; Röthlisberger, *et al.*, 2005). The choice of Fab fragments for use in this study was due primarily to studies indicating better performance in biosensor-based assays (Townsend *et al.*, 2006) and their increased stability in various matrices which is an important characteristic for environmental and veterinary residue diagnostics.

3.1 Aims

The aims of the research described in this chapter were:

a) to construct a recombinant antibody fragment library from an anti-TBZ monoclonal antibody-secreting hybridoma cell line received from Dr. David Brandon of the United States Department of Agriculture (USDA),

b) to screen the library against the drug protein carrier conjugate, TBZ-BSA, to isolate an antibody specific for TBZ, and

c) to improve the sensitivity of the antibody by incorporating a double helix (dHLX) gene upstream of the anti-His tag and down-stream of the gene III of the pComb3xTT vector thus increasing the valency of the antibody.

3.2 Results

3.2 Anti-TBZ antibody-secreting hybridoma cell line screening, purification and characterisation.

3.2.1 Screening of the anti-TBZ antibody-secreting hybridoma cell line, antibody titre and isotyping.

Upon receipt of the anti-TBZ monoclonal antibody-secreting hybridoma cell line stocks were cultured, maintained and stored, as described in section 2.8.1. Following the continuous culture of the cell line, anti-TBZ monoclonal antibody supernatants were screened for binding activity by direct ELISA, as described in section 2.9.13 and represented in the schematic in Figure 3.2.1.

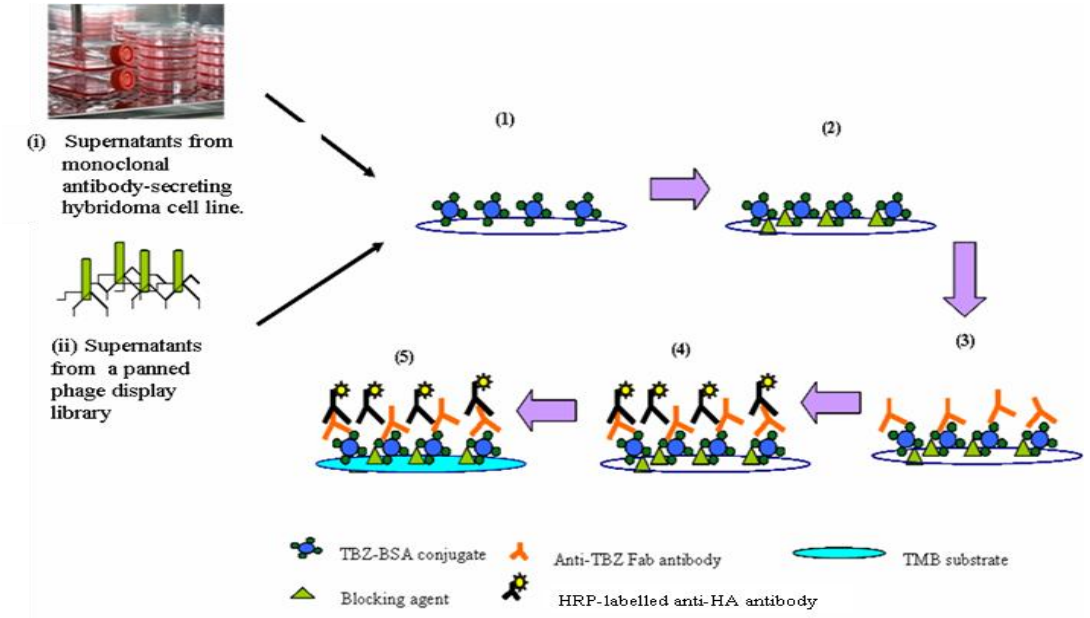


Figure 3.2.1 Schematic diagram of the direct ELISA format that was used to determine the anti-TBZ monoclonal antibody supernatant titre and to screen for positive antibody-producing clones during the bio-panning process. (1) A 96 well plate was coated with 1 µg/mL TBZ-BSA conjugate; (2) the plate was 'blocked' with a solution of 5 % (w/v) milk marvel in PBS. (3) supernatant from the antibody-secreting hybridoma cell line or lysates from positive phage-derived clones were added to the plate; (4) the anti-TBZ mAb was detected using a horse-radish peroxidase (HRP)-labelled anti-mouse IgG and the recombinant phage-derived Fab was detected with a HRP-conjugated anti-HA antibody; (5) TMB HRP-substrate was added to a 96 well plate and developed at R.T. and the reaction was quenched with the addition of 50 µL of 10 % (v/v) HCl.

To determine the un-purified anti-TBZ monoclonal antibody titre, a direct ELISA (Figure 3.2.2) was performed, as described in section 2.9.13. A titre is a measurement of how much antibody is contained in the serum of an antibody-producing animal or in the supernatant of an antibody-secreting hybridoma cell line and is expressed as the greatest dilution or reciprocal that still gives a positive result. The molarity of the PBS and PBST used for dilutions throughout was 10 mM, with a pH of 7.5, unless otherwise stated.

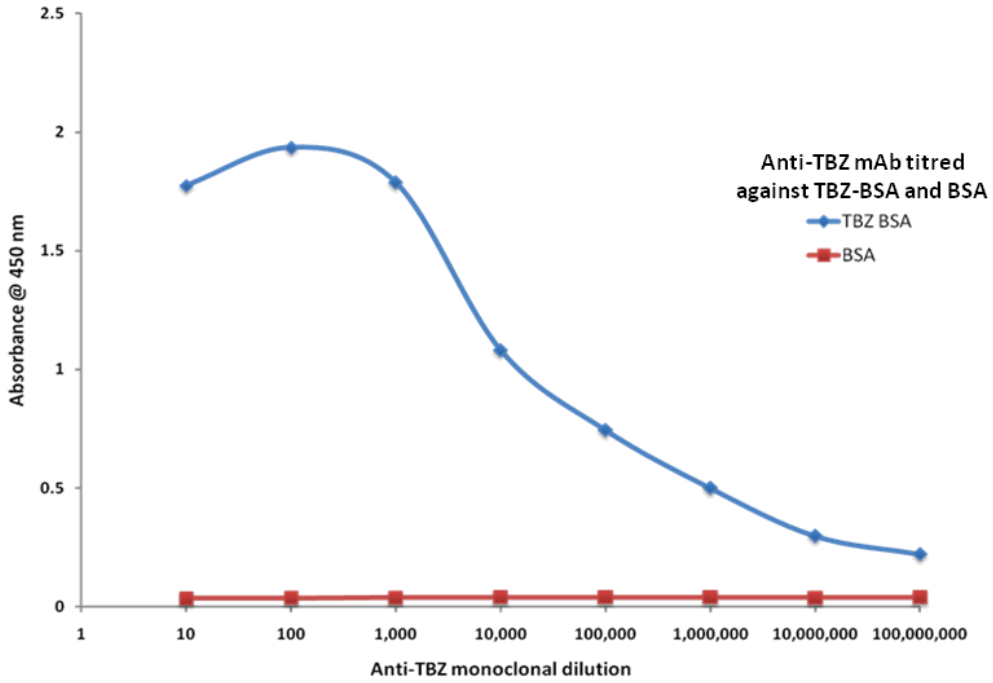


Figure 3.2.2: Plot of the absorbances obtained from the direct ELISA for the titration of the anti-TBZ monoclonal antibody supernatant against the conjugate TBZ-BSA and the carrier protein BSA. The plate was coated overnight with 1 µg/mL of TBZ-BSA or BSA. The plate was subsequently blocked with 5 % (w/v) milk marvel in PBS, and the anti-TBZ supernatant was serially diluted 1/10-1/100,000,000 in 0.5 % (w/v) milk marvel PBS. The detection antibody used was a HRP-labelled anti-mouse IgG.

Titration of the mAb against BSA confirmed that the antibody was specific only for the target TBZ as the absorbances recorded were approximately zero. The antibody was also titred against uncoated wells and these background absorbance values were subtracted from the figures obtained from the coated wells to give the specific binding response. At an antibody dilution of 1/10,000,000 against TBZ-BSA there is a significant response thus indicating that the mAb titres out at approximately a 1/10,000,000 dilution. Following this initial

characterisation, the isotype of the antibody was determined (section 2.8.2) to select the best purification strategy, as knowledge of its class and isotype permits selection of the most suitable affinity ligand to be applied. The class and subclass of the monoclonal antibodies was found to be IgG and $IgG_{1,}$ respectively, and the antibody was found to have kappa light chains (Figure 3.2.3).

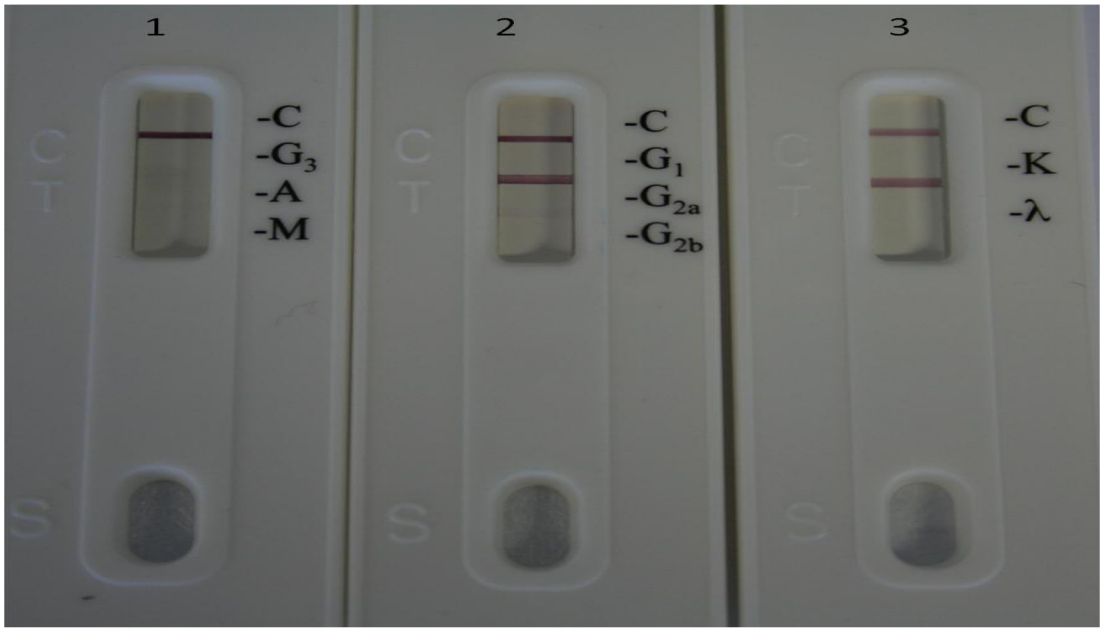


Fig 3.2.3: Picture of the isotyping of the anti-TBZ mAb. Cassette 1 shows that the anti-TBZ monoclonal antibody is not of the IgG_3, IgM or IgA isotype. Cassette 2 shows the anti-TBZ monoclonal antibody to be of the subclass $IgG_{1.}$ Cassette 3 shows anti-TBZ monoclonal antibody has kappa chains.

3.2.2 Purification of the anti-TBZ mAb via protein-G affinity chromatography.

The anti-TBZ mAb was purified using protein-G affinity chromatography (section 2.8.4). The purification of the anti-TBZ mAb was confirmed by SDS PAGE (Figure 3.2.4, section 2.9.2).

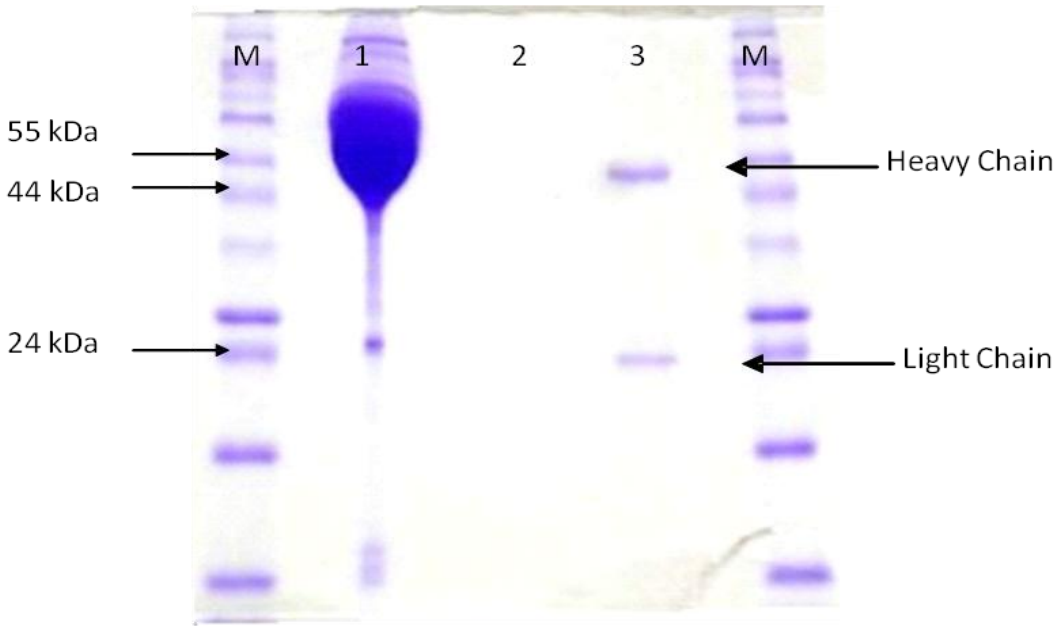


Figure 3.2.4: An SDS-PAGE gel of the purified anti-TBZ monoclonal antibody is shown. The gel was stained with Coomassie blue. Lane M (on both sides of the gel), represents the Sigma wide standard molecular weight markers; Lane 1 is the 'flow-through' diluted 1:20 in PBS; Lane 2 is the column wash diluted 1:2 in PBS, and Lane 3 is neat protein-G affinity-purified pooled anti-TBZ mAb fraction. The heavy chain and light chains are indicated at 50 kDa and 25 kDa, respectively.

3.2.3 Checkerboard ELISA for the optimisation of TBZ-BSA coating concentrations and anti-TBZ mAb dilution.

Following purification of the anti-TBZ mAb, an initial checkerboard ELISA was performed to determine the optimal concentration of TBZ-BSA conjugate to use for titre determination and further assay development (Figure 3.2.5).

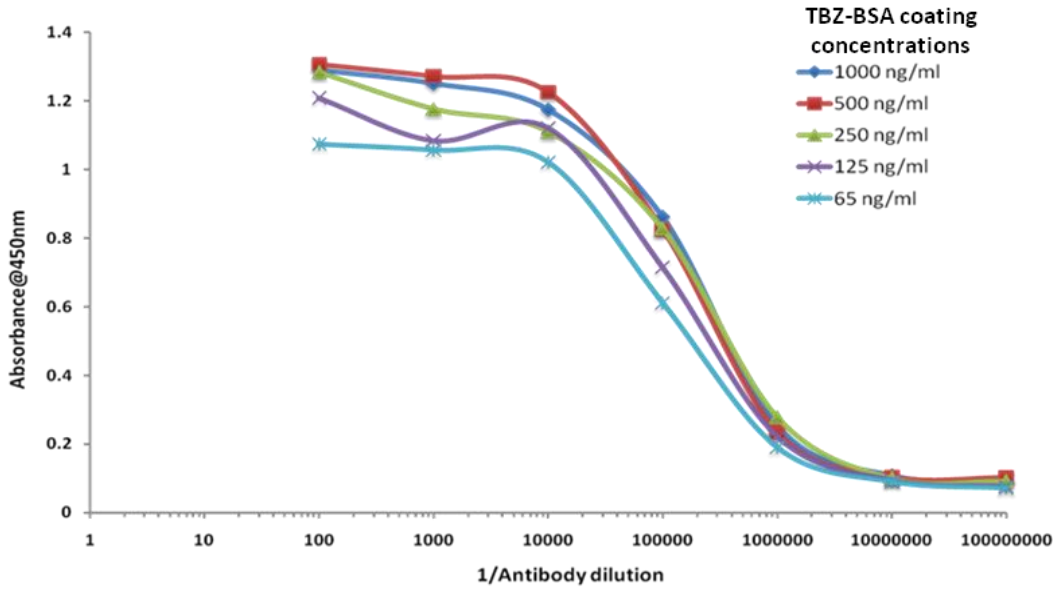


Figure 3.2.5: Plot of the absorbances obtained from the checkerboard ELISA for the determination of the optimal performance characteristics of the anti-TBZ protein G- purified mAb. The ELISA plate was coated with different concentrations of TBZ-BSA (1 µg/mL to 0.06 µg/mL) in PBS. Wells were subsequently blocked with PBS; containing 5 % (w/v) skimmed milk powder. Protein G-purified monoclonal anti-TBZ antibody, serially diluted (ranging from 1/10 to 1/100,000,000) in 0.5 % (w/v) milk marvel in PBST, was then added. The detection antibody used was a HRP-labelled anti-mouse IgG.

The 250 ng/mL coating concentration of the TBZ-BSA conjugate was chosen as the most favourable concentration to give an optimum absorbance of 1 at 450 nm as the responses using the two higher concentrations of 1000 and 500 ng/mL, coupled with a high dilution factor (1/50,000-1/100,000), were almost identical. The use of a higher concentration of antibody would have been wasteful. Wells were also blocked with BSA and the absorbance measured was subtracted from the response generated from the absorbance obtained from the wells coated with varying concentrations of TBZ-BSA.

3.2.4 Anti-TBZ mAb competitive assay for the detection of TBZ

Following this a competitive assay (schematic shown in Figure 3.2.6) was performed to establish an IC_{50} of the purified anti-TBZ mAb (Figure 3.2.7, section 2.9.18). An IC_{50} (half maximal inhibitory concentration) is a measure of the effectiveness of a compound to inhibit a biochemical reaction such as antigen/antibody binding. This value can be extrapolated from

an inhibition/competitive curve. In this case the IC_{50} value refers to the concentration of free TBZ required to give 50 % inhibition of the binding of the mAb to the immobilised TBZ-BSA.

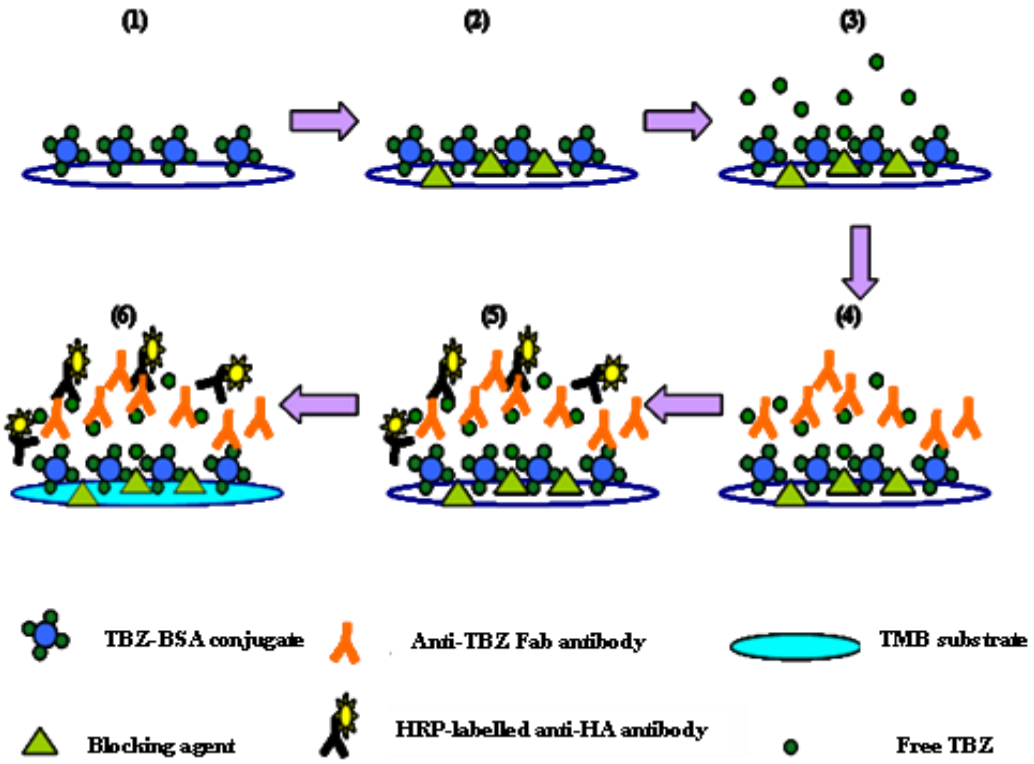


Figure 3.2.6: Schematic diagram of competitive ELISA used to screen for positive clones and for assay development. (1) a 96 well plate was coated with the TBZ-BSA conjugate; (2) the plate is 'blocked' with a solution of 5 % (w/v) milk marvel in PBS, pH 7.5; (3) free TBZ was added to each well; (4) lysate supernatant containing anti-TBZ Fab, or purified mAb, was added to each well; (5) the recombinant phage-derived Fab was detected with a horse-radish peroxidase (HRP)-conjugated anti-HA antibody; (6) TMB HRP-substrate was added to a 96 well plate and developed at R.T. and the reaction was quenched with the addition of 50 µL of 10 % (v/v) HCl. The absorbance was measured at 450nm.

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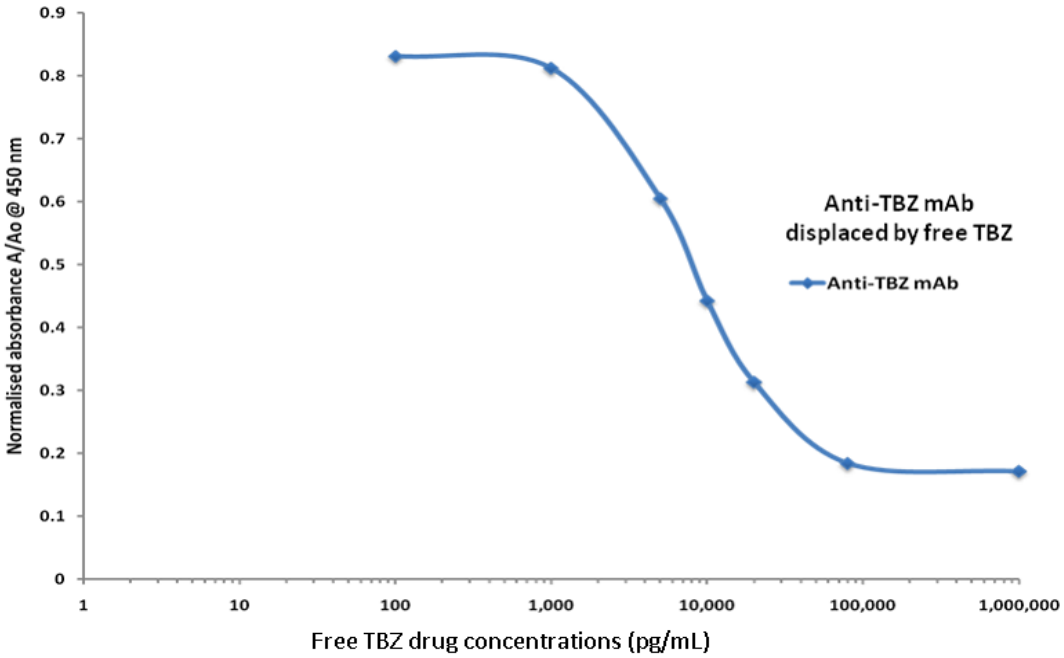

Figure 3.2.7: Normalised plot of the absorbances obtained with the competitive assay using protein G-purified anti-TBZ mAb. The plate was coated with 250 ng/mL of TBZ-BSA and subsequently blocked with 5 % (w/v) milk marvel in PBS, Free TBZ concentrations (1-100,000 pg/mL) were prepared in 0.5 % (w/v) milk marvel in PBST -and added to the plate followed by a 1/10,000 of anti-TBZ antibody in 0.5 % (w/v) milk marvel in PBST. The detection antibody used was a HRP-labelled anti-mouse IgG.

The IC_{50} of this assay was determined as 50,000 pg/mL using an equation based on the four parameter logic fit. The equation is as follows; IC_{50} = (Absorbance @ Zero drug concentration-(Absorbance at zero drug concentration-Absorbance @ maximum drug concentration))/2. This antibody can detect TBZ below the required MRL of 100 µg/kg, and, thus, there is no requirement for further enhancement. However, the possibility of the production of a functional recombinant antibody expressed in bacteria offered a number of advantages over the maintenance of a hybridoma cell line. Bacterial stocks may be stored at -80 indefinitely and can be rapidly thawed and produced in large-scale with the use of bioreactors and fermentation procedures. Additionally, their further manipulation at a genetic level is feasible with recombinant DNA technology. Advantages of this technology includes, (i) antibodies can be produced in bacteria, yeast or plants, and (ii) intrinsic properties such as immunogenicity, affinity, specificity and stability of antibodies can be improved by various mutagenesis technologies (Pansri *et al.*, 2009). One of the main aims of this research was to

generate a recombinant antibody fragment against TBZ and, as a monoclonal antibody-secreting anti-TBZ hybridoma cell line that had a high affinity to TBZ was readily available, it was decided to use this as the functional source of immunoglobulin genes to produce a Fab antibody, thus eliminating the requirement of immunisation of an animal. A Fab fragment was chosen for construction for several key reasons:

a) It was necessary to generate antibodies for a high sensitivity BIAcore™-assay.

b) A Fab has uniform binding kinetics, is smaller in size than other IgG counterparts and has greater stability, thus making it ideal for use in a high sensitivity assay format.

c) Such a recombinant antibody, as discussed above, has the capacity for re-engineering to enhance its specificity or to amplify its selectivity for a broader range of TBZ metabolites.

3.3 The construction of the anti-TBZ antibody binding fragment (Fab) library.

The cDNA generated from the RNA extracted from the hybridoma (section 2.8.5) was utilized for the initial amplification of the murine variable light (V_λ) and variable heavy (V_H) chains. The PCR reaction was carried out using the combination primers, described in section 2.9.1, and the components and reaction programme, outlined in section 2.9.2.

The MSCVK sense primer mixture was combined with a number of MHybJK reverse primers to amplify the variable light genes from the cDNA. The variable light sense primers have a 5´sequence tail that contains the *Sfi*1 site. This sequence is further recognized by the extension primers of the subsequent PCR rounds for the final amplification of the 1,500 bp gene product. The reverse primers have human C_κ sequence tails that create the chimeric light-chain–pelB fragments.

A mixture of MHyVH sense primers was combined with a mixture of MHyIgGCH1-B reverse primers to amplify V_H gene segments from cDNA. Each sense primer has a 5´sequence tail that corresponds to the 3´ end of the pelB leader sequence and is recognized by the sense extension primer (leadVH) in the second-round PCR. The reverse primer contains a human C_H1 sequence tail that is used in the overlap extension PCR to create the chimeric heavy chain fragment. All PCR reactions were resolved on a 1 % (w/v) agarose gel (Figure 3.3.1).

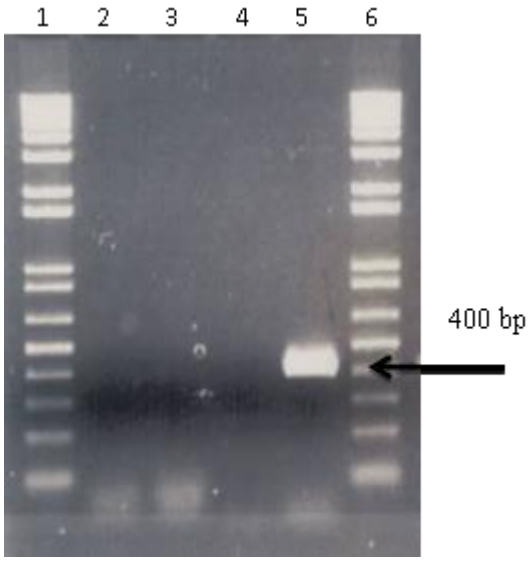

Figure 3.3.1: Amplification of the murine variable light and variable heavy chains using the cDNA obtained from the anti-TBZ monoclonal antibody-secreting cell line. Lanes 1 and 6: 1 Kb Sigma DNA ladder; Lane 2: V_λ chain amplification; Lane 3: V_H chain amplification; Lane 4: negative control and Lane 5: positive control.

There was no amplification for either of the variable chains. The DNA template used for the positive control was a previously generated anti-amphetamine Fab fragment prepared 'in-house'.

Following this, PCRs were performed varying the concentration of $MgCl_2$ used for the amplification of both the variable light and heavy chains. However, there was no amplification of either variable chain. In the case of murine genes mouse variable antibody regions are generated through somatic DNA recombination. The V_κ light chain in mice is the dominant light chain and is generated by the combination of one of many V_κ genes with four J_κ genes (J (joining) genes are the joining segments of polypeptide chains which link the variable regions to the constant regions of both the heavy and light chains). The variable regions of the heavy chain are formed by the combination of one of several hundred V_H genes with one of about 30 D (Diversity) segments and one of six J_H segments. The cloning of the murine antibody repertoire requires many V-specific oligonucleotide primers thus leading to a potential loss of diversity and the inability of the some primers to amplify the gene (Barbas *et al.,* 2004). Successful amplification of the variable light chains (Figure 3.3.2) was achieved using the combination of reverse primers and individual MSCVK primers numbers 2, 3, 4, 5, 9, 11 and 17. The same approach was used for the amplification of the variable heavy chains

(Figure 3.3.3) where successful amplification was accomplished with the MhyVH 8, 9, 10 and 11 primers.

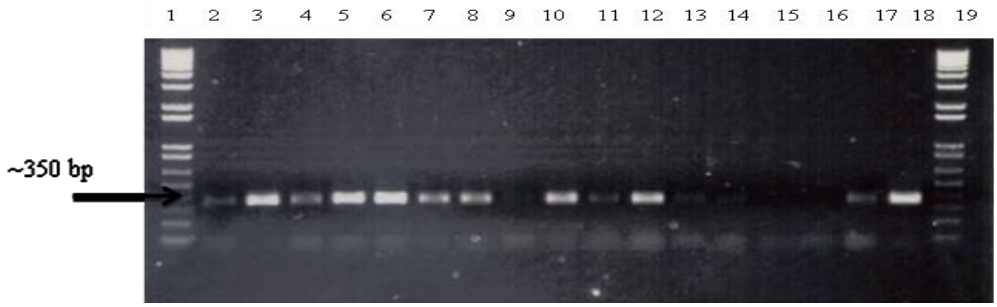

Figure 3.3.2: Optimisation of the variable light kappa chain using one of each forward primer from the MSCVK 1-17 primer combination. Lanes 1 and 19: 1 Kb Sigma DNA ladder and Lanes 2-18: MSCVK 1-17. Products at 350 bps were successfully amplified using the forward primers MSCVK 2, 3, 4, 5, 9, 11 and 17.

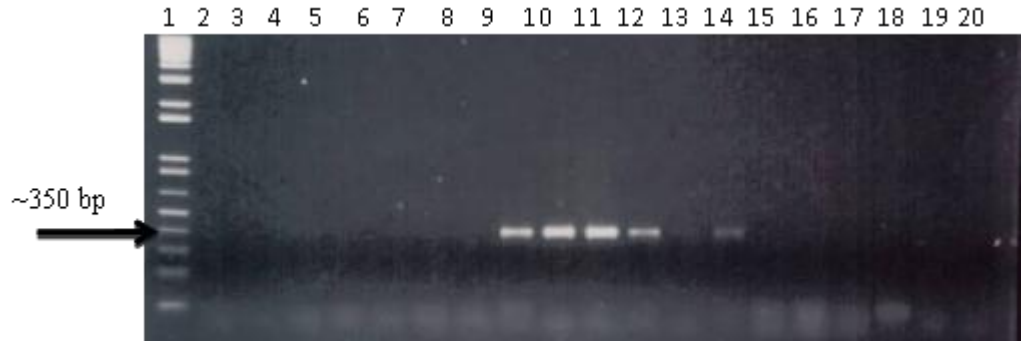

Figure 3.3.3: Optimisation of the variable heavy chain using one of each forward primer from the MhyVH 1-19 primer combination. Lanes 1 and 20: 1 Kb Sigma DNA ladder and Lanes 2-19; MhyVH 1-19. Products of 350 bps were successfully amplified using primers 8, 9, 10 and 11.

Subsequently, a 3 x PCR reaction was performed using each primer pair which yielded a band using the reaction mix and programme, as described in section 2.9.2. All amplified variable light and heavy chains were pooled together, ethanol precipitated and gel-purified.
The Cκ-pelB leader and C_H1 sequences were amplified from pComb3xTT using the reaction mix and PCR programme, as described in 2.9.3 and 2.9.4, respectively. The forward primer HKC amplified the 5´ region of human Cκ. This is the region used in the overlap extension PCR to create the chimeric light chain. The reverse primer Lead B is specific for the 3´end of the pelB leader sequence. The forward primer, HIgGCH1-F, for the amplification of the C_H1

products is specific for the overlap extension that creates the heavy chain Fd region. The reverse primer, dpseq, is specific for the decapeptide region of the pComb3xTT.

A band of the expected size of 400 bp was observed for the C_k-pelB product and the C_H1 product (Figure 3.3.4). A large-scale PCR was carried out for both, and the resulting bands were ethanol-precipitated and gel-purified.

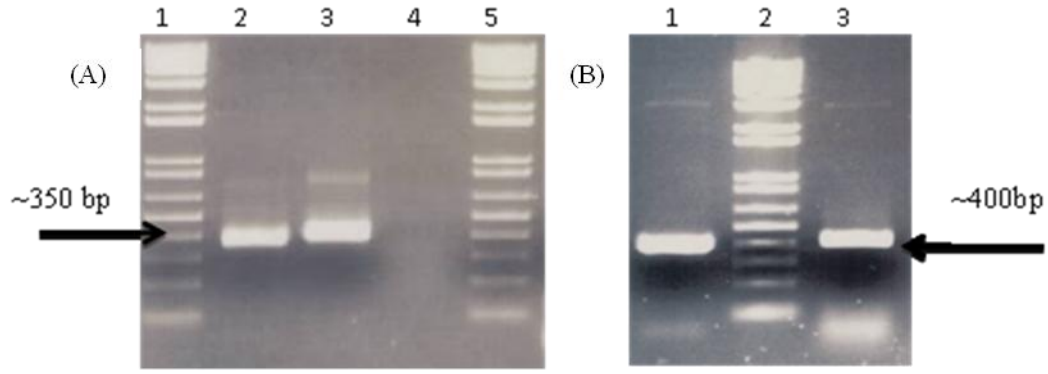

Figure 3.3.4: Amplification of the V_λ and V_H chains (A) and the C_k-pelB and C_H1 (B). The PCR reactions and programmes are outlined in 2.11.3 and 2.11.5, respectively. Gel (A) Lanes 1 and 5: 1 Kb Sigma DNA ladder; Lane 2: variable light chain; Lane 3: variable heavy chain and lane 4: negative control. Gel (B) Lane 1: constant kappa chain; Lane 2: 1 Kb Sigma ladder and Lane 3: constant heavy chain.

Following successful amplification of the variable and constant domains the variable light chains were fused with the constant region using PCR to generate the chimeric V_L. A chimeric heavy chain was similarly constructed. The PCR was carried out, as described in section 2.9.6, and the expected band sizes of 800 bp and 750 bp were observed for the chimeric light chain and heavy chain, respectively (Figure 3.3.5 (A)). A final PCR was performed to combine the chimeric light chain-pelB fragment to the Fd fragment resulting in a murine chimeric Fab gene product. This was carried out by splice by overlap extension, as described in section 2.9.7. A band at 1,500 bps was observed that corresponds to the correct band size for a chimeric Fab gene (Figure 3.3.5 (B)).

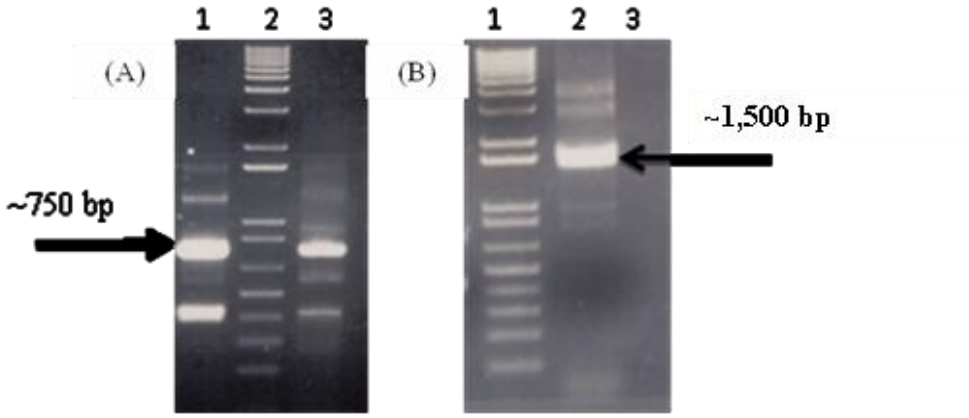

Figure 3.3.5: Initial overlap of the variable and constant domains (A) and the final splice by overlap extension of the chimeric light chains and chimeric heavy chain (B). (A) Lane 1: chimeric light chain (800 bp); Lane 2: 1 Kb Sigma DNA ladder and Lane 3: chimeric heavy chain (750 bp). (B) Lane 1: 1 Kb Sigma DNA ladder and Lane 2: murine chimeric Fab gene product (1,500 bp).

3.4: Transformation and subsequent bio-panning of the anti-TBZ Fab library against TBZ-BSA.

A large-scale (500µl) amplification of the final SOE product was carried out. The PCR product was concentrated using ethanol precipitation, quantified using the Nanodrop ND-1,000 ™ and subsequently cloned into the pComb3xSS vector. The directional cloning was performed using *Sfi*1 and was successfully ligated into the digested pComb vector (section 2.9.8). The ligation was ethanol-precipitated and transformed into *E. coli* XL1-Blue electro competent cells (section 2.9.9).

The library size obtained was 1.4 x 10^7. A colony pick PCR was performed, as described in section 2.9.10, to ensure that the gene insert was present at 1,500 bps (Figure 3.4.1). The transformed library was subjected to three rounds of panning against varying concentrations of the TBZ-BSA conjugate immobilised on a solid support (immuno-tube) (section 2.9.12). The phage input and output titres of each round of panning are shown in Table 3.4.1.

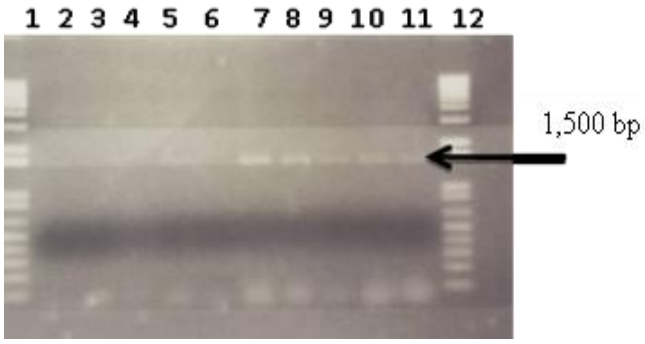

Figure 3.4.1: Colony pick PCR for the amplification of the Fab gene insert from the transformed anti-TBZ Fab library. Lanes 1 and 12: 1 Kb Sigma DNA ladder and Lanes 2-11: amplification of TBZ Fab gene from 10 different colonies.

Table 3.4.1: The phage input and output titres for all three rounds of bio-panning against TBZ-BSA.

Round of bio-panning	Colony forming units (CFU)
Input 1	6.3×10^{11}
Output 1	7.3×10^{8}
Input 2	4.4×10^{12}
Output 2	5.6×10^{7}
Input 3	6.4×10^{12}
Output 3	6.7×10^{7}

Colonies (96) were selected from each round of panning and cultured overnight in 200 µL of media containing 100 µg/mL carbenicillin. All clones were sub-cultured and the expression of the Fab was induced with 1 mM IPTG, (section 2.9.1.4). The lysates obtained from the Fab clones were used to analyse their specificity to TBZ-BSA immobilised on a microtitre plate and for binding to antigen in solution for competitive ELISA (section 2.9.13). A number of controls were used in this experiment to ensure all components of the ELISA were functioning. 1) wells were coated with TBZ-BSA, subsequently blocked and a 1/20,000 of the anti-TBZ mAb was added, followed by the addition of a HRP-labelled anti-mouse IgG to ensure the conjugates specificty. 2) wells were coated with an amphetamine conjugate, subsequently blocked and a 1/100 dilution of an anti-amphetamine Fab added, followed by

the addition of HRP-labelled anti-HA antibody to determine that this antibody was specific for the detection of the HA tag from the Fab-secreting clones. (3) All clones were screened against BSA (1 µg/mL) to ensure that all positives were not specific to BSA (data not shown). Figure 3.4.2 shows the eleven clones that were deemed positive. These clones included A1, A10, B11, C9, D7, E6, F2, F5, F9, H1, H4, and H9.

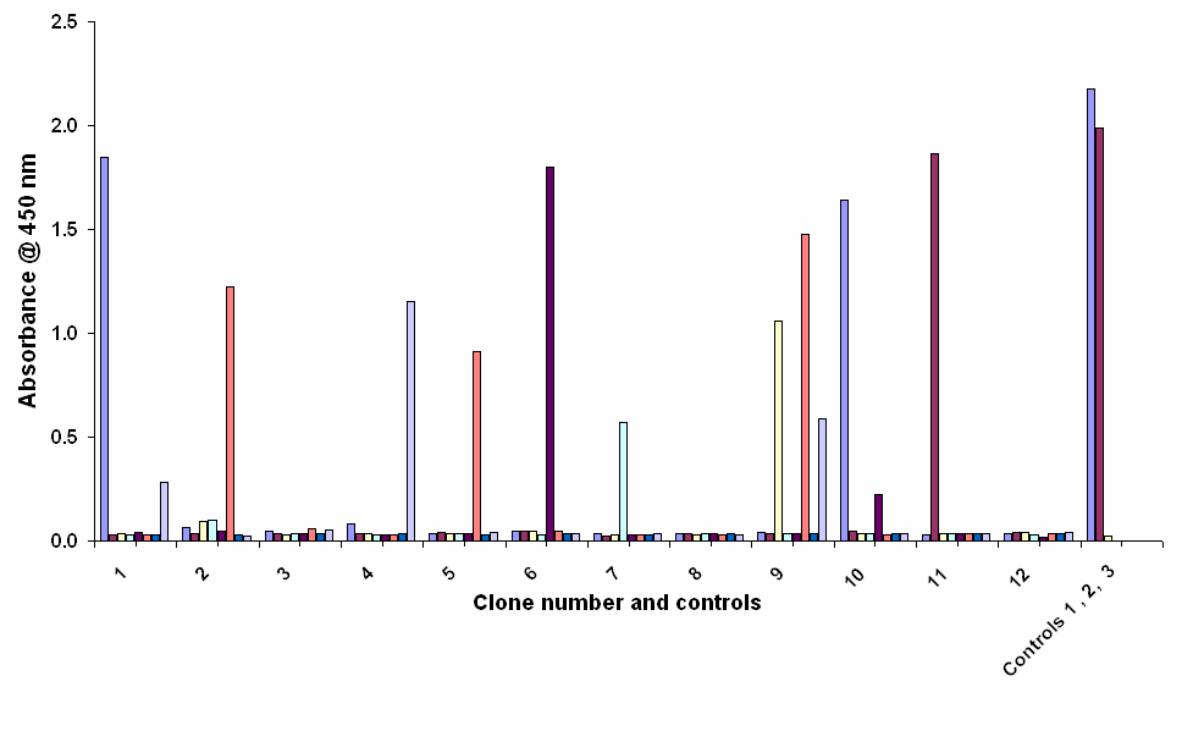

Figure 3.4.2: The plot of the absorbances from the positive clones obtained from round one of the panning of the anti-TBZ library against TBZ-BSA. Plates were coated with 1 µg/mL of TBZ-BSA and subsequently blocked with 5 % (w/v) milk marvel in PBS, Supernatant lysates (100 µL) obtained from the growth of each colony (section 2.12) were added to their corresponding wells. This was followed by a 1/2,000 dilution of a HRP-labelled anti-HA antibody. Any clone that gave an absorbance value of above 0.2 at 450 nm was selected for further characterisation. Controls used (1, 2 and 3) were as explained previously to verify the conjugate's (1) and secondary antibody's (2) specificity. Control 3 was used to determine if there was a high level of binding between the Fab-secreting clone and the carrier protein BSA.

Overnight cultures were prepared of all 12 clones that had an absorbance above 0.2. These were sub-cultured the following day and allowed to grow until the O.D reached 0.6. Expression was induced with 1 mM IPTG and grown O/N at 30°C. Lysates were prepared as

in section 2.9.14. Each of the Fab-secreting clones was screened by direct ELISA to determine their titre against 1 µg/mL of TBZ–BSA immobilised on the surface of the plate (Figure 3.4.3).

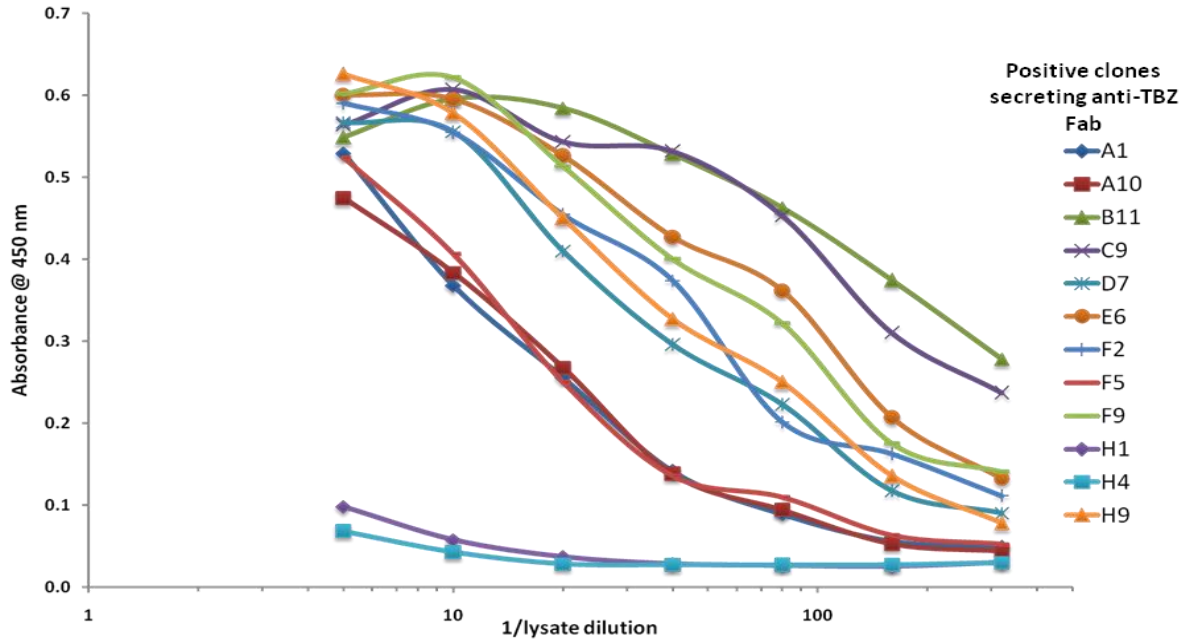

Figure 3.4.3: Plot of the absorbances obtained from the antibody titres of lysates of 12 positive clones. TBZ-BSA was coated on the plate which was subsequently blocked with 5 % (w/v) milk marvel in PBS. Serial dilutions (1:2) were performed on all antibody lysates and 100 uL of each dilution was added to the plate. This was followed by a 1/2,000 dilution of a HRP-labelled anti-HA secondary antibody. The numbers in the legend correspond to the location of each clone on the ELISA plate.

The binding profile of each positive anti-TBZ Fab-secreting clone is illustrated above in Figure 3.4.3. During this screening process clones H1 and H4 exhibited low binding to the TBZ-BSA conjugate and thus they were discarded. Fresh lysates from the ten remaining positive clones were prepared, as lysates stored for longer than 24 hr at 4°C sometimes lose their sensitivity. A competitive assay was performed using the freshly prepared lysates and serial dilutions of free TBZ. Six of the nine clones showed low levels of displacement by the addition of free TBZ (Figure 3.4.4), and, therefore, they were eliminated from further characterisation and purification. Three clones (D7, E6 and F2) were selected for further study.

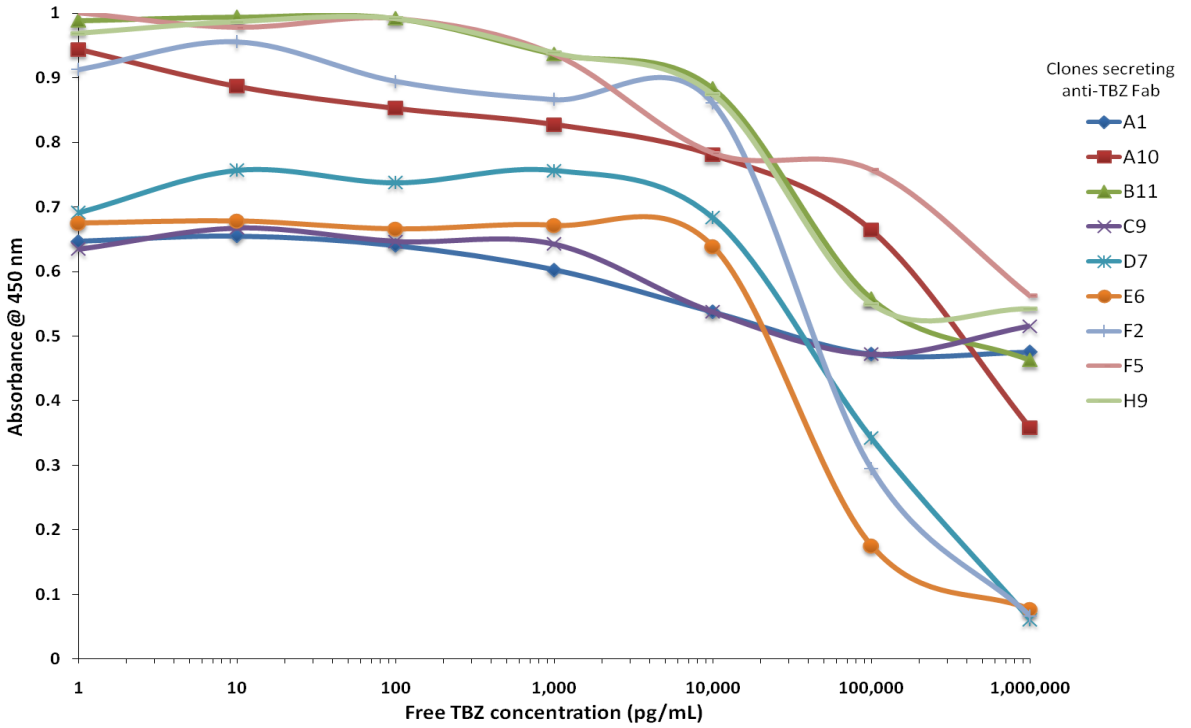

Figure 3.4.4: Plot of the absorbance's obtained from the competitive assay for the clones secreting anti-TBZ Fab. The plate was coated with 1 µg/mL of TBZ-BSA and subsequently blocked with 5 % (w/v) milk marvel in PBS. Serial dilutions of free TBZ were prepared (1-1,000,000 pg/mL) and added to their corresponding wells followed by a 1/10 dilution of each of the antibody lysates. Detection antibody was a HRP-labelled anti-HA antibody

3.5 Purification of the anti-TBZ Fab and the development of a competitve assay for the detection of TBZ.

3.5.1 Purification and antibody titre for the anti-TBZ titre

The clones that were significantly displaced by the addition of free TCB were D7, E6 and F2. The IC_{50} of the assay from Figure 3.4.4 that incorporated the three clones secreting anti-TBZ Fabs was approximately 20,000 pg/mL. These clones were grown on a large-scale, as described in section 2.9.14, and subsequently purified via IMAC (section 2.9.15). A problem in the large-scale expression of the three clones led to the ineffective purification of F2 and D7. However, clone E6 was successfully purified. Following IMAC purification of this anti-TBZ Fab it was titred against the TBZ-BSA conjugate to determine the working dilution to be used for further assays (Figure 3.5.1).

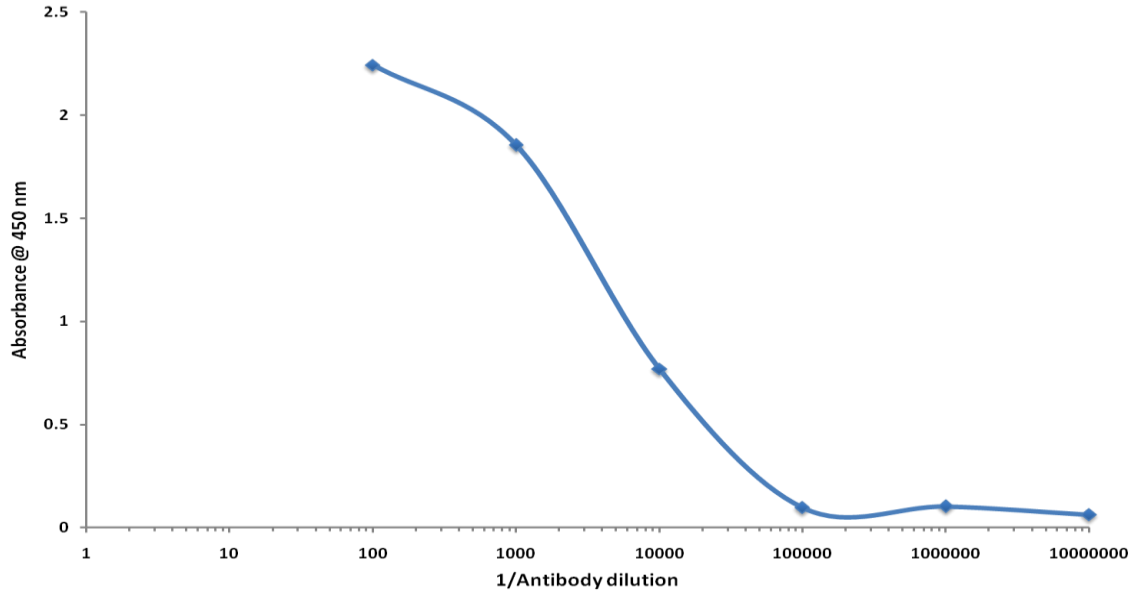

Figure 3.5.1: Plot of the titration of the anti-TBZ purified Fab against TBZ-BSA. The ELISA plate was coated with 1 µg/mL of TBZ-BSA and subsequently blocked with 5 % (w/v) milk marvel in PBS. Dilutions (1/100-1/10,000,000) of the anti-TBZ Fab were prepared in 0.5 % (w/v) milk marvel in PBST. The detection antibody was HRP-labelled anti-HA.

3.5.2 Intra and inter-day assays for the anti-TBZ mAb for the detection of TBZ in spiked PBS.

A dilution of 1/1,000 was used for any subsequent assays. When incorporated into a competitive assay a 1/500 dilution was mixed with the analyte to give a final working dilution of 1/1000 which is the optimum dilution. Inter-day assays were used to evaluate the performance of the assay over a period of days. Repeatability (intra-day; Figure 3.5.2)) was determined by carrying the assay out three times on the same day using the same batches of sample, antibody, analyte and diluents and three replicate measurements of antibody plus each free TBZ drug dilution were used. These absorbance values (A) were then divided by the absorbance measurement at a zero free drug concentration (A_0) to give normalised values (A/A_0). Using the BIAcore™ evaluation approach based on a 4 parameter best fit equation it was possible to calculate the mean, standard deviation, coefficient of variation (CV) and percentage accuracies for both the intra and inter-day assays (Tables 3.5.1 and 3.5.2, respectively).

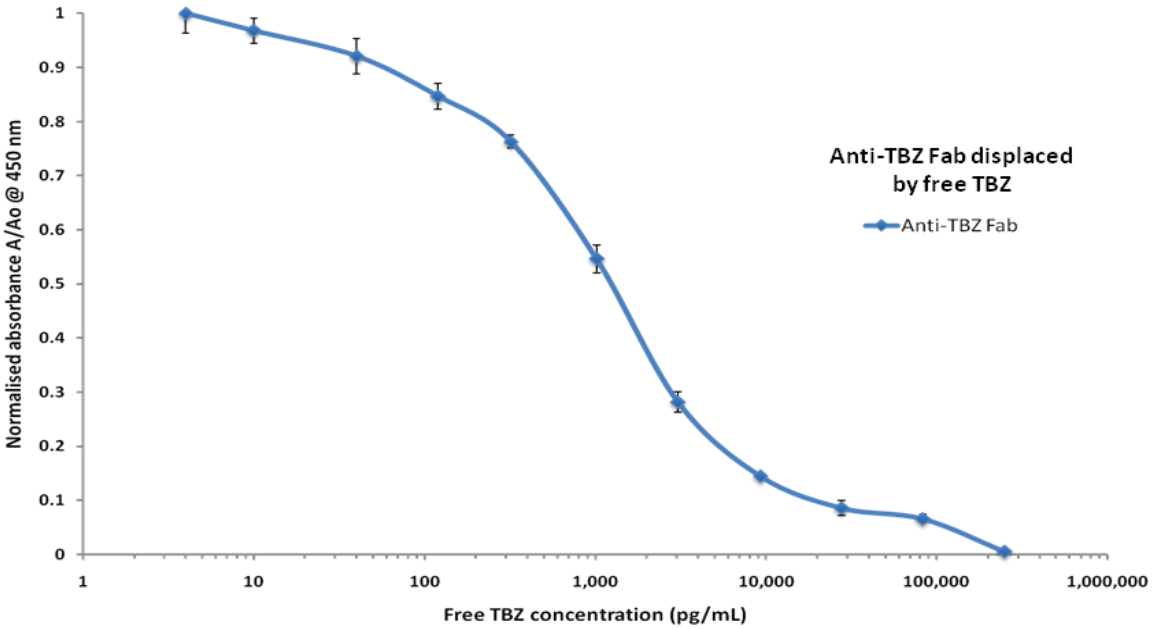

Figure 3.5.2: Plot of the absorbances obtained from the intra-day competitive assays using the anti-TBZ Fab for the detection of free TBZ in PBS. A coating concentration of 1 µg/mL of TBZ-BSA, an antibody concentration of 1/1000 and free TBZ dilutions of 2,500-0.041 ng/mL were used. The detection antibody was HRP-labelled anti-HA. The plotted results represent the mean response ± SD of three replicates.

Table 3.5.1: Tabulated values for TBZ concentration (pg/ml) of 'spiked' PBS, back calculated values, and percentage recoveries (% recoveries) obtained for the anti-TBZ antibody intra-day assay in 'spiked' PBS. Eleven standards were analysed in triplicate three times on the same day.

TBZ concentration (pg/ml)	Back calculated TBZ concentration (pg/mL)	Percentage accuracies (%)
250,000	248,500	100
83,000	67,988	120
27,700	29,672	93
9,250	10,065	92
3,050	3,448	88
1,020	909	89
320	295	105
120	161	74
40	69	58
10	14	66
4	4	88

The inter-assay variation was calculated by performing the assay on five separate occasions. The mean, standard deviation, percentage CV's and back calculated accuracy of all five assays were determined and the results are shown in Table 3.5.2.

Table 3.5.2: Tabulated values for TBZ concentration (pg/ml) of 'spiked' PBS, back calculated values, percentage coefficient of variation (% CV) and percentage recoveries (% recoveries) obtained for the anti-TBZ Fab antibody inter-day assay in 'spiked' PBS. Eleven standards were analysed in triplicate on three separate days.

TBZ concentration (pg/ml)	Back calculated TBZ concentration (pg/mL)	Percentage accuracies (%)	Coefficients of variation (CVs), (%)
250,000	245100	98	10
83,000	72675	91	3
27,700	28750	103	5
9,250	9758	105	6
3,050	3215	105	8
1,020	1012	99	7
320	314	98	10
120	125	104	4
40	38	95	4
10	9	90	2
4	4	100	3

3.5.3 Cross reactivity and limit of detection studies for the anti-TBZ mAb.

The specificity of the antibody was determined by evaluating its cross-reactivity to the TBZ metabolite TBZ-NH_2. In some instances cross reactivity with similar compounds indicate that the antibody is sufficiently specific, however, when used for the detection of drugs that are metabolised a high percentage of cross reactivity to major metabolites may be beneficial. To determine the antibodies cross reactivity, the same competitive approach was used, as previously described in section 2.9.18, with the free TBZ-NH_2 metabolite. This was directly compared with the parent molecule as shown in Figure 3.5.3.

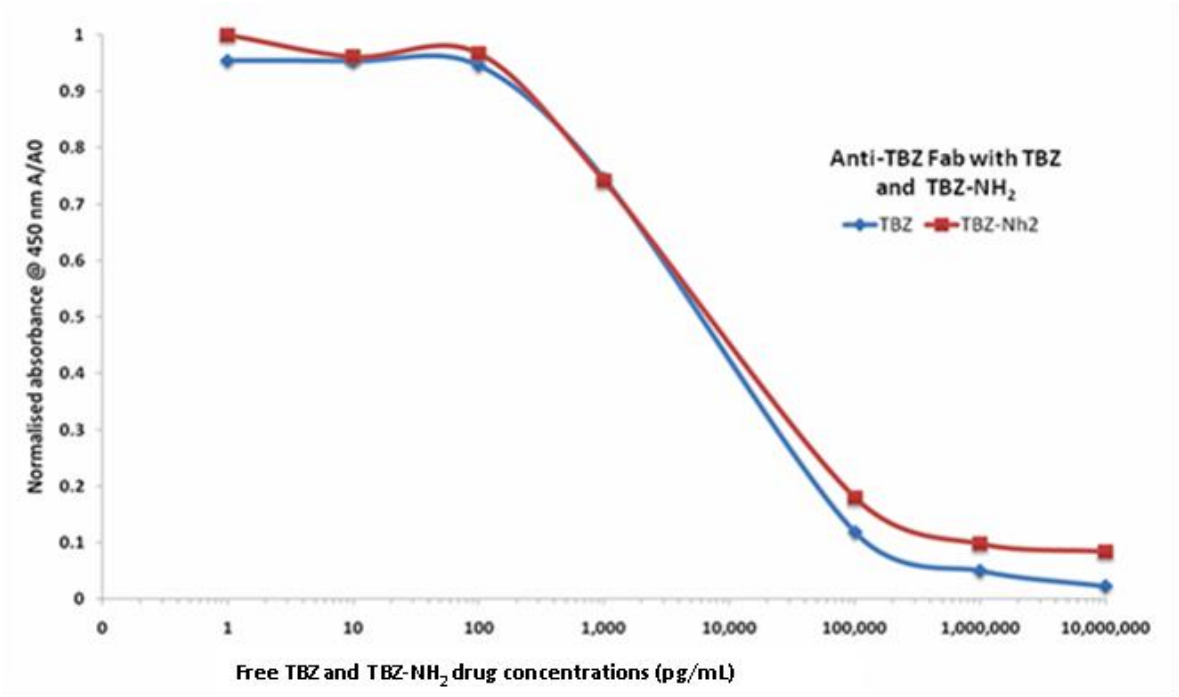

Figure 3.5.3: Plot of the absorbance's obtained from the cross-reactivity studies for the anti-TBZ Fab. Plates were coated overnight with 1 µg/mL TBZ-BSA and subsequently blocked with PBS, containing 5 % (w/v) milk marvel. Dilutions of free TBZ and free TBZ-NH_2 were prepared in PBST containing 5 % (v/v) methanol. The anti-TBZ antibody was diluted 1/1000 in PBST containing 0.5 % (w/v) milk marvel. The detection antibody used was the HRP-labelled anti-HA.

The percentage cross reactivity was calculated using the following formula:

$$\left(\frac{IC50\ TBZ}{IC50\ TBZ - NH_2} \right) x100$$

The IC_{50} of TBZ and TBZ- NH_2 thus have an approximate value of 8 ng/mL for both drugs. There indicates almost 100 % cross reactivity of the Fab with TBZ-NH_2.

For the development of an assay for the detection of veterinary residues, two important performance characteristics of such confirmatory methods are taken into account, the detection capability (CCβ) and the decision limit (CCα) or the limit of detection (LOD) (Van Loco, 2007). The definition of the decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. According to Armbruster and Pry (2008) the LOD is defined as the lowest TBZ concentration, likely to be

reliably distinguished from the limit of the blank (LOB) and at which detection is feasible. This can be calculated with the formula LOD = LOB + 1.645 (SD of the lowest concentration sample) where LOB is the highest analyte concentration expected to be found when replicates of blank are tested (Armbruster and Pry, 2008). Detection capability (CCβ) or the Lowest Level of Quantification (LLOQ) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of 5 %. The LOB and LOD assays were performed as described in section (2.9.19) The LOD of detection was estimated as between 2.5 and 5 ng/mL (µg/kg). The CCβ was established by fortifying 20 blank samples with 2.5, 5 and 7.5 ng/mL of free TBZ and comparing these to 20 blank samples (Figure 3.5.4) and ensuring that they can be detected with 95 % confidence. These assays were carried out to fully characterise the assays performance in order to understand its capability and limitations, and to ensure that it is 'fit-for-purpose'.

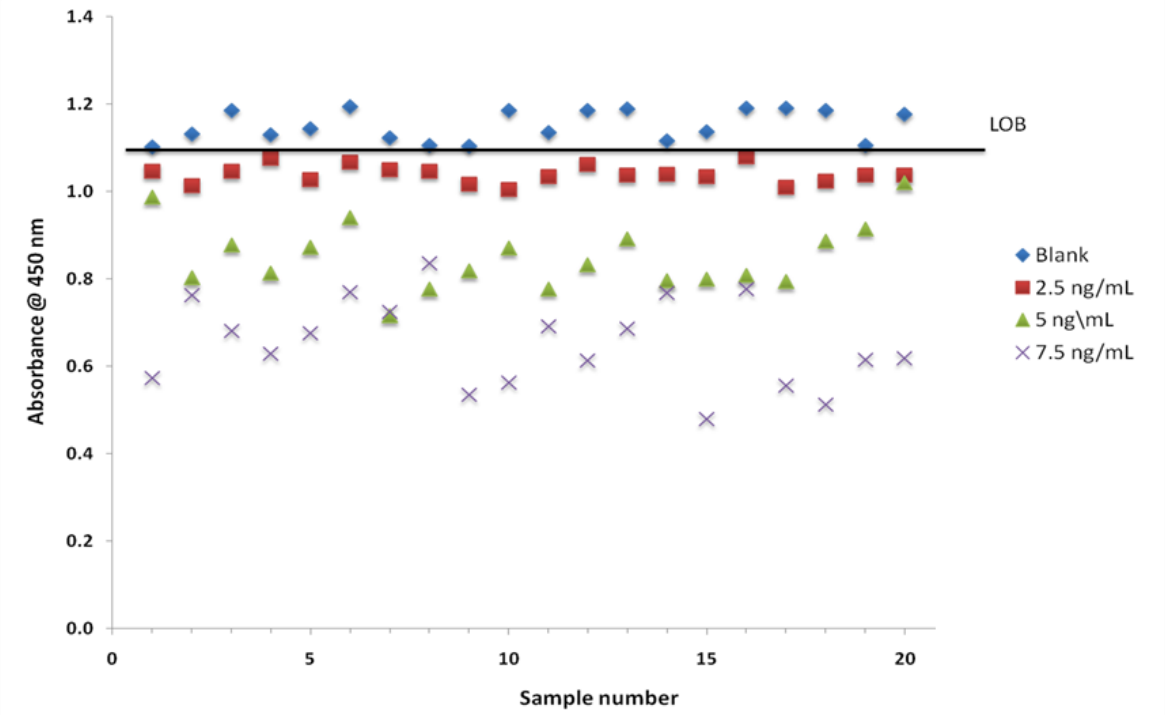

Fig 3.5.4: Graphical representation of the limit of the blank and limit of detection of the anti-TBZ Fab. The plates were coated with 1 µg/mL of TBZ-BSA overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS. PBS containing 5 % (v/v) methanol was fortified with 7.5, 5 and 2.5 ng/mL of free TBZ and 50 µL was added to the corresponding wells followed by 50 µL of anti-TBZ Fab in 5 % (v/v) methanol. The detection antibody was HRP-labelled anti-HA.

3.6 Construction of the modified double helix (dHLX) pComb3xTT Vector

One of the aims of this research was to generate an 'in-house' vector that allowed for the rapid enhancement of any recombinant antibody construct using some simple cloning steps. The vector was designed to alter the valency of the antibody thus increasing functional affinity therefore increasing the sensitivity of the assay to which the antibody is applied. Any antibody fragment generated "in-house" could be inserted into the vector via a restriction digest, followed by a ligation, and finally transformed, into a bacterial strain of choice. To achieve this, the pComb3xTT vector was modified by the insertion of the double helix (dHLX) gene. This dHLX domain was amplified from the pAK500 (plasmid vector belonging to the Krebber vector series (Krebber *et al*., 1997) using a set of specifically designed primers. The dHLX forward primer acccgtacggacccaaacctagcacccccct was designed to amplify the N-terminus of the dHLX domain for cloning into pComb3xTT via the *BsiW*I site. The dHLX-back primer gcgccgcaccatcatcaccatgttccggacta was designed to

amplify the C-terminus of the dHLX domain including the 5 histidines from pAK500 and for cloning into pComb3xTT via the *BspE*I site in pComb3xTT (deleting the HA tag) (see Figure 3.6.1).

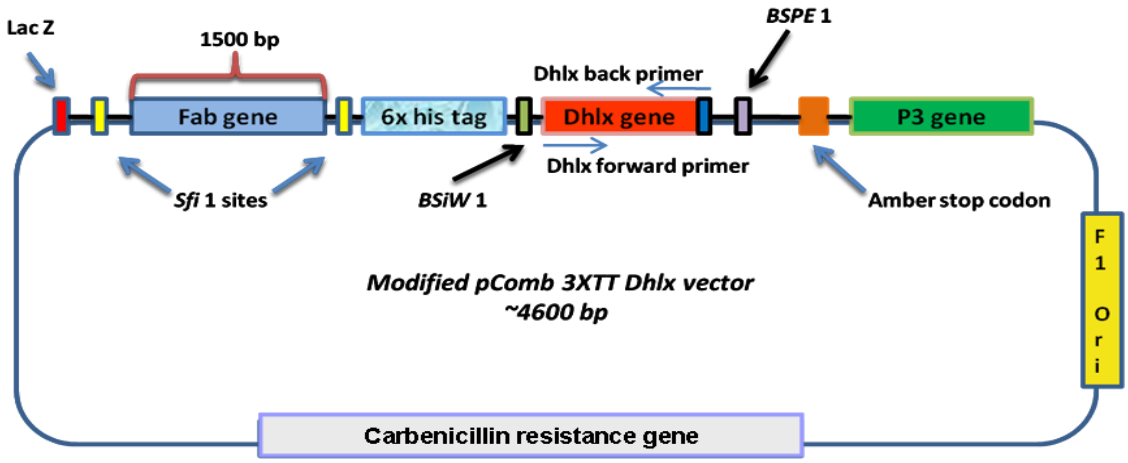

Figure 3.6.1: Modified pComb3xTT vector. This vector contains two *Sfi*1 sites that flank either sides of the Fab gene region assisting the removal and insertion of other gene fragments. The poly His tag was maintained for ease of purification via immobilised metal affinity chromatography (IMAC). The HA tag was removed by enzymatic digestion of the vector with *BsiW*1 and *BspE*1, and replaced by the double helix gene fragment followed by the amber stop codon and pIII gene. The origin of replication and the carbenicillin resistance gene remained intact.

3.6.1 Modification of the Pcomb 3xTT vector for the insertion of the dHLX gene by PCR.

The first stage of vector modification was the amplification of the dHLX gene from the pAK500 plasmid (Figure 3.6.2 (A)) using a set of designed primers (section 2.10.2). The 200 bp product was resolved on a 1 % (w/v) agarose gel and subsequently gel purified. In parallel to this the anti-TBZ Fab fragment (Figure 3.6.2 (B)) was amplified from the pComb3xSS vector using the same PCR reaction, as in section 2.9.12.

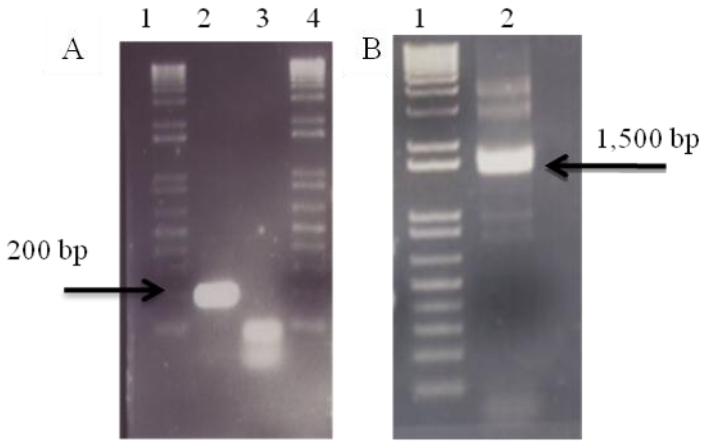

Figure 3.6.2: Amplification of the dHLX gene (A) by PCR as described in section 2.10.2.1, and the *Sfi*1 digested Fab fragment (B).

(A) Lanes 1 and 4: 1 Kb Sigma DNA ladder; Lane 2: 200 bp dHLX gene fragment and lane 3: negative control where water was used as the template.

(B) Lane 1: 100 bp Sigma DNA ladder and Lane 2: *Sfi*1 digested 1,500 bp Fab fragments.

These fragments were gel-purified and digested with the restriction enzyme *SfI*1 followed by an ethanol precipitation and a gel purification (Figure 3.6.3). The Fab fragment was then re-cloned into the pComb3xTT vector by ligation for further modifications. The ligated vector was transformed into XL1 Blue cells. Positive clones were selected via colony pick PCR (Figure 3.6.4) and the vector containing the Fab gene was carried forward for the insertion of the dHLX gene fragment upstream of the amber stop codon.

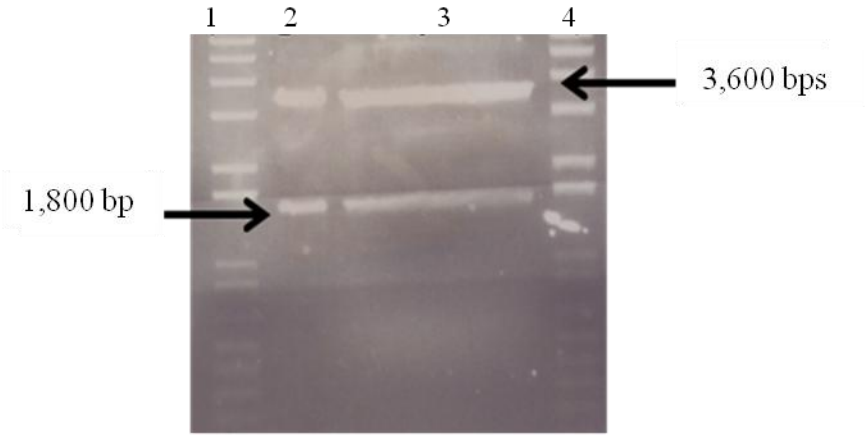

Figure 3.6.3: Restriction digest of the pComb3xTT vector using the enzyme *SfI*1 which yields a stuffer fragment at ~ 1,800 bp and the vector at 3,600 bp. Lanes 1 and 4: 1 Kb Sigma DNA ladder and Lanes 2 and 3: digested pComb vector.

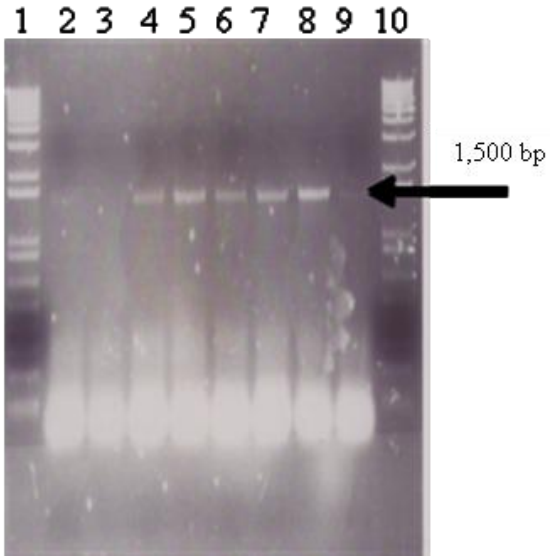

Figure 3.6.4: Colony pick PCR for verification that the Fab gene fragment has been successfully inserted into the pComb 3xTT vector. Lanes 1 and 10: 1 Kb Sigma DNA ladder and Lanes 2-9: PCR amplification of 8 positive colonies selected.

The second stage of modification included the sequential digestion of both the dHLX gene and the pComb3xTT vector containing the Fab with the enzymes *BsiW*1 and *BspE*1, is shown in Figure 3.6.5. This was followed by the ligation of the dHLX gene into the digested vector. Positive clones were established by PCR amplification of the dHLX gene from selected colonies (Figure 3.6.6).

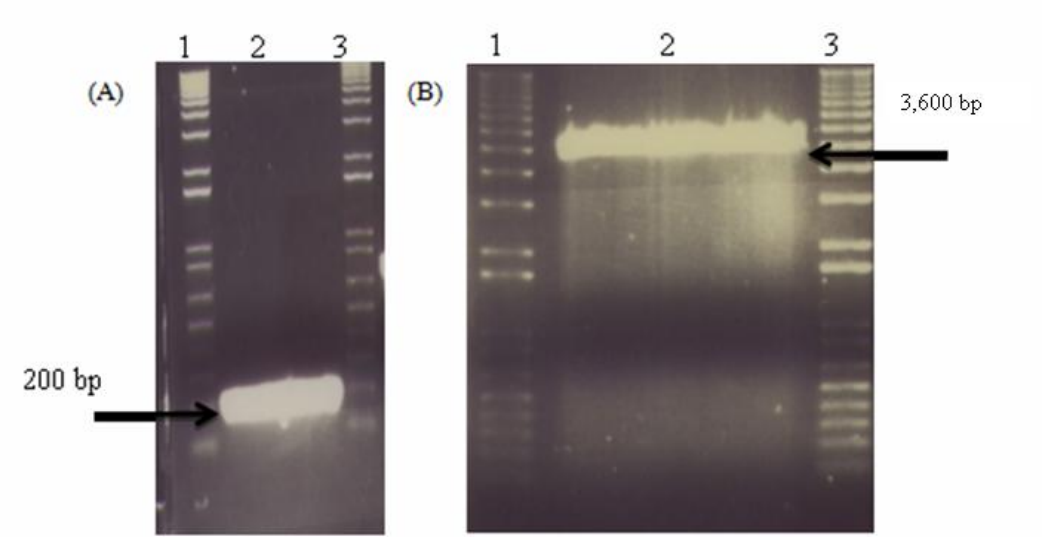

Figure 3.6.5: Restriction digest of the dHLX gene insert (A) and pComb3xTT (B) with the restriction enzymes *Bspe*1 and *Bsw*1. Gel (A) Lanes 1 and 3: 1 Kb Sigma DNA ladder and Lane 2: 200 bp digested insert. Gel (B) Lanes 1 and 3: 1 Kb Sigma DNA ladder and Lane 2: 3,600 bp shows the digested vector.

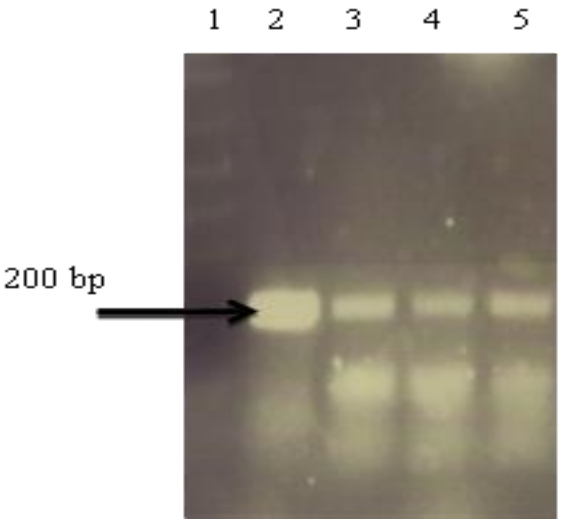

Figure 3.6.6: The PCR amplification of the dHLX gene from 4 positive clones. Lane 1: 1 Kb Sigma DNA ladder and Lanes 2-5: dHLX positive clones.

3.6.2 Purification of the anti-TBZ dHLX Fab and its implementation into a competitive assay for the enhanced detection of TBZ.

The pComb3xTT now contains the dHLX gene, enhancing the Fab so that the sensitivity and affinity was improved. All five clones were purified by affinity chromatography as the poly His tag was still intact. Only one Fab-secreting clone was specific to TBZ. This purified antibody was titred against the TBZ-BSA conjugate and an optimum dilution of 1/500

dilution was established (Figure 3.6.7). This dilution was used in the competitive assay format for the detection of TBZ and for the intra and inter-assays to determine reproducibility and accuracy (Figures 3.6.8 and Tables 3.6.1 and 3.6.2).

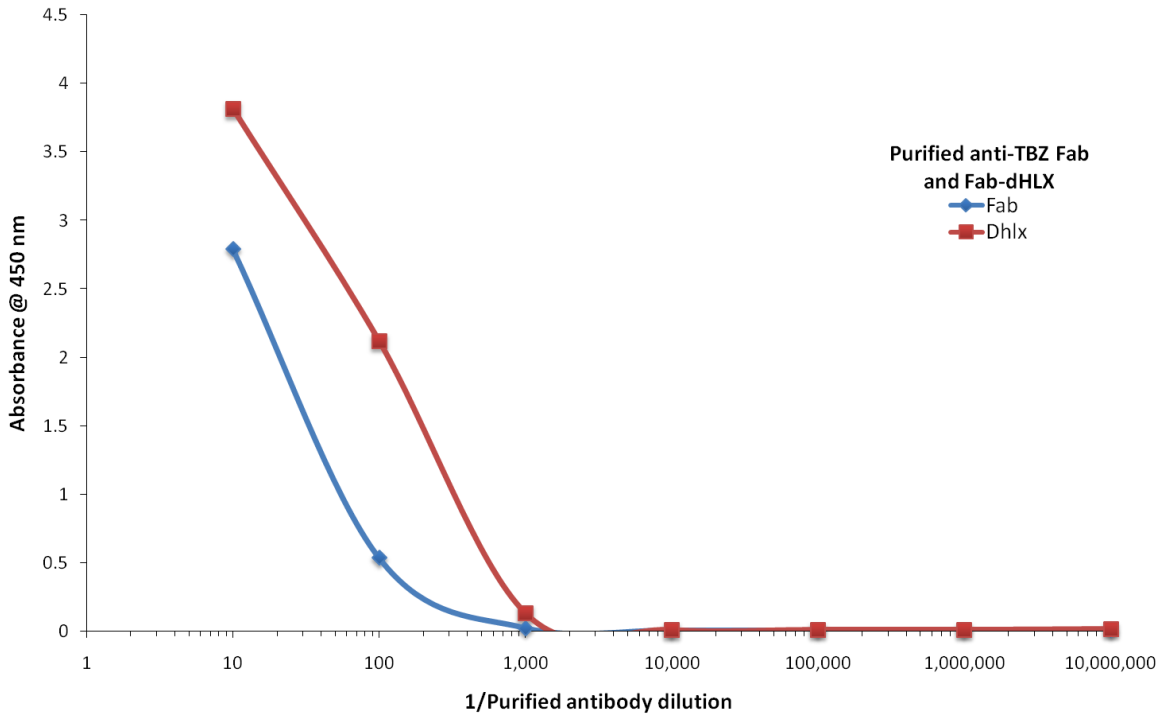

Figure 3.6.7: Plot of the absorbance's obtained from the titre of the purified anti-TBZ Fab and anti-TBZ Fab-DHLX The plate was coated with 1 µg/mL of TBZ-BSA overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS. The IMAC-purified antibody preparation was diluted in PBST containing 0.5 % (w/v) milk marvel. The detection antibody used was HRP-labelled anti-HA.

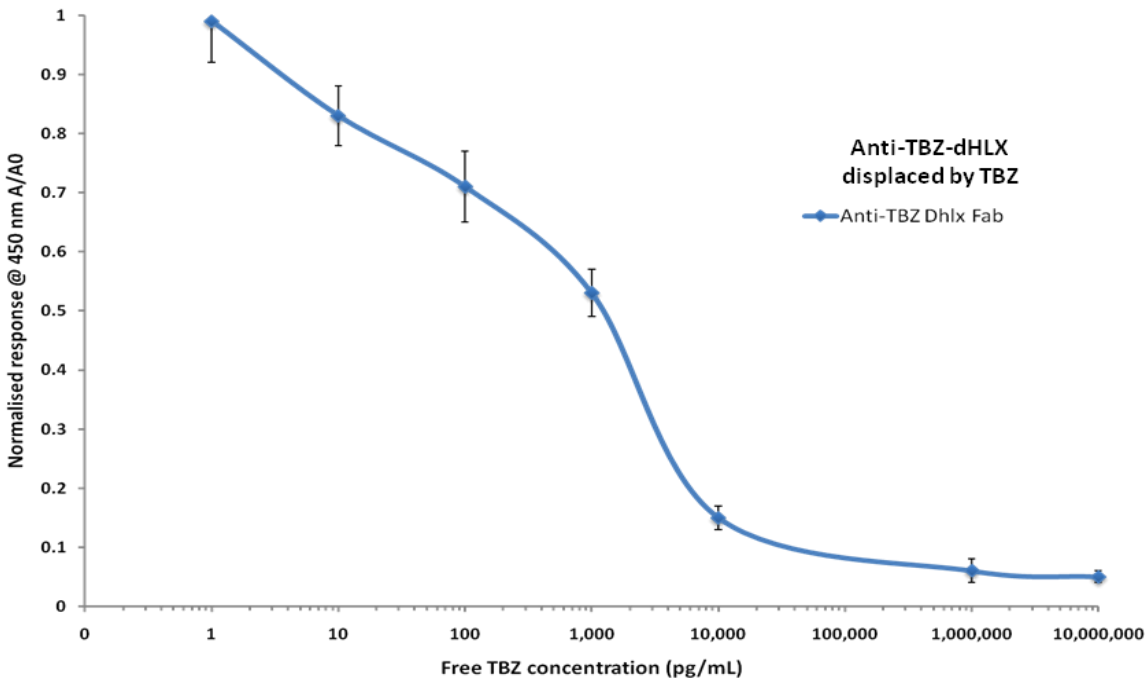

Figure 3.6.8: Plot of the intra-day assays for the anti-TBZ dHLX construct. Plates were coated with 1 µg/mL of TBZ-BSA overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS. Free TBZ dilutions (1µg/mL- 1 pg/mL) were prepared in 5 % (v/v) methanol in PBST. The antibody was diluted 1/500 1 % (w/v) milk marvel.

Table 3.6.1: Tabulated values for TBZ concentration (pg/ml) of 'spiked' PBS, back calculated values, and percentage recoveries (% recoveries) obtained for the anti-TBZ dHLX Fab antibody intra-day assay in 'spiked' PBS. Seven standards were analysed in triplicate, three times on the same day.

TBZ concentration (pg/mL)	Back-calculated TBZ concentrations (pg/mL)	Percentage Accuracies (%)
1,000,000	989,000	101
100,000	101,260	99
10,000	10,765	92
1,000	989	101
100	112	89
10	11	90
1	1	100

Table 3.6.2: Tabulated values for TBZ concentration (pg/ml) of 'spiked' PBS, back calculated values, percentage coefficient of variation (% CV) and percentage recoveries (% recoveries) obtained for the anti-TBZ dHLX Fab antibody inter-day assay in 'spiked' PBS. Seven standards were analysed in triplicate over three days.

TBZ concentration (pg/mL)	Back-calculated TBZ concentrations (pg/mL)	Percentage Accuracies (%)	Coefficients of variation (CVs), (%)
1,000,000	1,012,602	98	11
100,000	119,273	83	11
10,000	99897	100	10
1,000	1076	93	8
100	99	101	9
10	9	101	7
1	1	100	1

The anti-TBZ Fab, the dHLX formatted vector expressing the anti-TBZ Fab, and the mAb were all directly compared via competive ELISA to illustrate the difference in binding capabilities to free TBZ between the three (Figure 3.6.9). The IC_{50} of the anti-TBZ dhlx Fab, Fab, and mAb was approximately 800, 1400 and 8000 pg/mL respectively.

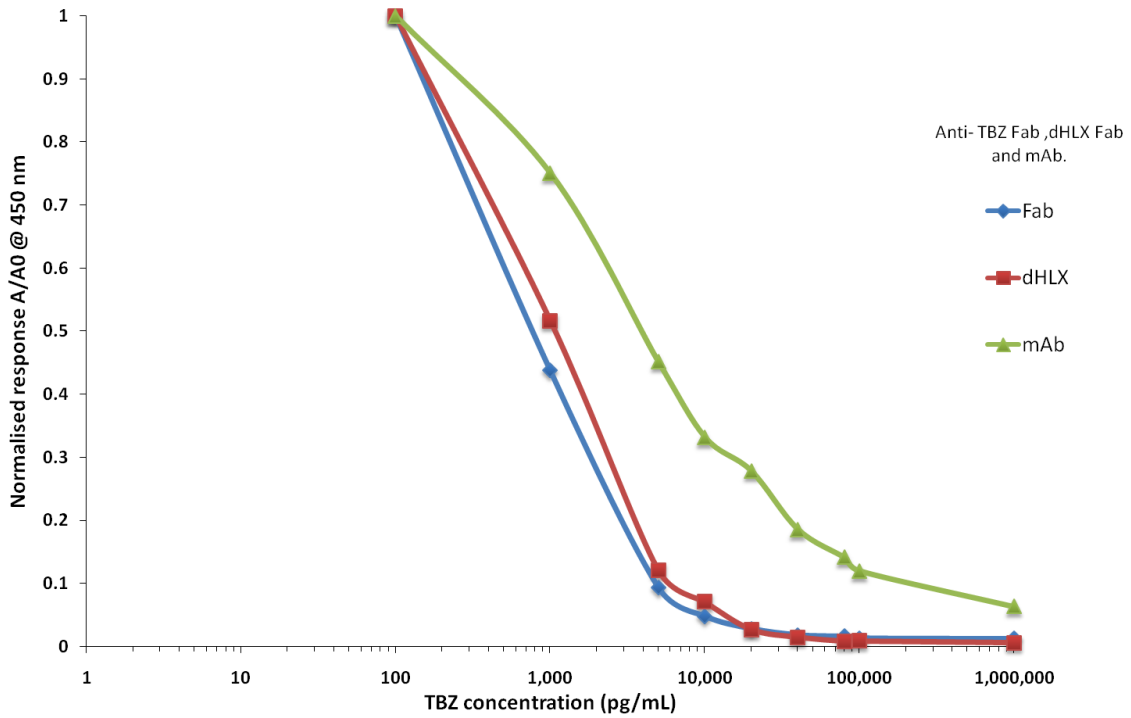

Figure 3.6.9: Plot of the absorbances obtained from the competitive ELISA for the comparison of the anti-TBZ Fab, mAb and dHLX. Plates are coated with 1 µg/mL of TBZ-BSA overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. Free TBZ dilutions (1µg/mL- 1 pg/mL) were prepared in 5 % (v/v) methanol in PBS,. Each antibody was diluted in 1 % (w/v) milk marvel. The Fab and the dHLX was detected with a HRP-labelled anti-His antibody, and the mAb with a HRP-labelled anti-mouse IgG.

To further confirm the bivalency of the dHLX Fab, HPLC was carried out directly comparing the Fab and the dHLX Fab using a size exclusion column as outlined in section 2.10.4. A calibration curve was prepared using a set of protein standards with given molecular weights (670 kDa Thyroglobulin, 150 kDa IgG, 44 kDa Ovalbumin, 17 kDa Myoglobin and 244 daltons Uridine). The log of these molecular weights were plotted against the retention time to give a linear correlation and from this the unknown molecular weights were interpolated using Prism™ graphpad software (Figure 3.6.10). The anti-TBZ Fab eluted with a retention time of 15.97 minutes equating in a molecular weight of approximately 64 kDa. The anti-

TBZ dHLX Fab eluted in two peaks with retention times of 13.634 and 20.24 correlating to molecular weights of 300 and 57 kDa respectively (Figure 3.6.11).

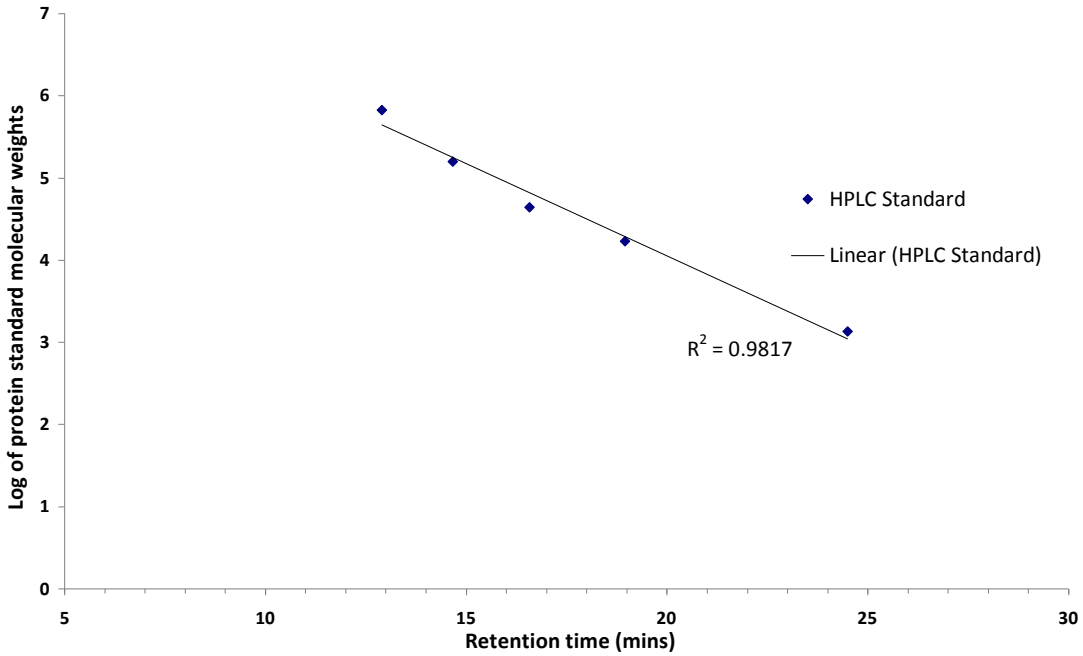

Figure 3.6.10: Calibration curve for the SEC HPLC of the anti-TBZ Fab and dHLX Fab. The log of the molecular weight of each standard was plotted against their corresponding retention times. Standards used were 670 kDa Thyroglobulin, 150 kDa IgG, 44 kDa Ovalbumin, 17 kDa Myoglobin and 244 daltons Uridine. The unknown samples were interpolated using Prism™ Graph pad.

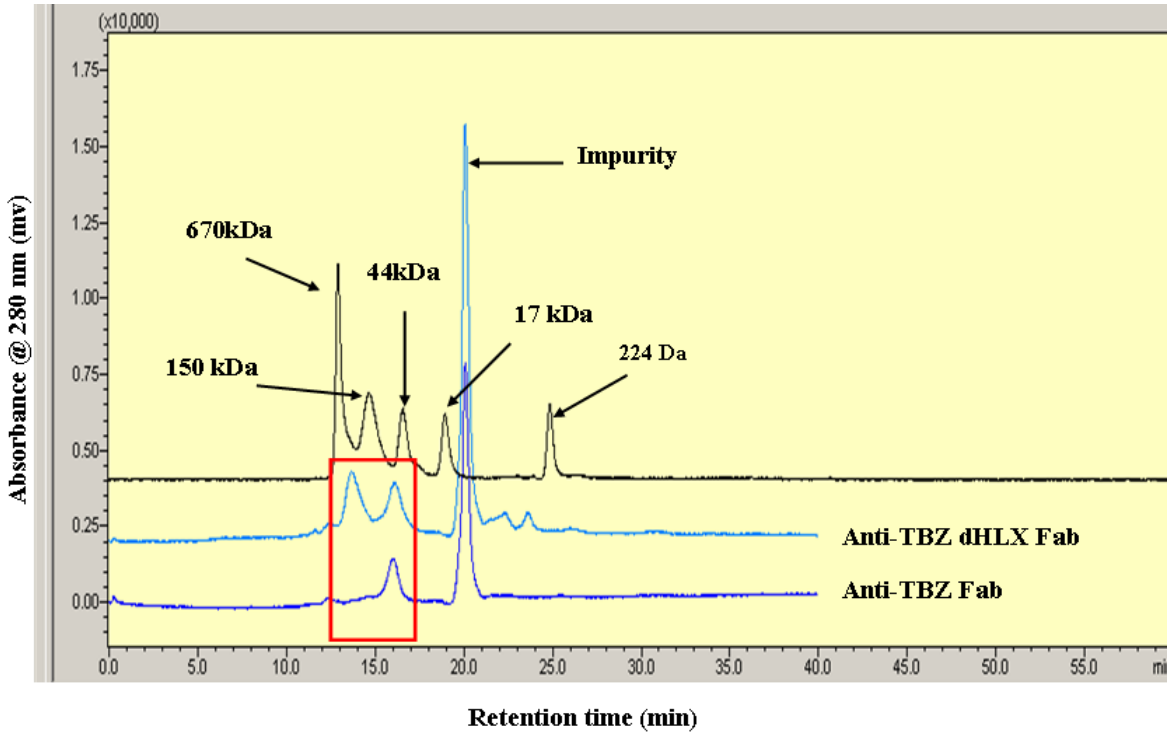

Figure 3.6.11: HPLC chromatogram of the purified anti-TBZ Fab and dHLX Fab. The molecular weight standards are indicated by the arrows (670 kDa Thyroglobulin, 150 kDa IgG, 44 kDa Ovalbumin, 17 kDa Myoglobin and 244 daltons Uridine). The molecular weights of the Fab (63 kDa) and the dHLX with 2 peaks (one at the 57 kDa 300 kDa) are highlighted by the red box surrounding the peaks

3.7 Discussion and conclusions.

In this research a large (1.4 x 10^7) Fab library was successfully constructed from an anti-TBZ hybridoma cell line with some primer optimisation. Following the screening and characterisation of the positive clones a highly sensitive Fab was produced and subsequently used in the development of an ELISA assay for the detection of TBZ. Prior to assay development the anti-TBZ Fab's tolerance to solvents was examined. Most benzimidazoles are not water soluble and are dissolved in solvents such as ethanol and methanol. Methanol is generally used as an extraction solvent for ELISA because it has less effect on antigen-antibody interactions and HRP activity than other solvents. In this study it was determined that methanol at a concentration of 5 % (v/v) was acceptable for the suspension of TBZ and had did not affect the antibody's interaction with the TBZ-BSA conjugate or free TBZ binding (results not shown). Limits of the blank were estimated by using blank samples (PBS, pH 7.5, 5 % (v/v) MeOH). Following this the LOD was determined by using blank material

which was fortified with TBZ at concentrations near the assays limit of detection at 2.5, 5 and 7.5 ng/mL. The LOD was established using the equation previously discussed. The lowest concentration of analyte that was detected 95 % of the time with 95 % confidence was 2.5 µg/kg (Fig 3.4.5).

According to European legislation (2002/657/EC) the inter-laboratory coefficient of variation (CV) for the repeated analysis of a reference or fortified material, under reproducible conditions should not exceed 20 % in the case of residues with established MRL's between 10 µg/kg–100 µg/kg. For this assay, inter-assay performance was determined on five different days, each sample being determined (in triplicate) with different concentration and batches of TBZ un-conjugated free drug. The coefficients of variation were established and were within regulatory limits with the inter assay ranging from 0.9-9.2 %.

In previous studies monoclonal and polyclonal antibodies have been generated and applied in ELISA assays for the detection and quantification of TBZ in different matrices. Brandon *et al.* (discussed in section 1.18) developed an assay using the anti-TBZ monoclonal antibody which had an LOD of 20 µg/kg for TBZ in potatos and apples (Brandon *et al.,* 1993). However, as shown in this research, an antibody derivative with an increase in sensitivity five times that of the parent monoclonal used in Brandon's study was generated that has a LOD of 2.5 µg/kg. This Fab also echibits solvent tolerance with 5 % (v/v) methanol, a property which is required for the analysis of hydrophobic compounds.

According to Korpimäki and co-workers, this increase in sensitivity of the Fab fragment is unusual in comparison to other antibody formats that have been developed from a monoclonal antibody such as scFv and scAb configurations (Korpimäki *et al.,* 2004). However, Itoh and colleagues (1999) generated a recombinant Fab fragment against pseudouridine and uridine that showed an increase in sensitivity compared to the parental monoclonal. They suggested that an increase in sensitivity may be due to the difference in the number of antigen binding sites between a Fab fragment and an mAb. Since the Fab fragment has only a single antigen binding site, the target antigen can be detected quantitatively. However, as the mAb has two antigen binding sites, it is possible for it to react with the adsorbed antigen even when one of the antigen binding sites is saturated with free target analyte (the antigen to be determined). This may be possible cause for a low sensitivity of a mAb-based inhibition ELISA systems (Itoh *et al.,* 1999).

When constructing a chimeric Fab library the light chain V regions (both λ and κ) of mice, chicken and rabbits are spliced onto a single human Cκ region. This is ostensibly due to the dominance of the Vκ in the immunoglobulin repertoires of these animals inferring that V_λ clones are likely to be infrequent in resulting immune recombinant antibody libraries (Barbas *et al.*, 2001). However, Townsend *et al*. observed that Fab light chain function and overall antibody binding activity were improved by combining the V_λ region (isolated from a murine scFv library) with a C_λ region, as opposed to Cκ, implying that the use of a C_λ scaffold in Fab library construction improves the isolation of lambda-associated Fabs and there structural stability, secretion and overall activity of the final Fab product (Townsend *et al*., 2006). In the research reported in this chapter the same may apply as only the Vκ region was spliced onto the Cκ domain thus leading to an increase in the binding activity of the anti-TBZ Fab compared to the mAb.

This sensitive anti-TBZ Fab may be used as part of a screening method for the detection of TBZ as the assay format is simple, rapid and offers high throughput relative to conventional methods used such as LC-MS and HPLC,

This chapter also describes the modification of the pComb vector. Bivalency is an effective means for increasing the functional affinity of an antibody to a surface (Pack *et al.*, 1992). Pack and Plückthun investigated ways to harvest the gain in avidity by engineering small dimerization domains. This allowed the assembly of bivalent "miniantibodies" in the periplasm of *E.coli* based on scFv fragments connected to a hinge peptide and an amphiphatic helix (Pack, 1993; Pack and Plückthun, 1992). There are two ways to infer dimerization. Both of these designs were evaluated by Pack and Plückthun. The first was the use a four-helix bundle designed by Eisenberg and McLachlan. In this approach using naturally occurring bundles, the arrangement is anti-parallel and the four helix bundle may be formed by four separate molecules, two helix-dimer molecules, or one molecule containing a four helix construct (Eisenberg and McLachlan, 1986). The second was the use of a leucine zipper that is found as part of a DNA binding domain in various transcription factors. It functions as a dimerization domain and its presence generates adhesion forces in parallel alpha helices. One leucine zipper consists of multiple leucine residues at seven residue intervals forming an amphiphatic alpha helix with a hydrophobic region for dimerization (Pack, 1993). These studies showed that homo dimerisation occurred when fusing antibody fragments to the

double helix motif resulting in a significantly higher avidity than when fused with the leucine zipper (Pack, 1993).

Herein, the double helix gene sequence was amplified from the pAk vector (amino acid sequence GELEELLKHLKELLKG-PRK-GELEELLKHLLELLKG). Using the restriction enzymes *BspE*1 and *BsiW*1 we were able to digest the vector at these restriction sites and slot in the dHLX gene region had been amplified from the pAK vector by the use of primers designed for the restriction sites and thus ligating them together so that the gene sequence reads through. This allows dimerization to occur, assemble *in-vivo* in *E.coli*, and secretion into the periplasmic space.

The IC_{50} of the dHLX inter and intra assays show that the engineered Fab can detect TBZ concentrations as low as .8 ng/mL, which is an approximate a 2 fold improvement on the detection limit of the original Fab. This modified vector has the ability enhance the avidity of an antibody construct. This was further confirmed by SEC HPLC where the multitvalency of the antibody was shown by the representation of the monomeric Fab at 56 kDa and the multivalent dHLX at 300 kDa in Figure 3.6.11. This modified vector system allows for the enhancement of avidity for any antibody fragment by simple directional cloning, thus improving the antibodies detection capabilities.

Chapter 4

The generation of avian recombinant and polyclonal antibodies against Triclabendazole and Albendazole.

4.0 Introduction: The generation of avian antibodies

Avian IgY is the major globular protein produced by chickens. It is continually synthesized by B cells, secreted into the blood stream and accumulated in the egg yolk (Warr, Magor and Higgens, 1995). The IgY of hens provides their progeny with immunity against avian pathogens until they have a fully matured immune system. Only three avian immunoglobulin subclasses have been identified, IgM, IgA and IgY (Dias da Silva and Tambpurgi, 2010). Like IgG, IgY consists of two heavy (65 kDa) and two light (18 kDa) chains. The IgY differs in that the heavy chain consists of one variable and four constant domains while the light chain is comprised of one variable and one constant region. The Fab region is similar to the IgG counterpart, where the Fc portion is responsible for complement activation, opsonisation, and mast cell activation. A large amount of IgY is prevalent in the blood (~5 mg/mL), however, there is a vast amount contained in the egg yolk (approximately 100-150 mg/yolk) (Dong *et al.*, 2008).

IgY is frequently harvested for use due to advantages such as: (i) the low cost of housing, handling and feeding hens, (ii) reduced animal usage as large quantities of eggs can be produced on a daily basis for long periods with regular immunisations, (iii) large quantities of highly specific IgY are easily collected, (iv) bleeding of the animal is avoided and (v) IgY is easily separated and purified from IgA and IgM (Dong *et al.,* 2008; Krief, Letteson and Billen, 2002; Tini *et al.,* 2002). Use of intact whole IgY or IgG is advantageous in the field of therapeutics as they have a long half-life, the ability to activate complement, and engage Fc receptor-mediated effector functions (Schade *et al.,* 2005). However, these Fc receptor functions are not required for diagnostic and technical applications as they do not rely on the natural effector functions of the antibody. Therefore, antigen binding fragments smaller than their IgG/IgY counterparts may be used (Röthlisberger, Honegger and Pluckthun, 2005).

Phage display technology, as discussed in section 1.9.3, may be applied to the generation of avian recombinant antibody fragments. The molecular diversity of the avian species allows for the straightforward and relatively successful construction of recombinant libraries compared to those from mammals (Andris-Widhopf *et al.,* 2000). The heavy and light chain loci in chickens consist of functional variable (V) region genes and joining region (J) and multiple diversity (D) segments in the heavy chain genes that are rearranged using standard V(D)J recombination's. To generate a large diverse antibody repertoire, the avian species utilises a further type of DNA recombination. Primers are designed that flank the conserved

regions around the functional V_H and V_L chains. These are used to amplify the complete range of rearranged variable fragments that facilitate the cloning of highly diverse chicken immunoglobulin libraries (Greunke *et al.,* 2008). Avian scFv fragments are often selected for the development of recombinant antibody libraries for a number of reasons, (i) they are serologically distinct from mammalian immunoglobulins, (ii) the avian antibody repertoire is easily and more readily accessed than any other mammals other than the camelids and (iii) immunisation of chickens is a convenient way of producing antibodies recognising conserved epitopes on mammalian molecules which, because of self-tolerance, are not obtained by immunising mice (Davies *et al.,* 1995). Therefore, the choice was made to produce avian scFvs against the benzimidazoles TCB and ABZ.

4.1 Chapter aims:

The principle aims for the research described in this chapter were:

a) to produce an avian IgY against the conjugates TCB-BTG and ABZ-HRP,

b) to generate a validated assay for the detection of TCB and ABZ, and

c) to construct a recombinant antibody fragment library from the RNA extracted from an immunised chicken and to screen the associated library against both TCB and ABZ conjugates to isolate specific scFvs.

4.2 Results

4.2 Generation of an avian polyclonal antibody against TCB and ABZ

4.2.1 Serum titres from TCB-BTG and ABZ-HRP immunised chicken

A serum titre was determined on the chicken following immunisation (section 2.11.1) with the TCB-BTG and ABZ-HRP conjugates. Titres of approximately 1/100,000 and 1.1,000,000 for the immunogens ABZ-HRP (Figure 4.2.1) and TCB-BTG (Figure 4.2.2), respectively, were determined indicating that the chicken was ready for removal of the bone marrow and spleen for the harvesting of RNA and the further construction of a recombinant antibody library.

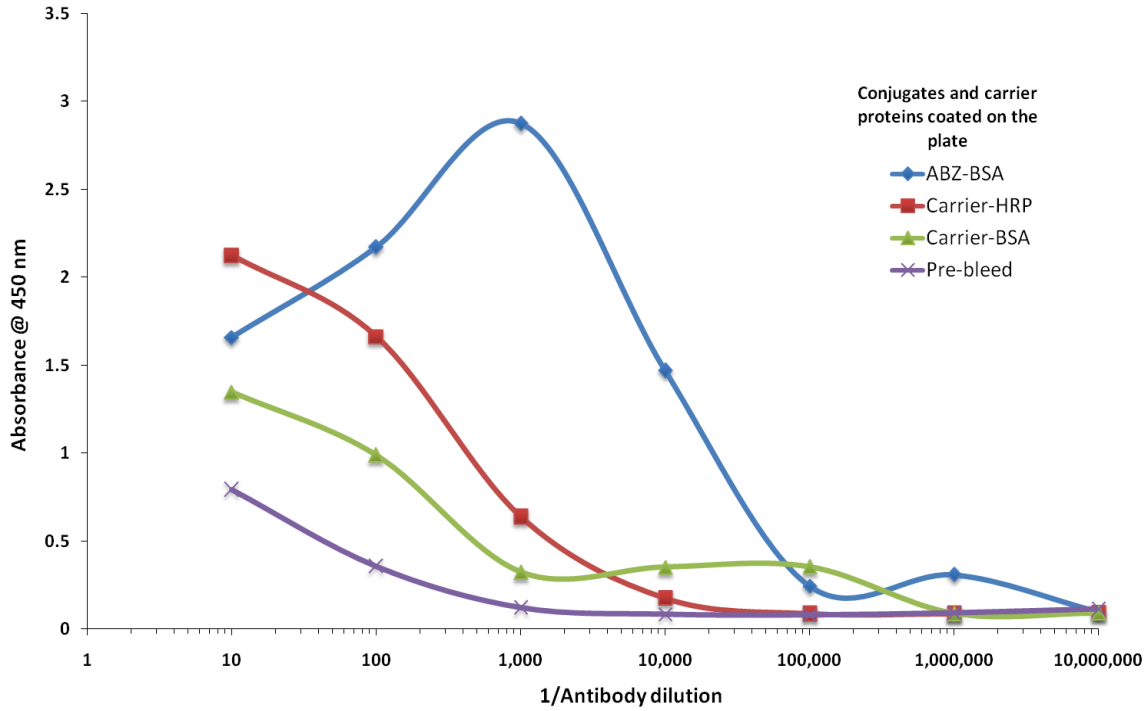

Figure 4.2.1: Avian serum titre plotted against ABZ-BSA. The plate was coated with 1 µg/mL of ABZ-BSA and the carrier proteins HRP and BSA and subsequently blocked with 5 % (w/v) milk marvel in PBS. The serum (antibody raised against ABZ-HRP) and the pre-bleed serum was diluted (1/10-1,000,000) in PBS containing 0.5 % (w/v) milk marvel. The detection antibody was AP-labelled anti-chicken IgY.

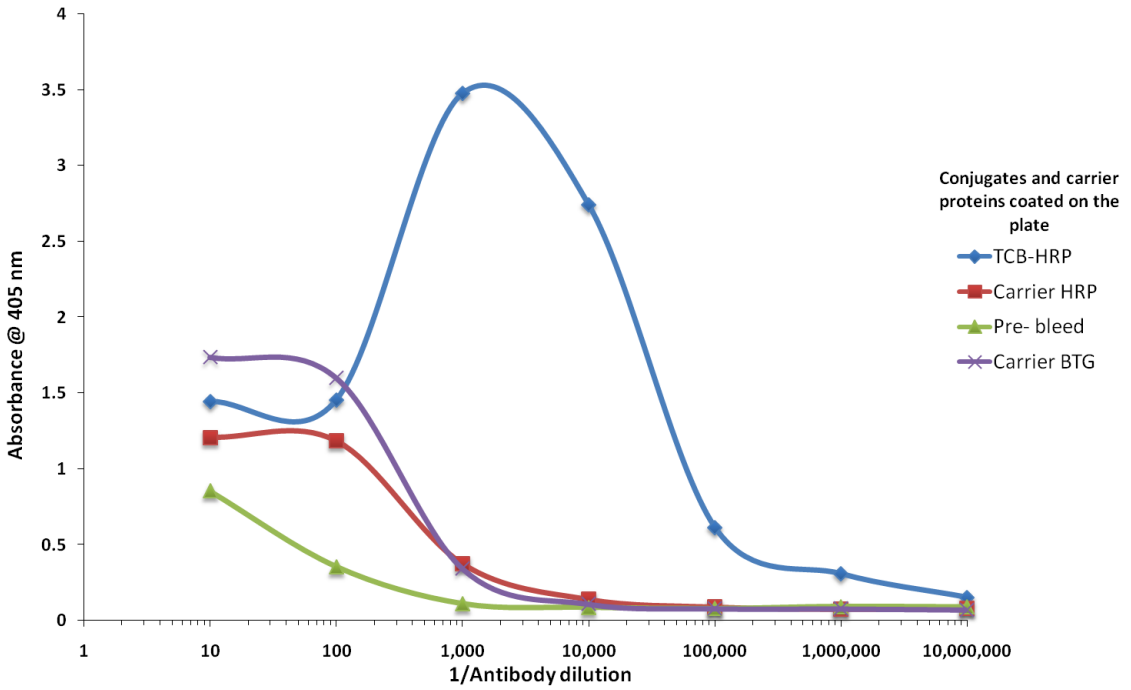

Figure 4.2.2: Avian serum titre plotted against TCB-HRP. The plate was coated with 1 µg/mL of TCB-HRP, or the carrier proteins HRP and BTG, and subsequently blocked with 5 % (w/v) milk marvel in PBS. The serum (antibody raised against TCB-BTG) and the pre-bleed serum was diluted (1/10-10,000,000) in PBS, pH 7.5, containing 0.5 % (w/v) milk marvel. The detection antibody was AP-labelled anti-chicken IgY.

There was a decrease in signal for the high dilutions of serum due to the phenomenon known as the Hook or high dose effect. This generates a low apparent response if the concentrations/ratios of antigen/antibody are 'off-scale'.

4.2.2 Purification of anti-TCB/ABZ from egg yolk

Eggs were collected throughout the immunisation period. The IgY was purified from the egg yolk (section 2.12.1) and the purity was confirmed by SDS-PAGE (Figure 4.2.3) (section 2.12.2). The purified anti-TCB/ABZ polyclonal antibody preparation was used for further assay development.

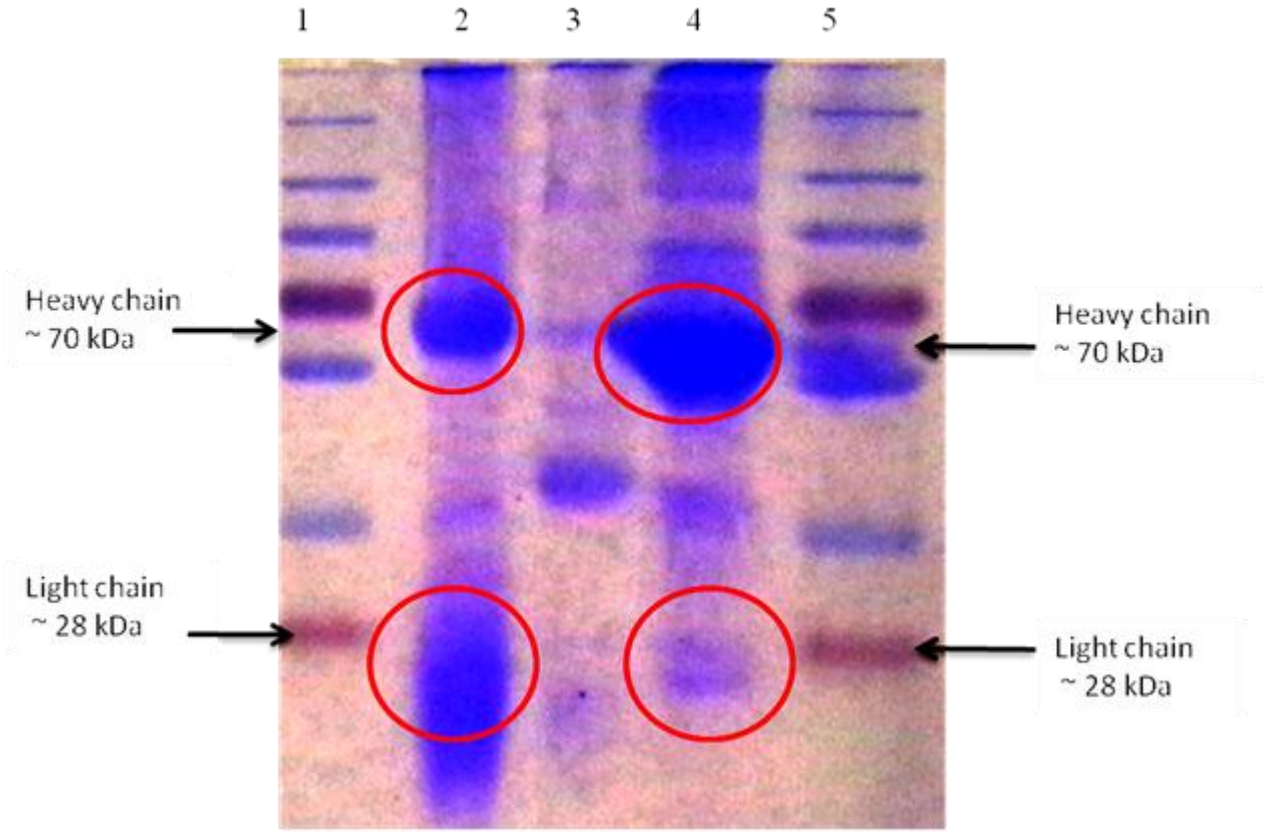

Figure 4.2.3: SDS-PAGE gel of the purified anti-TCB/ABZ IgY. Lanes 1 and 5: Fermentas Page Ruler™ molecular weight markers; Lane 2: purified anti-TCB/ABZ IgY with bands shown at the 72 kDa and 28 kDa; Lane 3: AP-labelled anti-chicken IgY; and lane 4: HRP-labelled anti-chicken IgY.

4.3 The development of a competitive ELISA assay using the anti-chicken pAb for the detection of TCB and ABZ.

4.3.1 Optimisation of coating buffers, conjugate and antibody concentrations.

Prior to assay development coating buffers and concentrations were established to determine the lowest concentration of conjugate and the optimal buffer that gave the maximum absorbance when using the purified anti-TCB/ABZ pAb at a dilution of 1/100 (section 2.12.3). The coating buffers used were citrate, pH 4.7, PBS, pH 7.5, TBS, pH 8.0, TBS, pH 7.4, carbonate, pH 10.3, and sodium acetate, pH 4.8. Plates were coated with dilutions of both the ABZ and TCB conjugates ranging between 54-7,000 ng/mL (Figures 4.3.1 and 4.3.2).

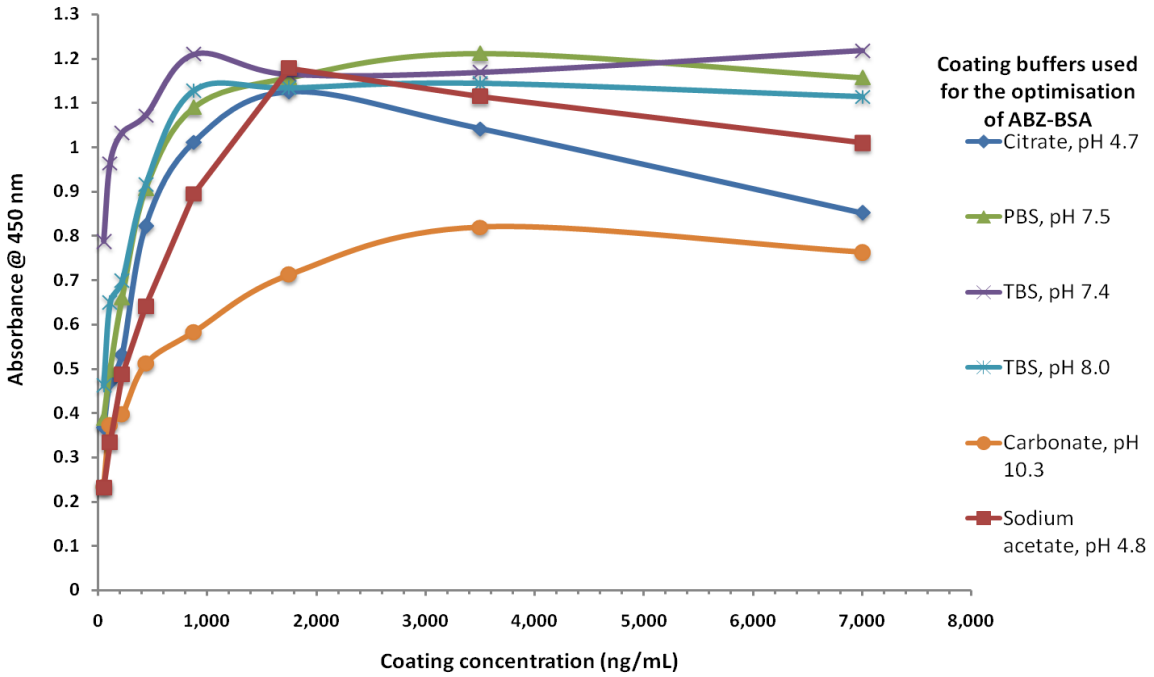

Figure 4.3.1: Plot of the absorbances obtained from the ELISA for the determination of the optimum antigen coating concentration and coating buffer for ABZ-BSA. The plate was coated with 54, 109, 218, 875, 1,750, 3,500 and 7,000 ng/mL of ABZ-BSA in the following buffers: citrate, pH 4.7; PBS, pH 7.5; TBS, pH 8.0; TBS, pH 7.4; carbonate, pH 10.3, and sodium acetate, pH 4.8. A 1/100 dilution of the anti-ABZ pAb was added to each well. The detection antibody was HRP-labelled anti-chicken IgY.

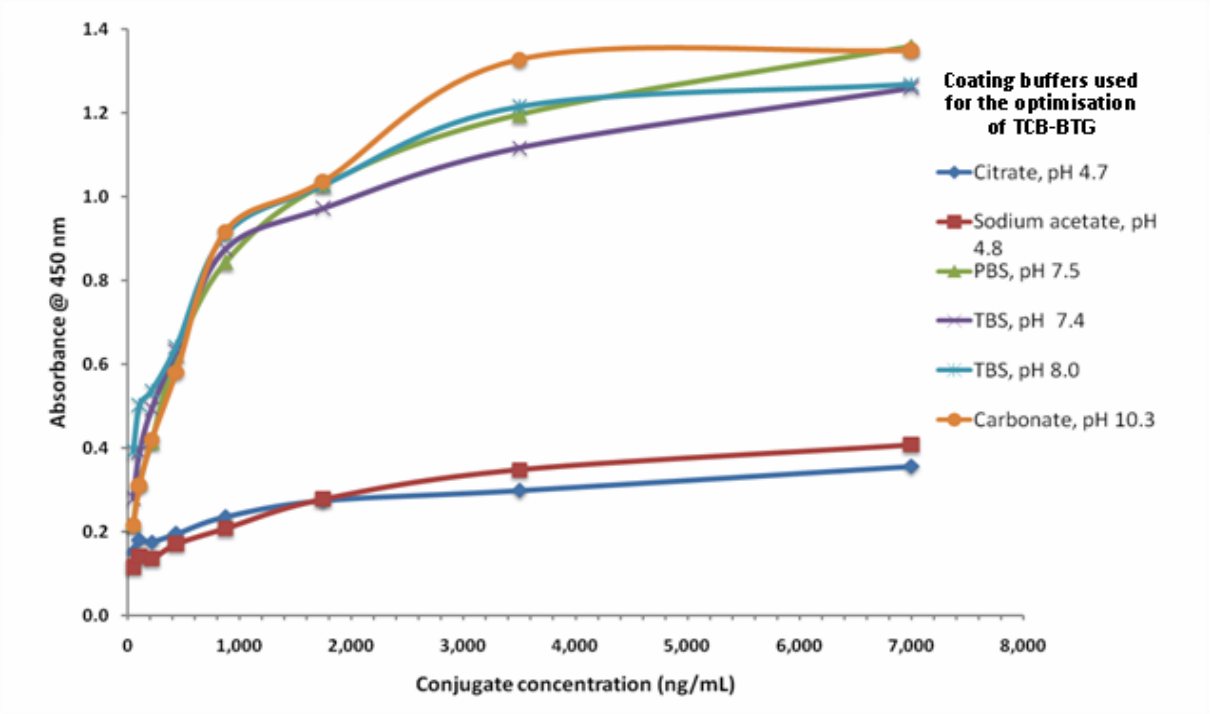

Figure 4.3.2: Plot of the absorbances obtained from the ELISA for the determination of the optimum antigen coating concentration and coating buffer for TCB-BTG. The plate was coated with 54, 109, 218, 875, 1,750, 3,500 and 7,000 ng/mL of TCB-BTG in the following buffers: citrate pH, 4.7; 1 x PBS, pH 7.5; TBS, pH 8.0; TBS, pH 7.4; carbonate, pH 10.3; and sodium acetate, pH 4.8. A 1/100 dilution of the anti-TCB pAb was added to each well. The detection antibody was HRP-labelled anti-chicken IgY.

Sub-optimal performance was determined with conjugate diluted in citrate and acetate buffers. There was also no significant differentiation in binding between the carbonate, 1 x PBS, pH 7.5 and TBS, pH 7.4 and 8.0 buffers when used for the coating of TCB-HRP to the plate. However, this antibody was to be used for a multi-analyte detection ELISA and, as shown in Figure 4.3.1, the carbonate buffer is not optimal for the coating of ABZ-HRP to the plate surface. There was no considerable difference between PBS, pH 7.5, TBS pH 7.4 or TBS pH 8.0. However, the buffer chosen was TBS pH 8.0, since in further studies implementing these three candidate buffers that there was a slight difference in antibody-antigen binding performance (results not shown). Following the selection of the optimum coating buffer, the optimal concentrations of the conjugate and the antibody were determined using a checkerboard ELISA (Figures 4.3.4, 4.3.5 and 4.3.6). The best performing conjugate

and antibody concentration pair while using the lowest concentration of both was selected. The values plotted in the following graphs are the absorbance minus the background.

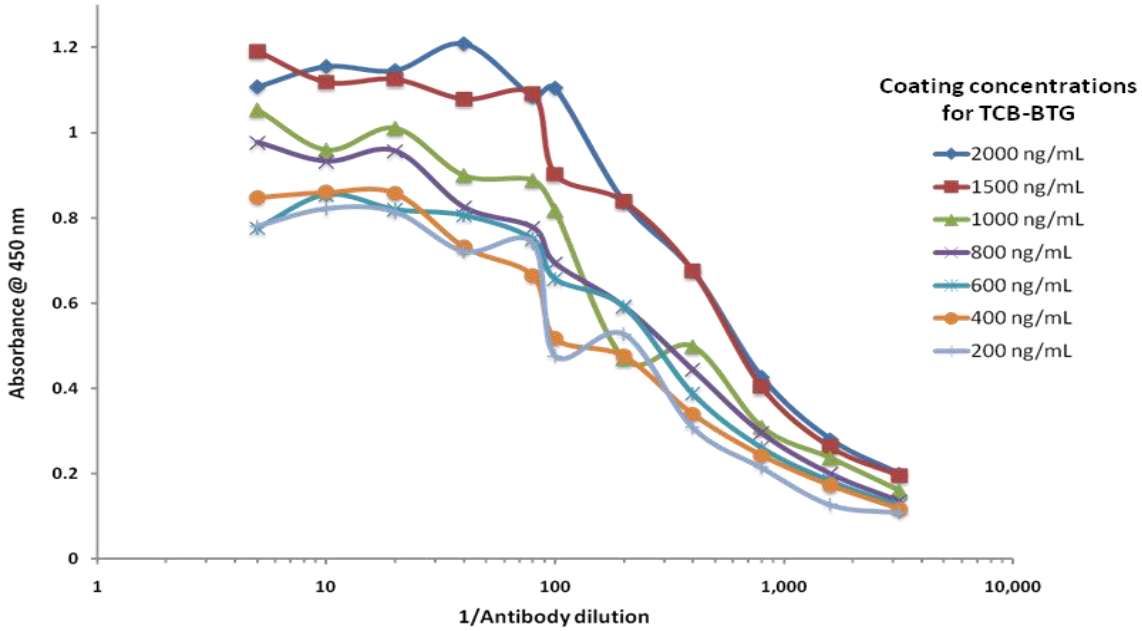

Figure 4.3.3: Plot of the absorbances obtained from the checkerboard ELISA for anti-TCB pAb. The plate was coated with TCB-BTG (2,000-100 ng/mL) and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. Dilutions of the antibody were prepared in the following increments 1/20, 1/40, 1/80, 1/100, 1/200, 1/400, 1/800, 1/1,600, 1/3,200, 1/64,000 and 1/128,000 in 0.5 % (w/v) milk marvel in PBST. The detection antibody was HRP-labelled anti-chicken IgY.

There was a significant difference shown in absorbance at 450 nm when using a concentration of 2,000 or 1,500 ng/mL compared to the lower concentrations. There was no apparent dissimilarity between 2,000 and 1,500 ng/mL when coupled with antibody dilutions of 1/200, 1/400 and 1/800. However, the antibody dilution and concentration chosen was 1/200 and 1,500 ng/mL, respectively. This gave an absorbance reading of almost 1 at 450nm.

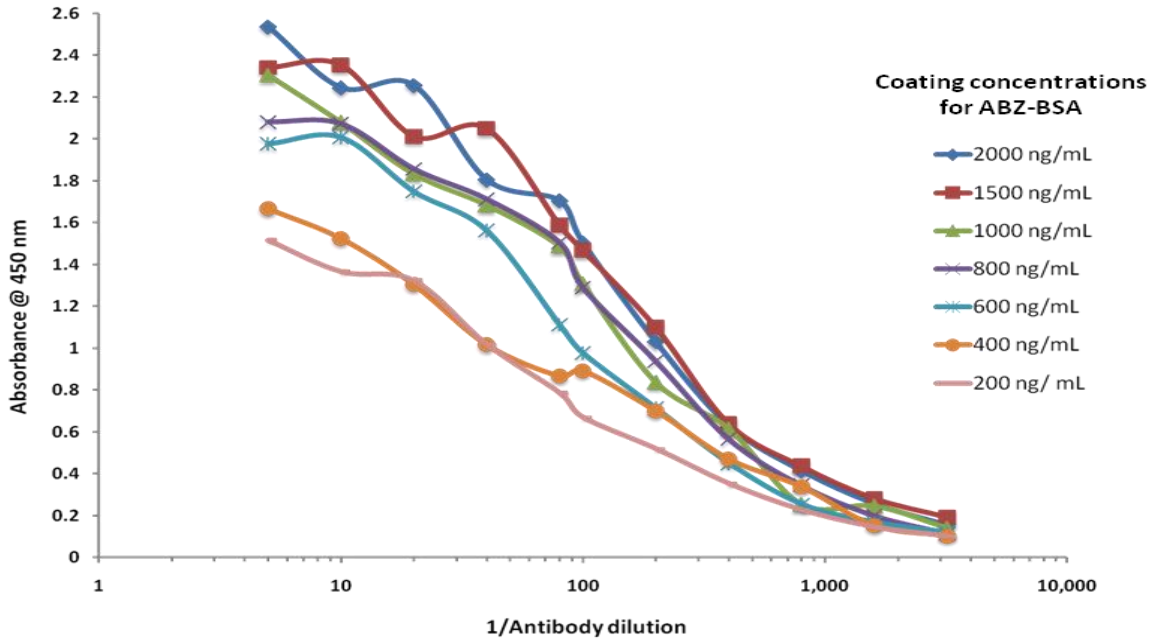

Figure 4.3.4: Plot of the absorbances obtained from the checkerboard ELISA for anti-ABZ pAb. The plate was coated ABZ-BSA (2,000-100 ng/mL) overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. Dilutions of the antibody were prepared in the following increments 1/5, 1/10, 1/20, 1/40, 1/80, 1/100, 1/200, 1/400, 1/800, 1/1,600, 1/3,200 in 0.5 % (w/v) milk marvel in PBST. The detection antibody used was the anti-chicken IgY.

There was no substantial difference shown in absorbance at 450 nm when using a concentration of 2,000, 1,500, 1,000 and 800 ng/mL. These concentrations gave a high absorbance reading of above 2 at 450 nm whereby a reading of 1 is sufficient. There was a notable difference between the concentrations 600, 400 and 200 ng/mL when using higher dilutions of antibody. However, when using a 1/100 dilution of the antibody the absorbance readings are almost identical. The concentration of the ABZ-BSA to be used for further assay development was 600 ng/mL as any lower may introduce a variation in concentration as pipetting small volumes of conjugate is not always accurate. A 1/100 dilution of the antibody was also selected.

4.3.2 Study to demonstrate the affect of various solvents on the anti-TCB/ABZ pAb

Once these parameters were established the antibody's stability and specificity in various solvents was investigated (Figures 4.3.5-4.3.7). The solvents used for these experiments were

methanol (MeOH), ethanol (EtOH) and dimethyl sulphoxide (DMSO). These solvents are the most popular routinely used reagents for the extraction of drug residues from meat and milk products (Keegan *et al.*, 2009; section 2.11.5).

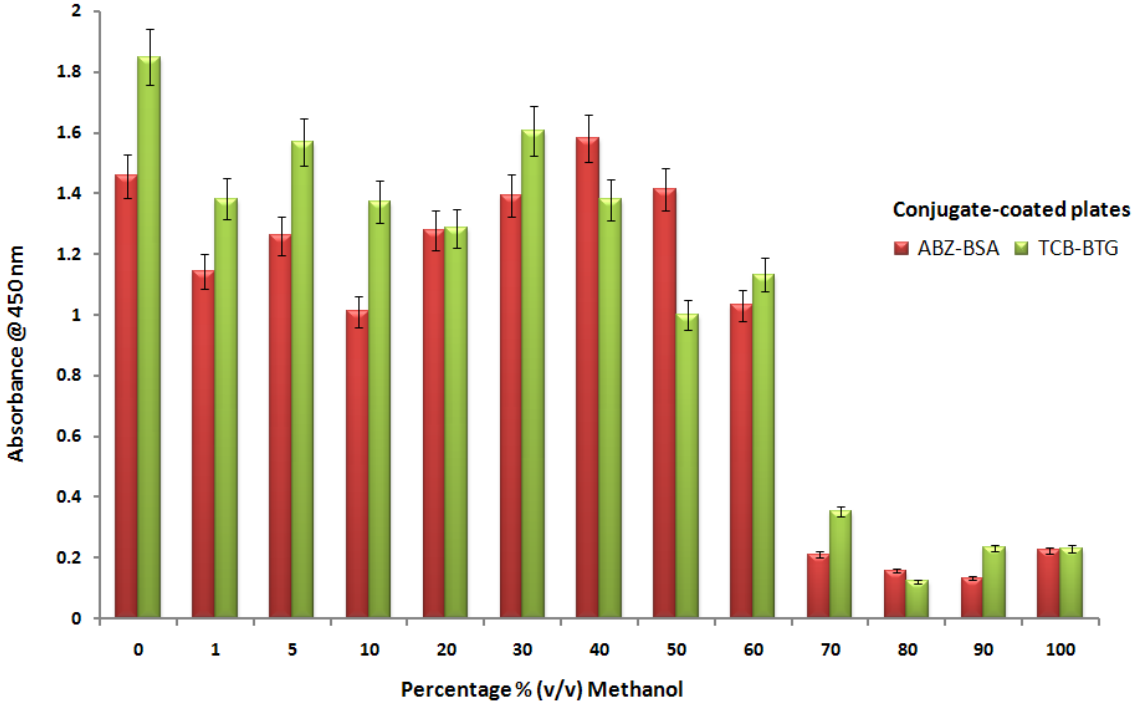

Figure 4.3.5: Results demonstrating the effect of methanol on the anti-TCB/ABZ pAb. Separate plates were coated with 1,500 and 600 ng/mL of TCB-BTG and ABZ-BSA, respectively. They were subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. Varying concentrations (0-100 % (v/v)) of methanol were added to the wells followed by a 1/100 dilution of the anti-TCB/ABZ pAb. The detection antibody was HRP-labelled anti-chicken IgY.

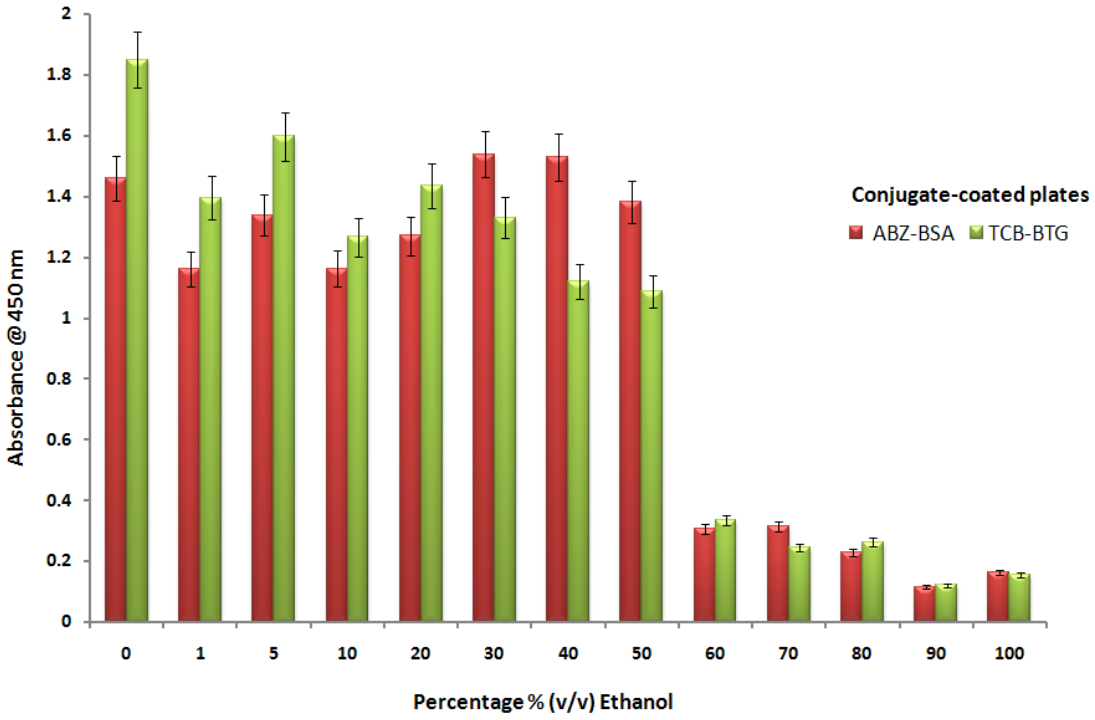

Figure 4.3.6: Results demonstrating the effect of ethanol on the anti-TCB/ABZ pAb. Separate plates were coated with 1,500 and 600 ng/mL of TCB-BTG and ABZ-BSA, respectively. They were subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. Varying concentrations (0-100 % (v/v)) of ethanol were added to the wells of both plates followed by a 1/100 dilution of the anti-TCB/ABZ pAb. The detection antibody was HRP-labelled anti-chicken IgY.

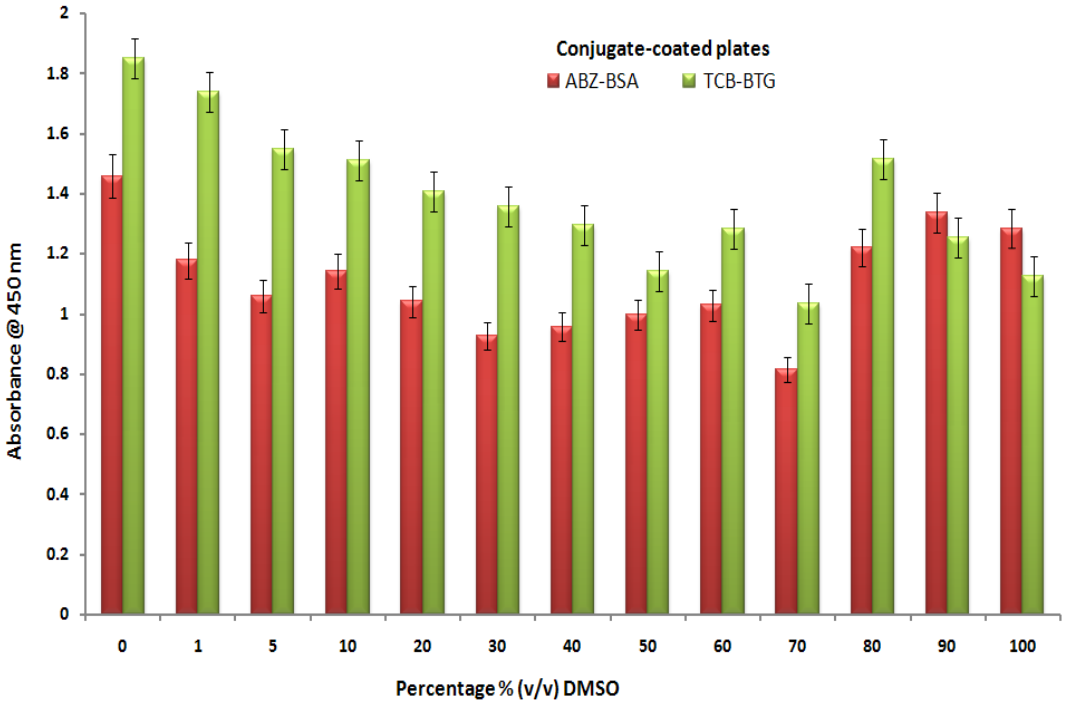

Figure 4.3.7: Results demonstrating the effect of DMSO on the anti-TCB/ABZ pAb. Separate plates were coated with 1,500 ng/mL and 600 ng/mL of TCB-BTG and ABZ-BSA respectively; and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. Varying concentrations (0-100 % (v/v)) of DMSO were added to the wells of both plates followed by a 1/100 dilution of the anti-TCB/ABZ pAb. The detection antibody was HRP-labelled anti-chicken IgY.

The studies in Figure 4.3.5-4.3.7 indicate that there is a slight decrease in absorbance when comparing the antibodies diluted in PBS with zero % solvent to when they are diluted in PBS containing solvent. This shows that the solvent has an affect on the binding of the antibody to the immobilised conjugate on the surface of the ELISA plate. However, an absorbance of above one is still obtained for the TCB/ABZ pAb binding to the ABZ-BSA and TCB-BTG conjugates when diluted in extraction buffers containing up to 60, 50 % (v/v) methanol and ethanol respectively. When the anti-TCB/ABZ pAb is diluted in PBS containing 30 % (v/v) DMSO this has an affect on the binding of the antibody to the conjugate ABZ-BSA shown by a drop in absorbance below one, however, with increasing percentage of DMSO (40-60 %) an absorbance of above one is obtained again. With > 70 % (v/v) DMSO the absorbance values continue to fluctuate. This pattern indicates that the stability of the antibody's binding to ABZ-BSA in DMSO may be hindered with the addition of concentrations above 30 % (v/v) DMSO. The antibody's binding to TCB-BTG

in DMSO decreases with increasing DMSO concentration up to 50 % and further fluctuates with the addition of > 60 % (v/v) DMSO.

4.3.3 Development of anti-TCB/ABZ pAb competitive assays for the detection of TCB and ABZ

Following this, the assays were performed in 0, 5 and 50 % to determine if the concentration of solvent would have any changes in the affinity of the antibody, either driving the antibody-antigen reaction towards complex binding or releasing bound antigen so that the response is lower. The TCB/ABZ pAb was titred in 5 % (v/v) MeOH, EtOH, DMSO and blank PBS (Figure 4.3.8 and 4.3.9).

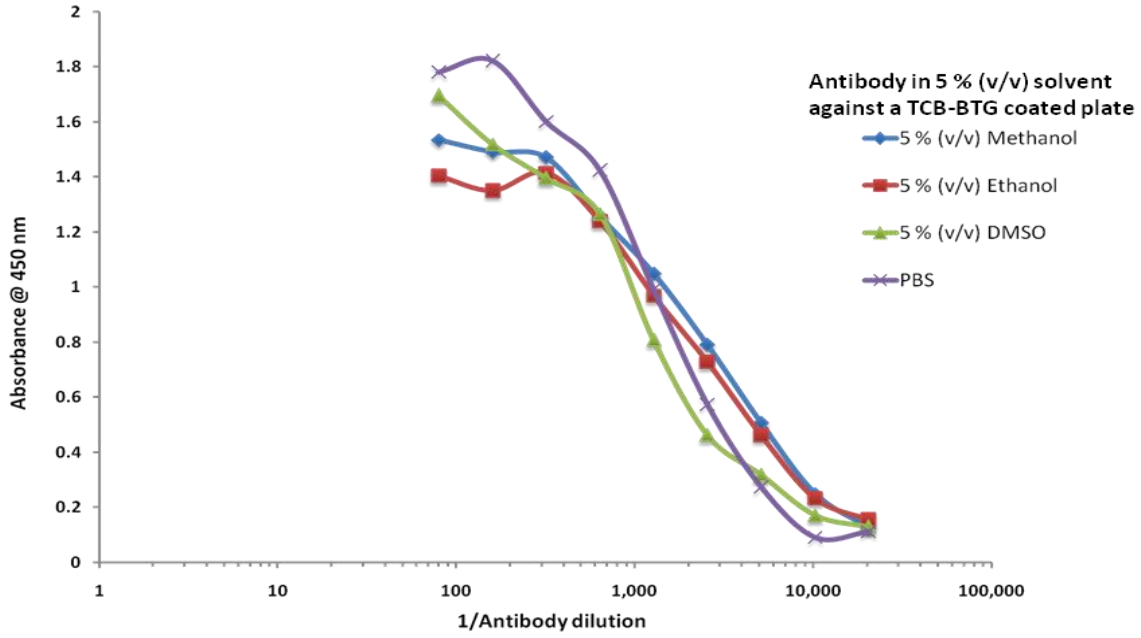

Figure 4.3.8: Plot of the TCB/ABZ pAb titre in 5 % (v/v) methanol, ethanol or DMSO. Plates were coated with 1,500 ng/mL of TCB-BTG overnight and subsequently blocked with 5 % milk marvel (w/v) in PBS, pH 7.5. The antibody was serially diluted (1/10-1/32,000) in PBS, pH 7.5, containing 5 % (v/v) of methanol, ethanol and DMSO. The detection antibody was HRP-labelled anti-chicken IgY. The titre was also performed in PBS, pH 7.5, to show the lack of affect of the solvents on the antibody binding.

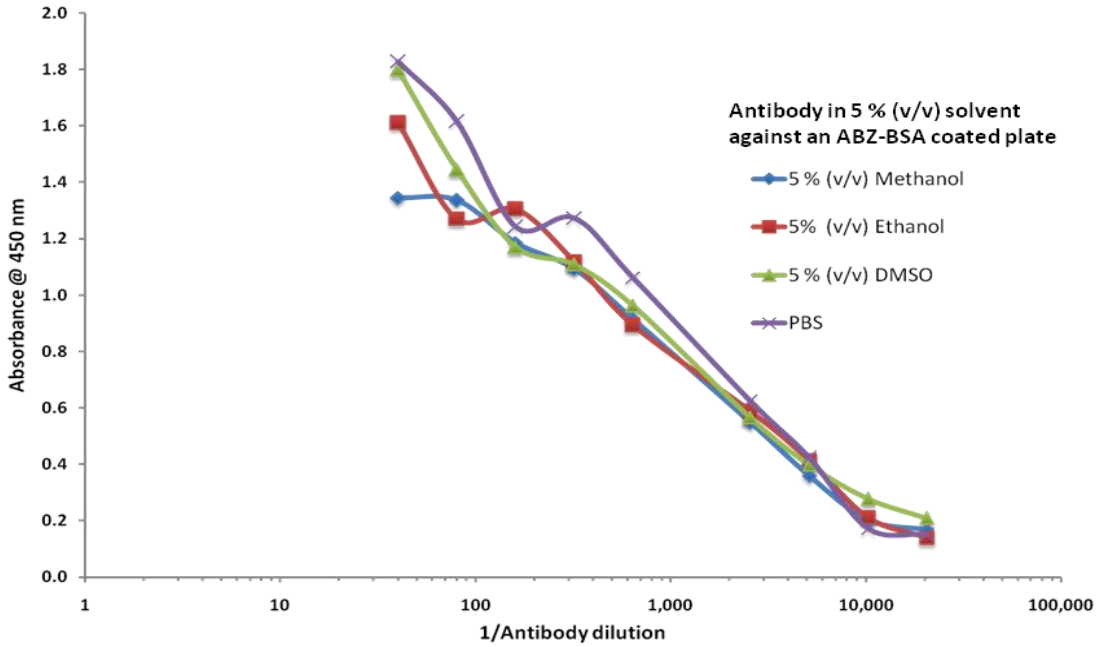

Figure 4.3.9: Plot of the TCB/ABZ pAb titre in 5 % (v/v) methanol, ethanol or DMSO. Plates were coated with 600 ng/mL of ABZ-BSA overnight and subsequently blocked with 5 % milk marvel (w/v) in PBS, pH 7.5. The antibody was serially diluted (1/10-1/32,000) in PBS, pH 7.5, containing 5 % (v/v) of methanol, ethanol and DMSO. The detection antibody was the HRP-labelled anti-chicken IgY. The titre was also performed in PBS, pH 7.5, to show the lack of affect of the solvents on the antibodies binding.

Upon analysis of the titres in figures 4.3.8 and 4.3.9, there appeared to be no significant variation of antibody-antigen binding between the three solvents. Therefore, it was decided to carry the work forward using only methanol as this is the major reagent used during the QuEChers method for the extraction of TCB and ABZ from milk. MeOH does not interfere with the antibodies binding to the analyte or HRP.

In addition to this, assays were carried out to examine the competitive nature of the anti-TBZ/ABZ pAb with free TCB and ABZ (Figure 4.3.10),

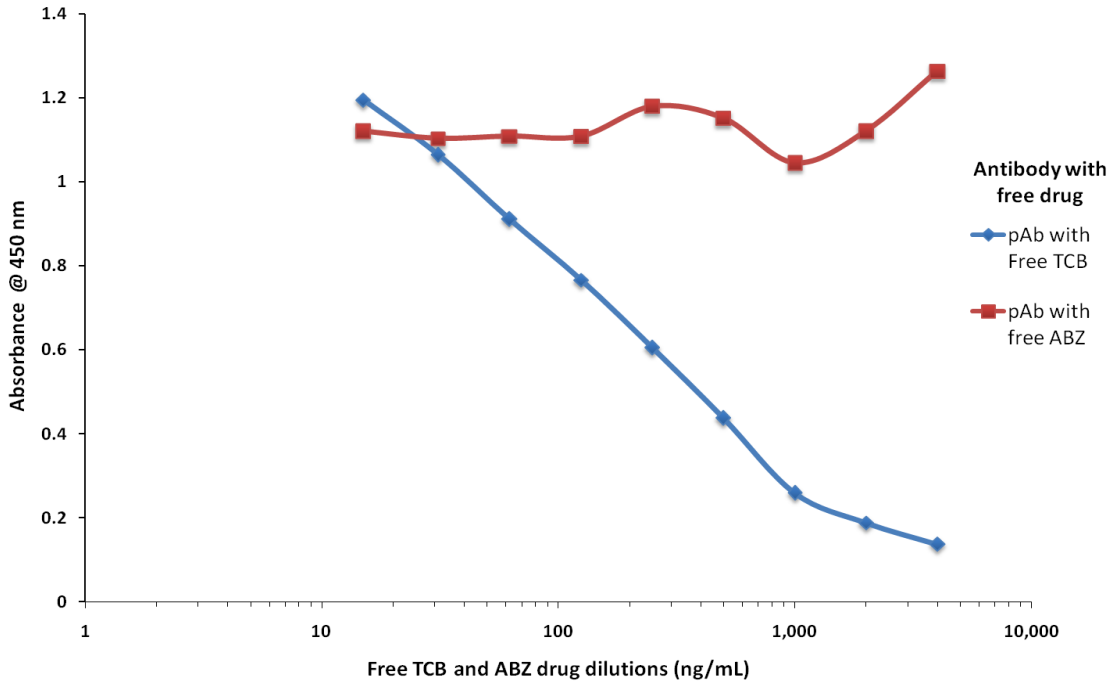

Figure 4.3.10: Plot of the absorbances obtained from the competitive assay for the detection of TCB and ABZ. Separate plates were coated with 1,500 ng/mL and 600 ng/mL of TCB-BTG and ABZ-BSA respectively overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. The pAb was diluted 1/2,560 for the TCB assay and 1/100 for the ABZ assay in PBS, pH 7.5, containing 1 % (w/v) milk marvel. Free TCB and ABZ were serially diluted (1-10,000 ng/mL) in 100 % (v/v) methanol. The detection antibody was HRP-labelled anti-chicken IgY.

There appeared to be significant displacement with the addition of free TCB with an approximate IC_{50} value of 200 ng/mL, however, there was no notable inhibition with the addition of free ABZ (Figure 4.3.10). Following this, competitive assays were performed using the free TCB and ABZ in 5 and 50 % (v/v) methanol in PBS, pH 7.5, (Figure 4.3.11 and 4.3.12).

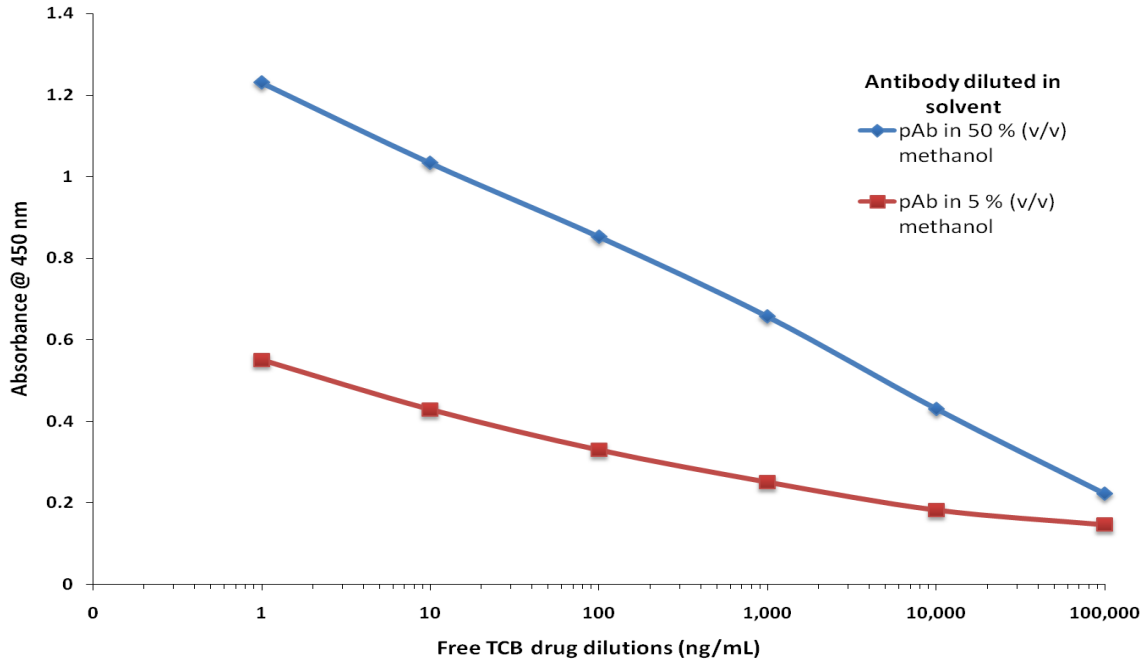

Figure 4.3.11: Plot of the absorbances obtained from the competitive assay for the detection of TCB in 5 and 50 % (v/v) in methanol. The plates were coated with 1,500 ng/mL of TCB-BTG overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5, The pAb was diluted1/2,560 in PBS, pH 7.5, containing 1 % (w/v) milk marvel. Free TCB and ABZ were serially diluted (1-10,000 ng/mL) in 10 and 100 % methanol. The detection antibody was HRP-labelled anti-chicken IgY.

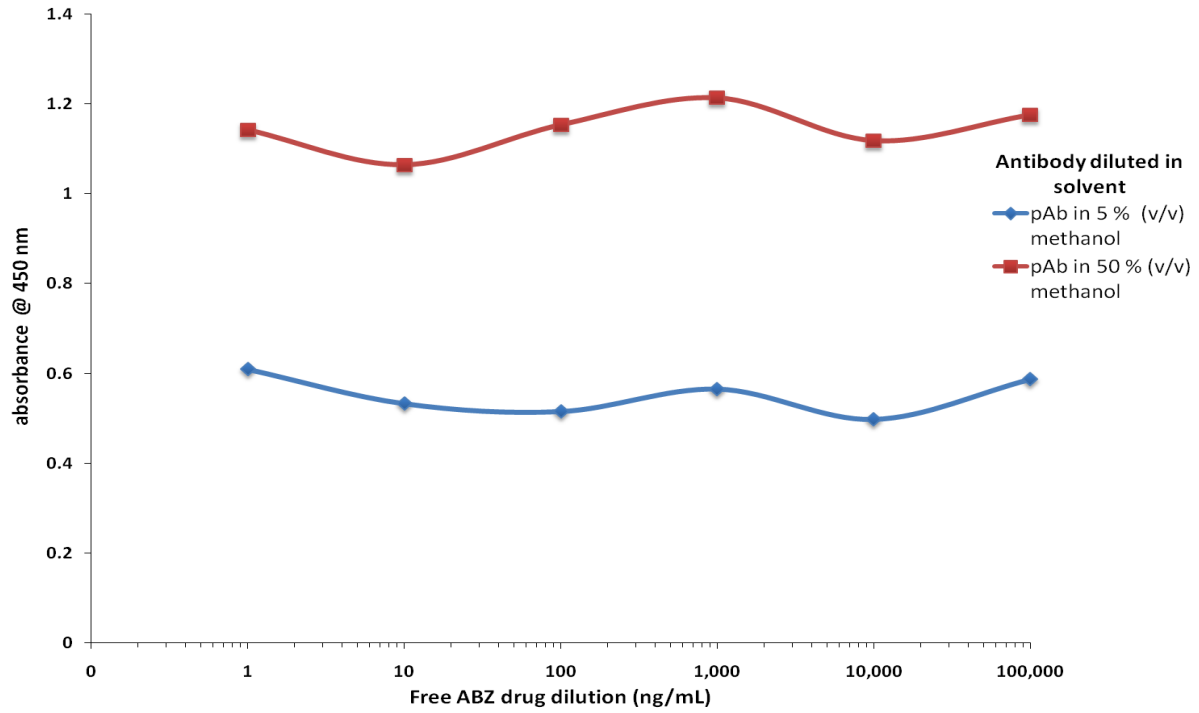

Fig 4.3.12: Plot of the absorbances obtained from the competitive assay for the detection of ABZ in 5 and 50 % (v/v) in methanol. The plates were coated with 600 ng/mL of ABZ-BSA overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. The pAb was diluted 1/100 in PBS, pH 7.5, containing 1 % (w/v) milk marvel. Free ABZ and the commercial ABZ endospec were serially diluted (1-10,000 ng/mL) in 10 and 100 % methanol. The detection antibody was HRP-labelled anti-chicken IgY.

The IC_{50} of this TCB assay (Figure 4.3.11) varies between the two solvent concentrations. When using 50 % (v/v) methanol it is approximately 1,000 ng/mL, in comparison when using 5 % (v/v) methanol it is slightly lowered at ~800 ng/mL, thus showing that there is a difference in affinity when varying the concentrations of solvent. The same approach was adopted with the free ABZ as shown in Figure 4.3.12, where the antibodies binding is decreased in 5 % (v/v) methanol. However, there is no significant amount of displacement of the antibody with the addition of free ABZ (4.3.12).

4.3.4 Cross reactivity studies of the anti-TCB/ABZ pAb with various TCB and ABZ metabolites.

The specificity of the antibody was determined by evaluating its cross-reactivity to the both the TCB and ABZ metabolites. To determine the antibodies cross reactivity, the competitive ELISA method (2.9.18) was used with different batches of TCB (1, 2, 3) and the TCB metabolites TCB-SO and TCB-SO_2 all in 5 and 50 % (v/v) methanol in PBS, pH 7.5, (Figure

4.3.13). The same approach was used for ABZ where different batches of ABZ (1, 2, 3) and the metabolites ABZ-NH_2, ABZ-SO and ABZ-SO_2 were tested for cross reactivity in both 5 and 50 % (v/v) methanol in PBS, pH 7.5, (Figure 4.3.14).

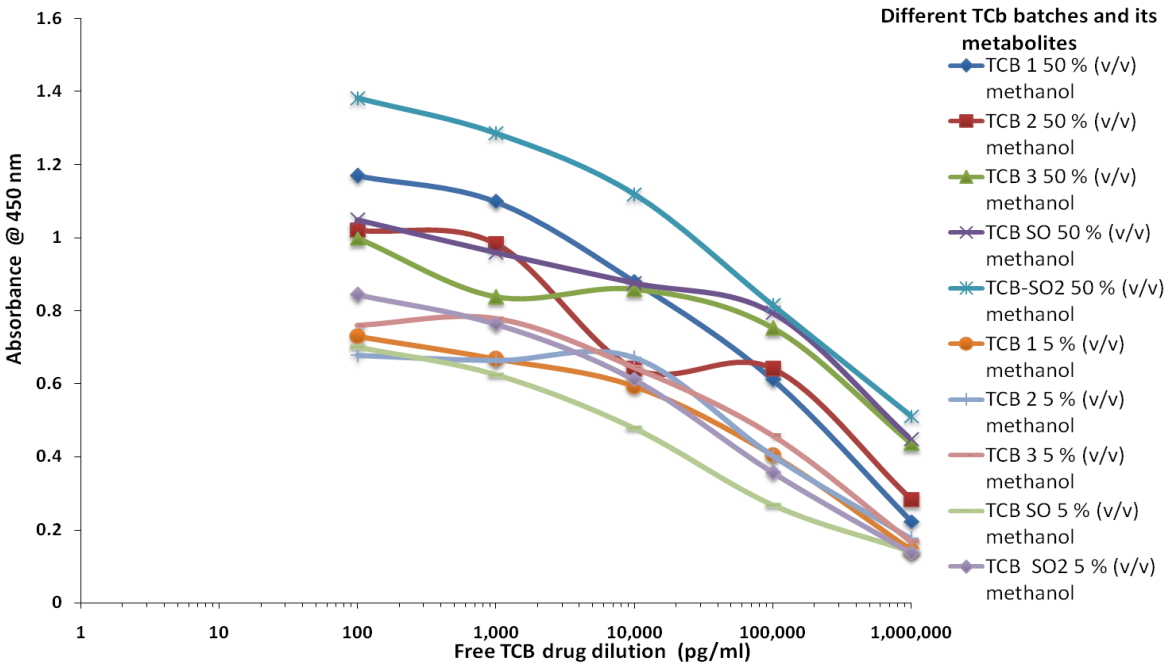

Figure 4.3.13: Plot of the absorbances obtained from the cross reactivity assay for the detection of TCB in 5 and 50 % (v/v) in methanol. The plates were coated with 1,500 ng/mL of TCB-BTG overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. The pAb was diluted 1/2,560 in PBS, pH 7.5, containing 1 % (w/v) milk marvel. Free TCB, TCB-SO and TCB-SO_2 were serially diluted (1-10,000 ng/mL) in 50 and 5 % (v/v) methanol. The detection antibody was HRP-labelled anti-chicken IgY.

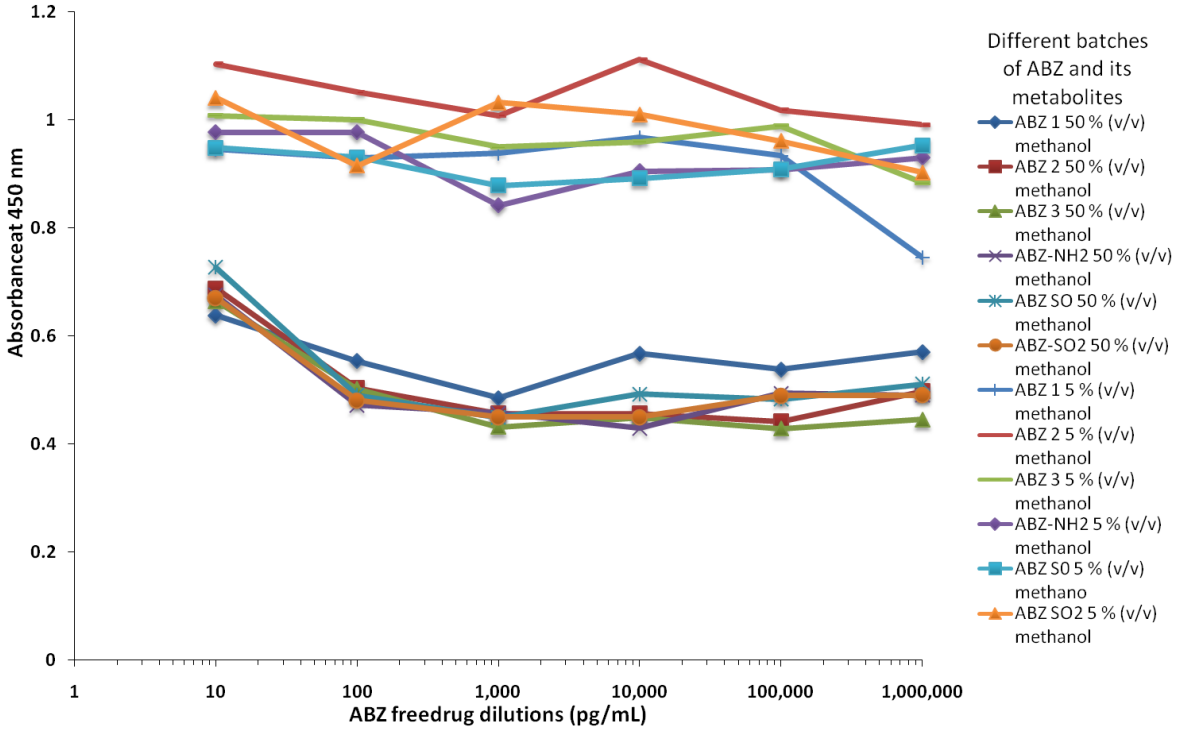

Figure 4.3.14. Plot of the absorbances obtained from the cross reactivity assay for the detection of ABZ in 5 and 50 % (v/v) in methanol. The plates were coated with 600 ng/mL of ABZ-BSA overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. The pAb was diluted 1/250 in PBS, pH 7.5, containing 1 % (w/v) milk marvel. Different stocks of free ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2 were serially diluted (1-10,000 ng/mL) in 100 and 10 % (v/v) methanol. The detection antibody was HRP-labelled anti-chicken IgY.

Upon observation of results shown in Figure 4.3.14, the anti-ABZ pAb lacks good competitive nature towards free ABZ and its metabolites, consequently it was decided to continue the validation with the TCB molecules only.

4.3.5 Intra and inter-day assays for the anti-TCB pAb for the detection of TCB in spiked PBS.

Inter and intra day assays were performed to ensure reproducibility, precision and accuracy. To determine the intra assay variation of the ELISA, three replicate measurements of each of the free TCB concentrations were used. These values (A) were again divided by the absorbance measurement at the zero free drug concentration (A_0) to give normalised values (A/A_0). From these curves the mean, standard deviation, CV and precision for the inter-assay measurements were calculated. The results are shown in Figure 4.3.15 and table 4.3.1.

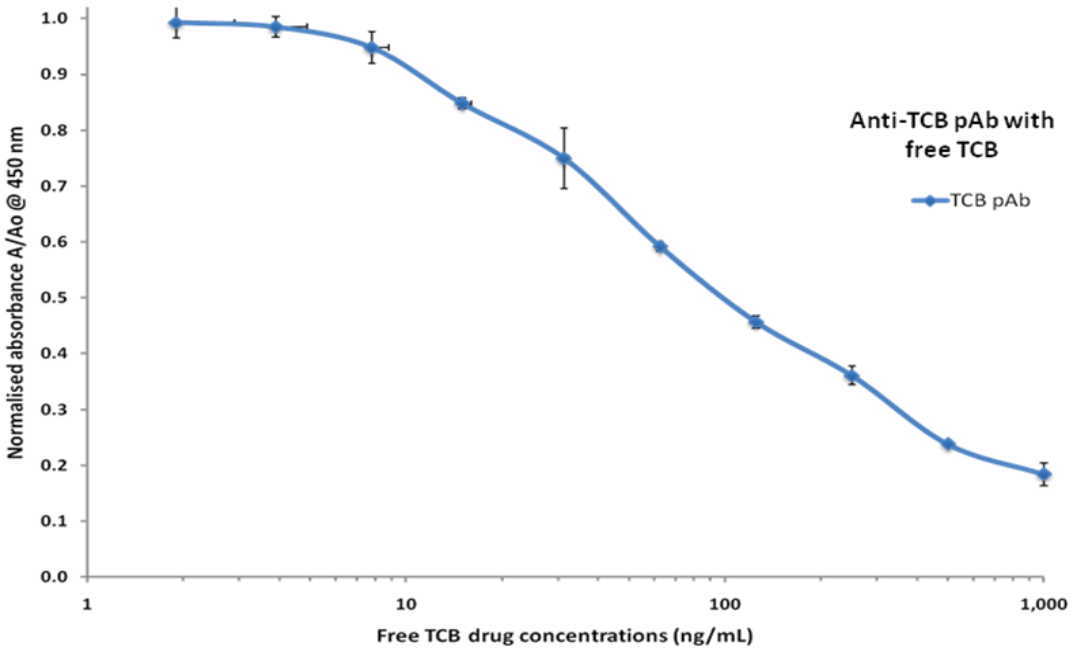

Figure 4.3.15: Intra-day assay calibration curve for the determination of TCB in PBS containing 50 % (v/v) MeOH. The competitive assay uses a coating concentration of 1,500 ng/mL of TCB-BTG, an antibody dilution of 1/2,560 and free TCB dilutions ranging between 1,000-1 ng/mL. The detection antibody was HRP-labelled anti-chicken IgY. The plotted results represent the mean response ± the SD (vertical error bars) of the three replicates of each sample during one day.

Table 4.3.1: Tabulated values for TCB concentration (pg/ml) of 'spiked' PBS, back calculated values, and percentage recoveries (% recoveries) obtained for the anti-TCB antibody intra-day assay in 'spiked' PBS. Ten standards were analysed in triplicate on the same day.

TCB concentration (pg/ml)	Back-calculated TCB concentration (ng/mL)	Percentage accuracies (%)
1,000	1,139	87
500	521	96
250	215	116
125	126	99
63	68	92
32	31	100
16	16	106
8	6	100
3	3	100
1	1	100

The inter-assay variation was calculated by performing the assay on five separate occasions. All readings were collated together and the mean, standard deviation, percentage CV's back calculated concentrations and percentage accuracies were determined (table 4.3.2).

Table 4.3.2: Tabulated values for TCB concentration (pg/ml) of 'spiked' PBS, back calculated values, percentage coefficient of variation (% CV) and percentage recoveries (% recoveries) obtained for the anti-TCB pAb antibody inter-day assay in 'spiked' PBS. Ten standards were analysed in triplicate on the five separate days.

TCB concentration (pg/ml)	Back-calculated TCB concentration (ng/mL)	Percentage accuracies (%)	Coefficients of variation (CVs), (%)
1,000	1,010	100	19
500	494	99	12
250	251	101	9
125	134	99	11
63	58	95	10
32	32	100	5
16	15	94	5
8	8	100	2
3	4	100	5
1	1	100	12

The inter-day studies show the proposed assay had good precision with CV's ranging from 19 % to 2 % between the assay range of 1 ng/mL and 1 pg/mL TCB. The percentage accuracies determined in both assays provide an accurate assessment of the measured TCB concentrations. Both assays had acceptable concentration values back-calculated from the curves.

As discussed in chapter three the LOD is defined as the lowest TCB concentration likely to be reliably distinguished from the blank, and at which detection is feasible. This can be calculated with the formula LOD = LOB ± 1.645(SD of the lowest concentration sample) where LOB is the highest analyte concentration expected to be found when replicates of the blank are tested.

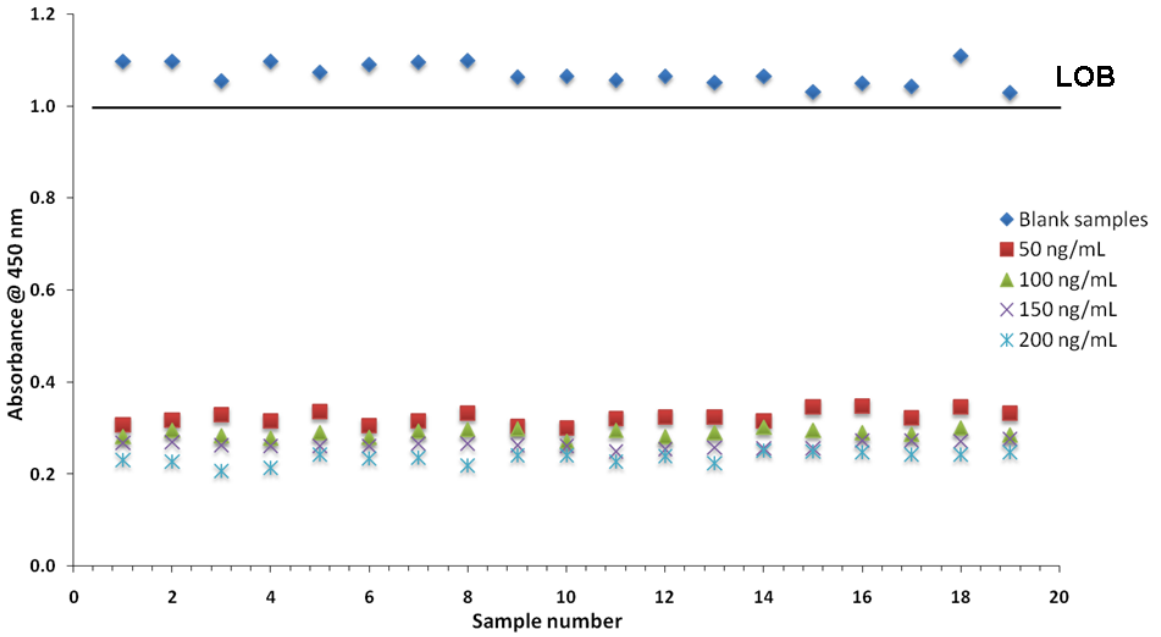

Figure 4.3.16: Graphical representation of the LOB and LOD of the anti-TCB pAb. The plates were coated with 1,500 ng/mL of TCB-BTG overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. PBS, pH 7.5, containing 50 % (v/v) methanol was fortified with 50, 100, 150, and 200 ng/mL of free TCB and 50 µL of each were added to the corresponding wells followed by 50 µL of a 1/2,500 dilution of anti-TCB pAb in 50 % (v/v) methanol. The detection antibody was a HRP-labelled anti-chicken IgY.

The mean and standard deviation of each sample was calculated and from this the LOB was established as 1.05 at an absorbance of 450 nm. The mean and standard deviation of the samples were calculated and subtracted from the LOB. This gave an LOD absorbance of 0.749. Reading this from the standard curve generated during the inter and intra day assays the "analytical" LOD is approximately 25 ng/mL.

4.3.5 Intra and inter-day assays for the anti-TCB pAv for the detection of TCB-SO and TCB-SO_2 in spiked PBS.

Inter and intra assay were also performed for the TCB metabolites TCB-SO and TCB-SO_2 in PBST containing 50 % (v/v) methanol. Reproducibility and accuracy studies were carried out as explained previously for both TCB-SO and TCB-SO_2 intra day (Figure 4.3..17 and table 4.3.3) and inter-day studies (Figure 4.3.18 and table 4.3.4). Additionally, cross reactivity

studies were performed and these were established as 100 % and 97.3 % for TCB-SO and TCB-SO_2 respectively.

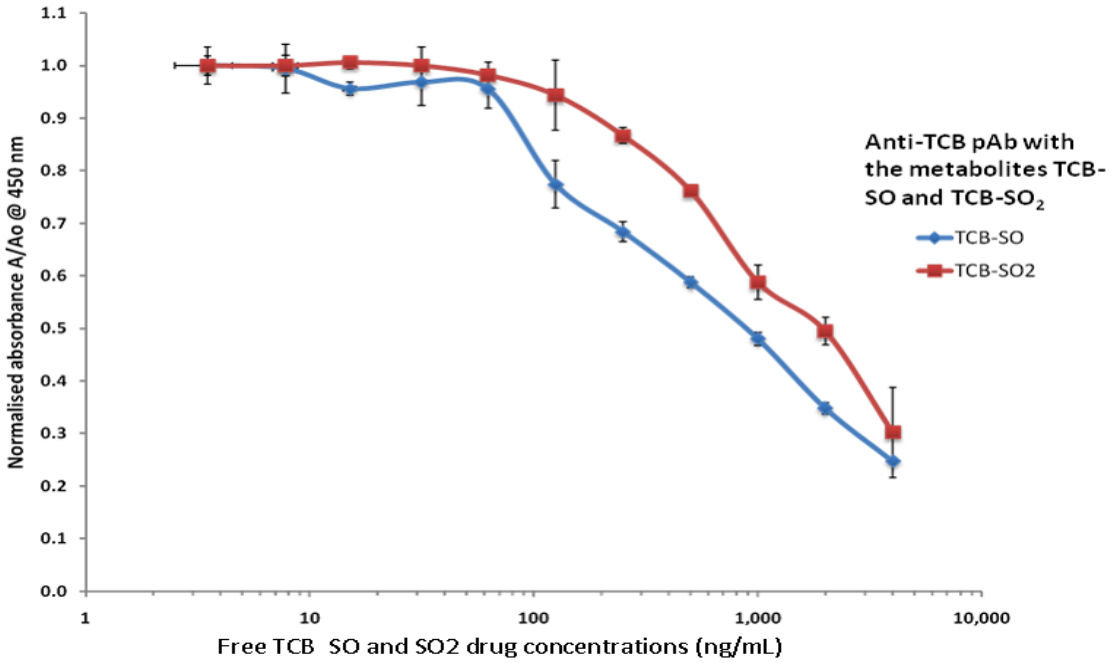

Figure 4.3.17: Intra-day assay calibration curve for the determination of TCB-SO and TCB-SO_2 in PBS containing 50 % (v/v) MeOH The competitive assay uses a coating concentration of 1,500 ng/mL of TCB-BTG, an antibody dilution of 1/2,560 and free TCB dilutions ranging between 4,000-3 ng/mL. The detection antibody was HRP-labelled anti-chicken IgY. The plotted results represent the mean response ± SD of the three replicates if the fortified samples carried out on the same day.

Table 4.3.3: Tabulated values for TCB-SO and TCB-SO_2 concentration (pg/ml) of 'spiked' PBS, back calculated values, and percentage recoveries (% recoveries) obtained for the anti-TCB antibody intra-day assay in 'spiked' PBS. Ten standards were analysed in triplicate on the same day.

TCB-SO and SO_2 concentration (pg/ml)	**Back-calculated TCB-SO concentration (pg/mL)**	**Percentage accuracies (%) TCB-SO**	**Back-calculated TCB-SO_2 concentration (pg/mL)**	**Percentage accuracies (%) TCB-SO_2**
4,000	4,050	99	4,259	94
2,000	2,125	94	1,809	101
1,000	1,061	95	1,148	88
500	489	98	497	100
250	253	99	242	103
125	121	103	125	100
62	64	97	72	86
31	32	97	32	101
15	16	94	14	93
8	9	88	7	106
3	3	100	3.5	86

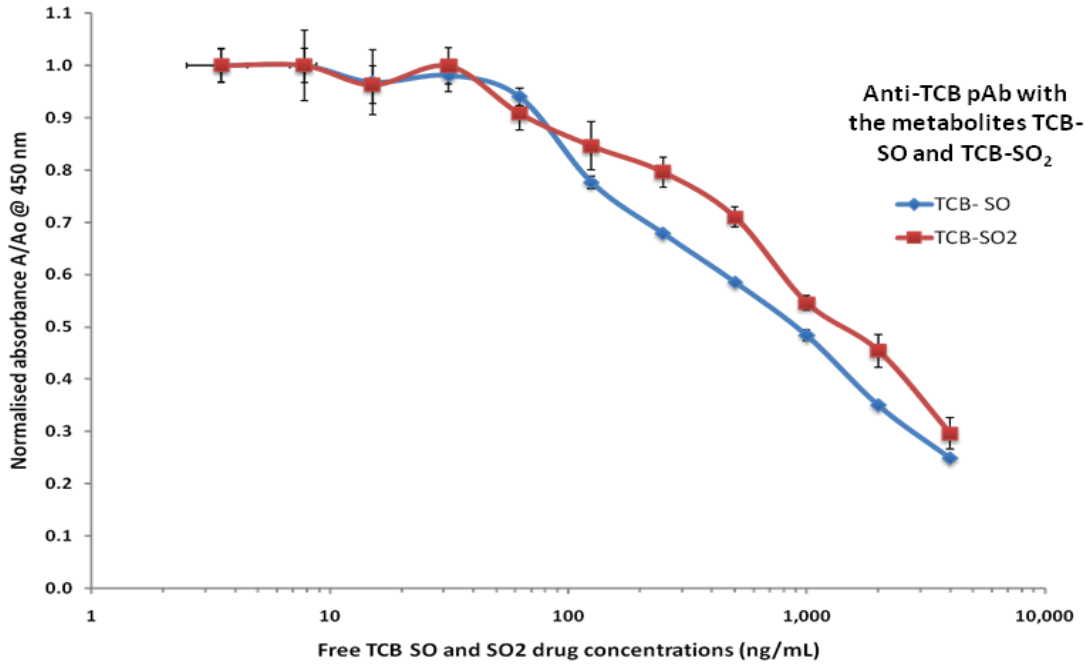

Figure 4.3.18: Inter-day assay calibration curve for the determination of TCB-SO and TCB-SO_2 in PBS containing 50 % (v/v) MeOH. The competitive assay uses a coating concentration of 1,500 ng/mL of TCB-BTG, an antibody dilution of 1/2,560 and free TCB-SO and TCB-SO_2 dilutions ranging between 4,000-3 ng/mL. The detection antibody was HRP-labelled anti-chicken IgY. The plotted results represent the mean response ± SD of the three replicates if the fortified samples carried out on the same day.

Table 4.3.4: Tabulated values for TCB-SO and TCB-SO_2 concentration (pg/ml) of 'spiked' PBS, back calculated values, percentage coefficient of variation (% CV) and percentage recoveries (% recoveries) obtained for the anti-TCB pAb antibody inter-day assay in 'spiked' PBS.. Ten standards were analysed in triplicate over three days.

TCB-SO and TCB-SO_2 concentration (pg/ml)	Back-calculated TCB-SO concentration (pg/mL)	Percentage accuracies (%)	Coefficients of variation (CVs), (%)	Back calculated TCB-SO concentration)pg/ml)	Percentage accuracies	Coefficients of variation (CVs), (%)
4,000	4263	106	1	4,012	100	7
2,000	1985	100	2	1,910	96	3
1,000	937	94	2	1,042	104	3
500	485	97	1	495	99	4
250	271	108	1	237	95	5
125	131	104	2	132	105	3
62	72	116	2	69	111	4
31	25	90	3	28	90	4
15	21	113	6	14	90	3
8	9	113	6	9	113	3
3	3	100	2	3	100	2

The competitive assay can detect low levels of TCB-SO and TCB-SO_2 (3 pg/mL) with acceptable % CV of 2 % and percentage accuracy values of approximately 100 %. The percentage accuracies determined in both assay formats provide an accurate assessment of the measured TCB-SO and TCB-SO_2 concentrations. Both assays had acceptable concentration values back-calculated from the curves.

Following the validation of the TCB assay the RNA previously extracted (section 2.
11.3) was used to construct an avian scFv library. This was panned against both TCB and ABZ hapten conjugates to isolate specific clones with high affinity to TCB and ABZ. The advantages of this system are discussed in section 4.6.

4.4 The library construction of the avian anti-TCB/ABZ scFv.

The cDNA generated from the RNA extracted from the spleen and bone marrow of the immunized chicken (section 2.11.3) was utilized for the initial amplification of the avian

variable light (V_λ) and variable heavy (V_H) chains. The PCR reaction was carried out using the primers described in section 2.11.4 and the components and reaction programme outlined in sections 2.11.4.1 and 2.11.4.2 respectively.

The short linker primer CSCVHo-F is paired with the CSCG-B reverse primer to amplify V_H fragments using the cDNA as a template. The sense primers have a sequence tail that is compatible to the linker sequence that is used in the overlap extension PCR. The reverse primer has a sequence tail containing an *Sfi*I site; this site is recognized by the reverse extension primer used in the second-round PCR.

The CSCVK sense primer is combined with the CKJo-B reverse primer to amplify the V_λ gene segments and a 5´ sequence tail that contains an *Sfi*I site that is recognized by the sense extension primer in the second-round PCR. The reverse primer has a linker sequence tail that is used in the overlap extension.

A gradient of $MgCl_2$ was required for the successful amplification of both the variable light (350 bp) and heavy chains (400 bp) from both the bone marrow and spleen cDNA (Figures 4.4.1 and 4.4.2).

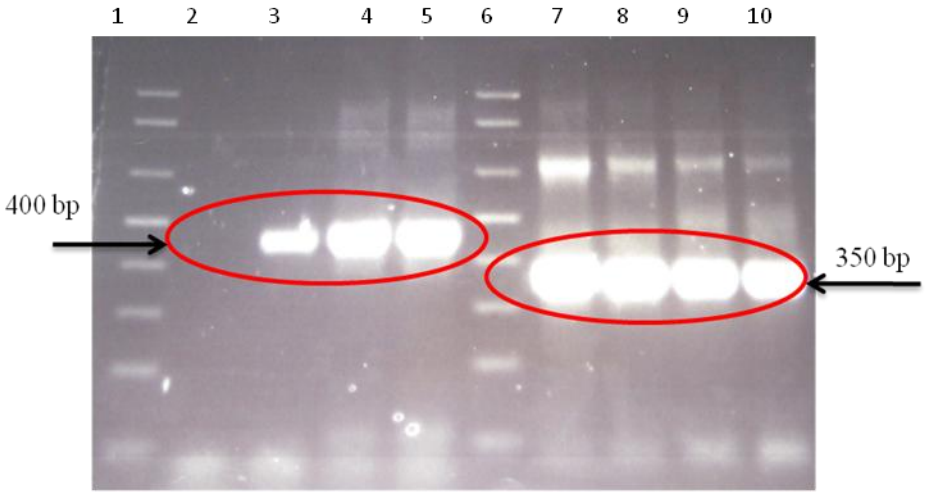

Figure 4.4.1: Amplification of the variable light and heavy genes from the cDNA generated from the TCB/ABZ avian spleen using a $MgCl_2$ gradient ranging from 0-3mM. Lanes 1 and 6: 1 Kb Sigma DNA ladder; Lane 2 and 7: 0 $MgCl_2$; Lanes 3 and 8: 1 mM $MgCl_2$; Lanes 4 and 5: 2 mM $MgCl_2$ and lanes 5 and 9: 3 mM $MgCl_2$.

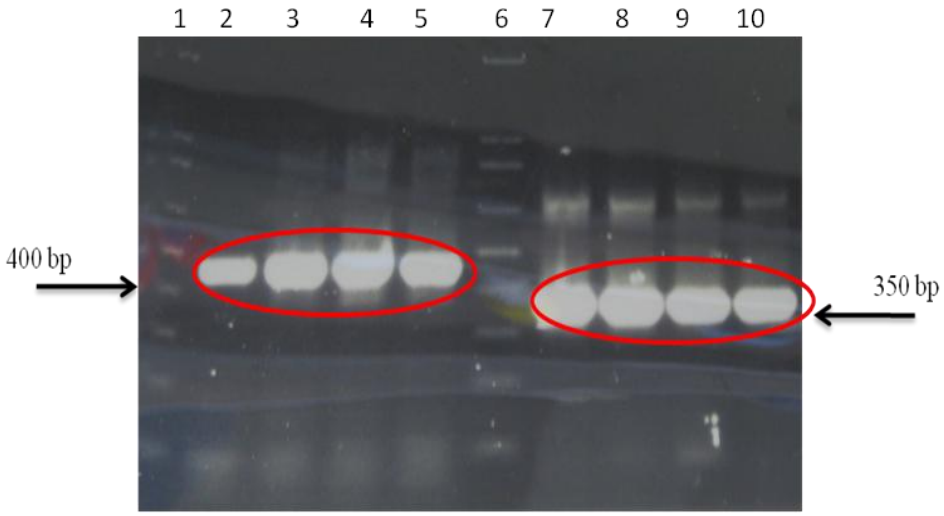

Figure 4.4.2: Amplification of the variable light and heavy genes from the RNA extracted from the TCB/ABZ avian bone marrow using 0-3 mM $MgCl_2$ gradient. Lanes 1 and 6: 1 Kb Sigma DNA ladder; Lane 2 and 7: 0 $MgCl_2$; Lanes 3 and 8: 1 mM $MgCl_2$; Lanes 4 and 5: 2 mM $MgCl_2$ and lanes 5 and 9: 3 mM $MgCl_2$.

All PCR products were resolved on a 1 % (w/v) agarose gel and purified (section 2.8.5). Following purification the overlap extension PCR was performed as outlined in table 2.11.5.1. The SOE PCR combines both the V_H and V_L fragments to construct the final scFv fragment. The sense and the reverse primers used (CSC-F and CSC-B) recognize the

sequence tails from the first round of amplifications. A $MgCl_2$ gradient was performed for the splice by overlap extension for both the spleen and the bone marrow yielding a 750 bp PCR product shown in Figure 4.4.3 and 4.4.4 respectively.

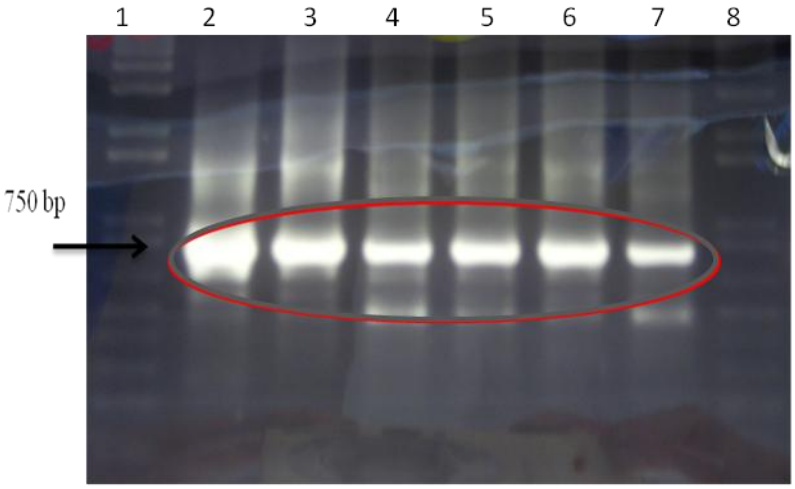

Figure 4.4.3: Final splice by overlap extension (SOE) (750 bp) of the anti-TCB/ABZ scFv gene fragment from the avian spleen. Lanes 1 and 8: 1 Kb Sigma DNA ladder; Lane 2: 0 $MgCl_2$; Lane 3:1 mM $MgCl_2$; Lane 4: 2 mM $MgCl_2$; Lane 5: 3 mM $MgCl_2$; Lane 6: 4 mM $MgCl_2$ and lane 7: 5 mM $MgCl_2$.

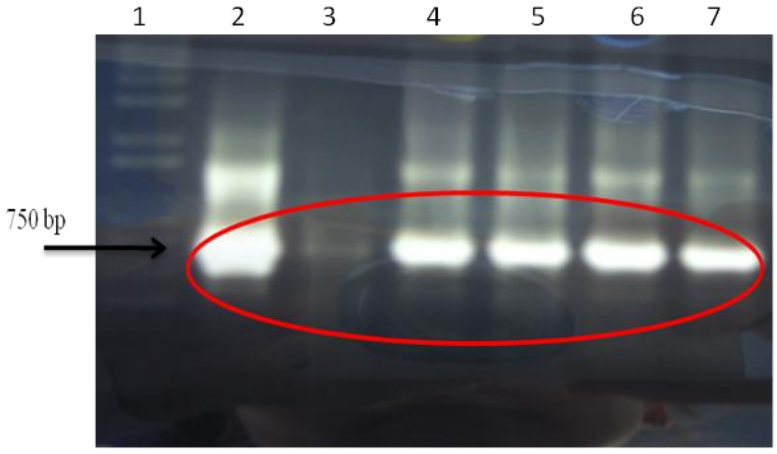

Figure 4.4.4: Final splice by overlap extension (SOE) (750 bp) of the anti-TCB/ABZ scFv gene fragment from avian bone marrow. Lane 1: 1 Kb Sigma DNA ladder; Lane 2: 0 $MgCl_2$; Lane 3: 1 mM $MgCl_2$; Lane 4: 2 mM $MgCl_2$; Lane 5: 3 mM $MgCl_2$; Lane 6: 4 mM $MgCl_2$ and lane 7: 5 mM $MgCl_2$.

Following the successful amplification of the 750 bp fragment, a 5 x reaction of both the spleen and bone marrow was performed. All bone marrow products and spleen products were pooled and ethanol precipitated. Both products were resolved on a 1 % (w/v) agarose gel and purified. The resulting 750 bp for the purified PCR product for the bone marrow is shown in Figure 4.4.5.

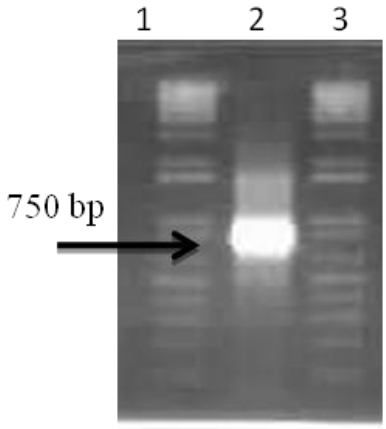

Figure 4.4.5: Final SOE for the avian scFv from the bone marrow, Lanes 1 and 3: 1 Kb Sigma DNA ladder and lane 2: bone marrow scFv fragment 750 bp

4.5 Transformation and subsequent bio-panning of the anti-TCB/ABZ scFV library against TCB-HRP and ABZ-HRP.

The PCR product was concentrated by ethanol precipitation (section 2.8.6), quantified using the Nanodrop ND-1,000™ and subsequently cloned into the pComb3xSS vector. The directional cloning was performed using the restriction enzyme *Sfi*1 (Figure 4.5.1) and was successfully ligated into the digested pComb vector. The ligation was ethanol precipitated and transformed into *E.coli* XL1-Blue electrocompetent cells (see section 2.8.6).

The library size for bone and spleen was 4.6 x 10^6 and 6.2 x 10^7 respectively. A colony pick PCR was performed to ensure that the gene insert was present at 750 bps (Figure 4.5.2). The transformed library was subjected to 5 rounds of bio-panning against the conjugates TCB-HRP and ABZ-HRP immobilised to a solid support (immuno-tube). The competitive elution strategy was employed (table 2.8.9.1).

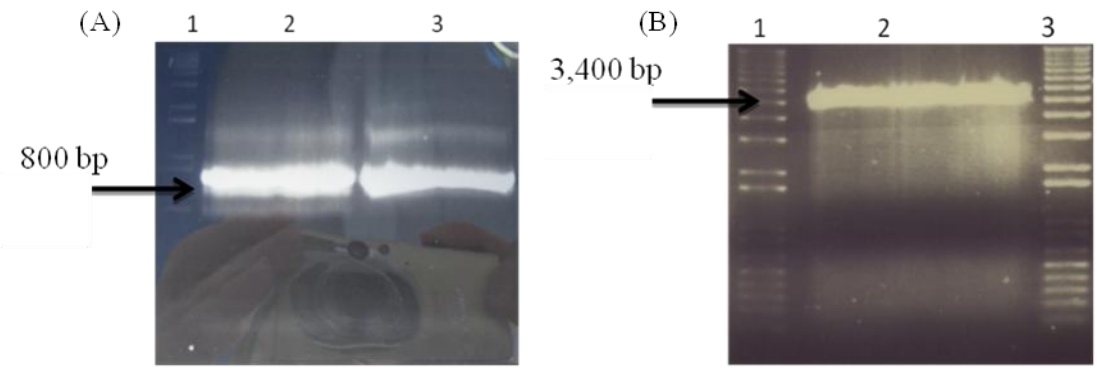

Figure 4.5.1: Digested 750 bp scFv gene product (A) and the digested pComb3xSS vector at 3,400 bp (B). Gel (A) Lane 1: 1 Kb Sigma DNA ladder and lanes 2 and 3: 750 bp digested PCR product. Gel (B) Lanes 1 and 3: 1 Kb Sigma DNA ladder and lane 2: digested pComb3xSS vector.

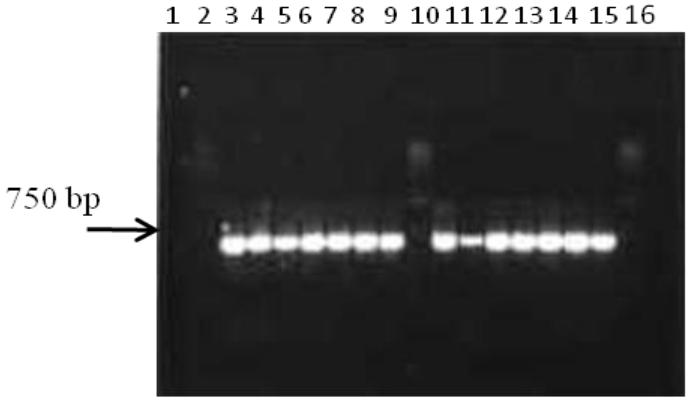

Figure 4.5.2: Amplification of the 750 bp gene insert by colony pick PCR for the anti-TCB/ABZ avian scFv. Lane 1, 9 and 17: 1 Kb Sigma DNA ladder and lanes 2-8: transformed scFv gene insert from the bone marrow library and lanes 11-16: transformed scFv gene insert from the spleen library.

Table 4.5.1: The phage input and output titres for all three rounds of bio-panning against TCB-HRP and ABZ-HRP.

Round of bio-panning	Colony forming units (CFU) for TCB	Colony forming units (CFU) for ABZ
Input 1	5.9×10^{11}	4.6×10^{11}
Output 1	7.3×10^{8}	2.6×10^{7}
Input 2	4.4×10^{12}	6.2×10^{11}
Output 2	1.1×10^{8}	1.8×10^{7}
Input 3	6.4×10^{12}	5.9×10^{11}
Output 3	3.8×10^{7}	2.1×10^{7}
Input 4	3.9×10^{11}	4.5×10^{11}
Output 4	7.5×10^{6}	6.9×10^{7}
Input 5	5.6×10^{11}	8.6×10^{11}
Output 5	9.4×10^{7}	2.3×10^{7}

Monoclonal phage ELISA's (Figures 4.5.3-4.5.7) were performed as per section 2.9.1. Any clone that gave an absorbance reading of below 0.2 was regarded as background and were not further analysed.

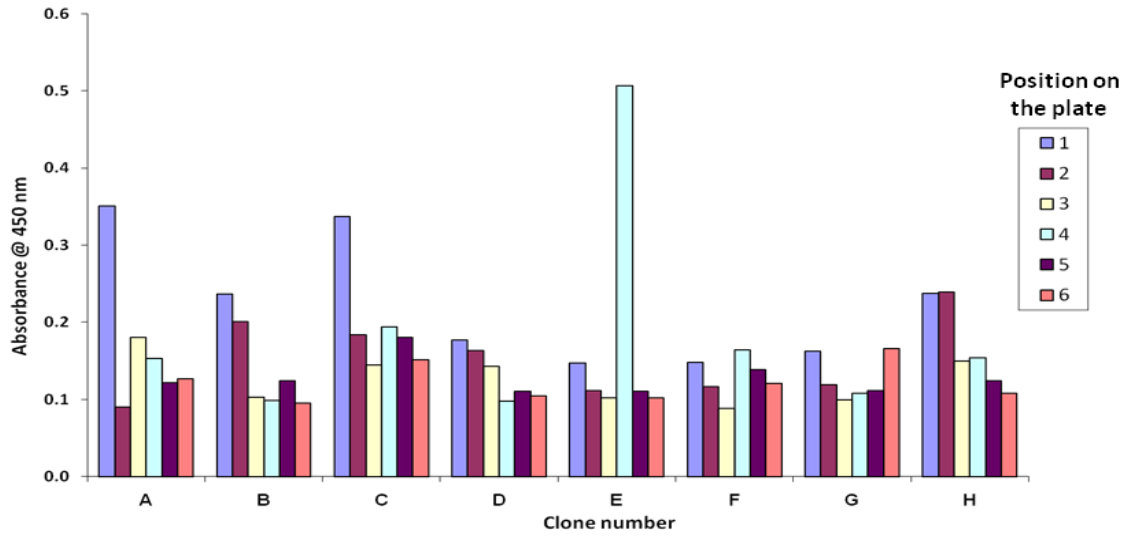

Figure 4.5.3: Plot of the absorbances obtained from the monoclonal phage ELISA of the anti-TCB scFv panned library round 3. The plates were coated with 1 μg/mL of TCB-BTG overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. The individual colony lysates (100 all) were applied to the wells and incubated for 1hr at 37°C. The detection antibody was the HRP-labelled anti-HA.

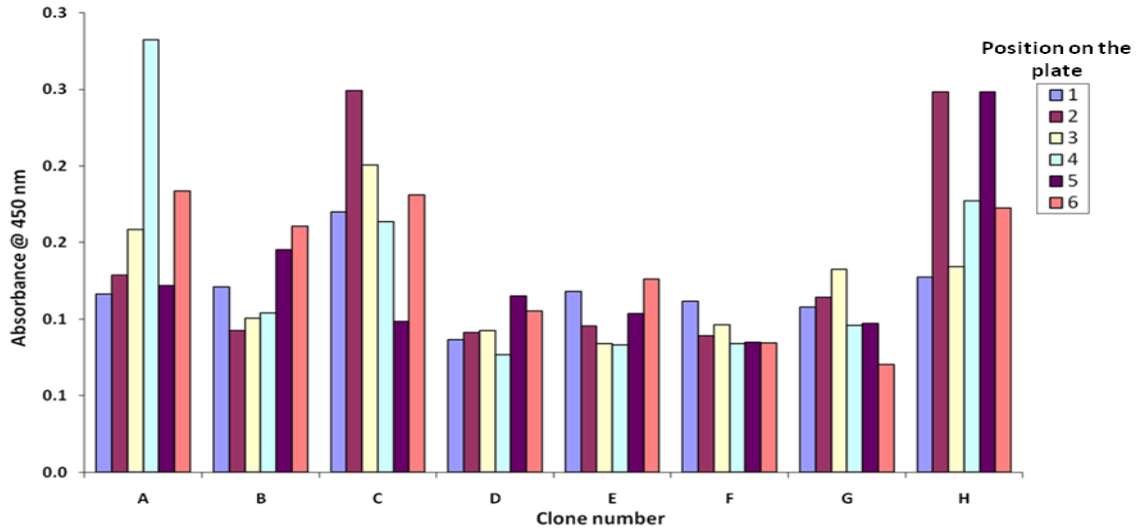

Figure 4.5.4 Plot of the absorbances obtained from the monoclonal phage ELISA of the anti-TCB scFv panned library round 4. The plates were coated with 1 μg/mL of TCB-BTG overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. The individual colony lysates (100 μL) were applied to the wells and incubated for 1 hr at 37°C. The detection antibody was the HRP-labelled anti-HA.

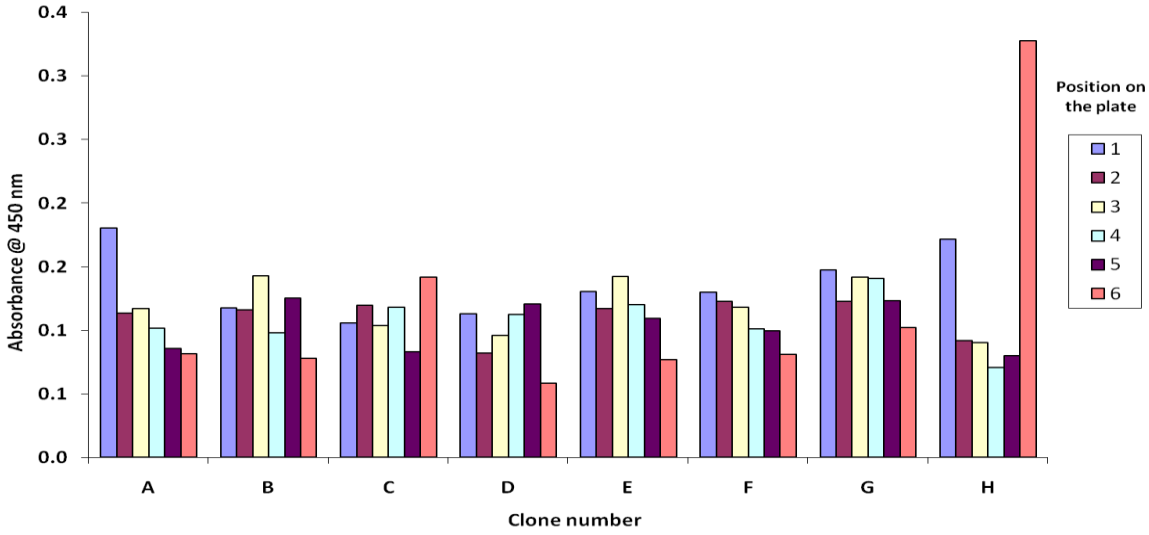

Figure 4.5.5: Plot of the absorbances obtained from the monoclonal phage ELISA of the anti-TCB scFv panned library round 5. The plates were coated with 1 µg/mL of TCB-BTG overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. The individual colony lysates (100 µL) were applied to the wells and incubated for 1 hr at 37°C. The detection antibody was the HRP-labelled anti-HA.

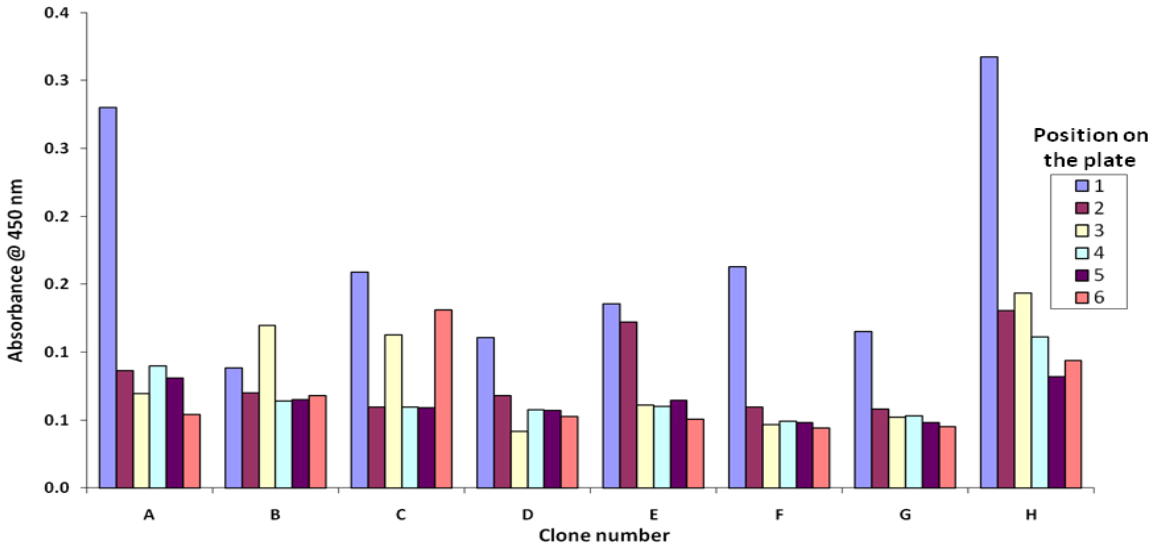

Figure 4.5.6: Plot of the absorbances obtained from the monoclonal phage ELISA of the anti-ABZ scFv panned library round 3. The plates were coated with 1 µg/mL of ABZ-BSA overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. The individual colony lysates (100 µL) were applied to the wells and incubated for 1 hr at 37°C. The detection antibody was the HRP-labelled anti-HA.

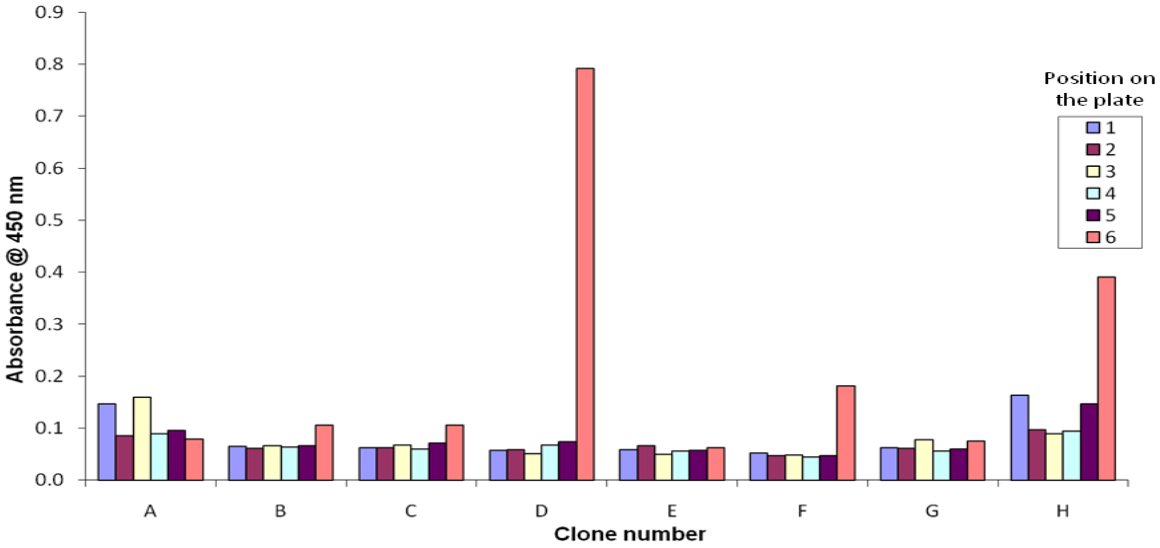

Figure 4.5.7: Plot of the absorbances obtained from the monoclonal phage ELISA of the anti-ABZ scFv panned library round 4. The plates were coated with 1 µg/mL of ABZ-BSA overnight and subsequently blocked with 5 % (w/v) milk marvel in PBS, pH 7.5. The individual colony lysates (100 µL) were applied to the wells and incubated for 1 hr at 37°C. The detection antibody was the HRP-labelled anti-HA.

The clones selected were as follows; TCB round three: A1, B1, C1, E4, H1, H2; round four; A4, C2, H2, H5 and round five: H6. The ABZ positive clones selected were as follows; round three: A1, H1 and round four H6, D6. These were amplified using the same PCR conditions as the colony pick PCR (Figure 4.5.8). All clones contained the gene of interest.

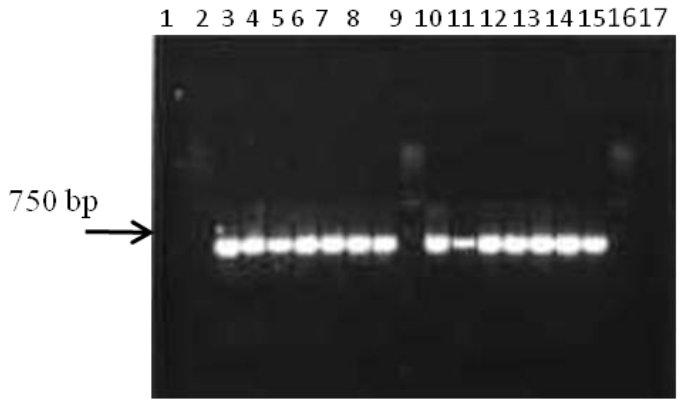

Figure 4.5.8: Gel picture of the gene inserts amplified from the positive clones selected from the bio-panning rounds. Lanes 1, 10 and 18: 1 Kb Sigma DNA ladder and lanes 2-9 and 11-17 are the gene inserts at 750 bp of the positive clones.

All positive clones were grown in 5 mL of SB (section 2.4), 100 μg/mL of carbenicillin. Following overnight incubation they were sub-cultured and allowed to grow until the O.D. reached 0.6. The cultures were induced with 1 mM IPTG and allowed to grow at 30^{o}C overnight. The resulting lysates were serially diluted and titred against the conjugates TCB-BTG and ABZ-BSA. There was no colorimetric signal observed on any of the plates. Following this observation, two clones TCB-E4 (round 3) and ABZ-D6 (round 4) were studied based on their binding profiles (absorbance values of above 0.5 and 0.7 respectively) in the initial monoclonal phage ELISA. Expression studies were performed as outlined in section 2.11.12. These studies showed that there was no expression at any time point after induction (1-6 hr and overnight) or at different temperature (25^{o}C or 30°C). It also verified the lysis method, either by freeze thaw or sonication does not affect the antibodies binding capabilities.

Plasmid preparations (section 2.10.1) of both clones were performed and they were transformed into TOP 10F´ and Rosetta cells. The presence of the gene of interest was confirmed by a colony pick PCR (Figure.4.5.9). Transformed colonies were selected and grown up and analysed as in section 2.11.12. There was no expression observed.

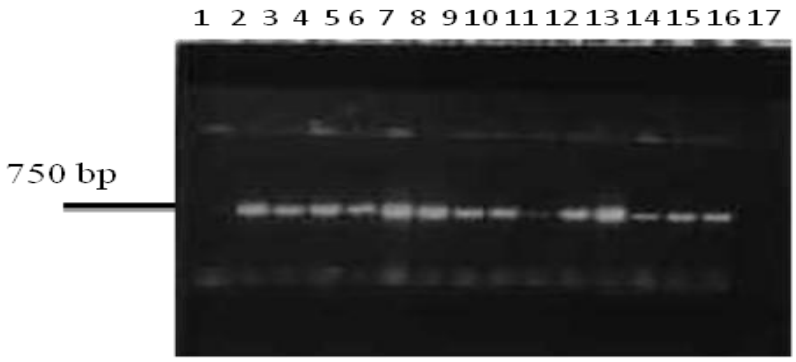

Figure 4.5.9: Colony pick PCR for colonies selected from the rosetta strain. Lanes 1 and 17: 1 Kb Sigma DNA ladder and lanes 2-16: positive gene inserts at 750 bps.

Finally, this library underwent construction twice more, followed by extensive rounds of panning. The stringency, elution and panning strategies were varied; however, no clones that secreted anti-TCB or anti-ABZ scFv were selected.

4.6 Discussion and conclusion.

The leghorn chicken gave a satisfactory immune response of over 1/100,000, indicating that the chicken was producing antibodies against TCB and ABZ. The TCB/ABZ specific IgY was purified from the egg yolk (5 mg/mL) and subsequently applied in the development of an assay for the detection of TCB and ABZ. Buffers for the optimum coating of the conjugates were investigated. TBS pH 8.0 and PBS pH 7.5 were selected for further coating of the conjugates. Following this, coating concentrations and optimal antibody dilutions were examined and it was found that for the conjugate TCB-BTG a coating concentration of 1,500 ng/mL and an antibody dilution of 1/2,500 was optimum. In relation to ABZ-BSA, the optimum conditions were 600 ng/mL for the coating concentration and the antibody dilution was 1/500. The pAb showed stability in PBS, pH 7.5, containing methanol (50 % (v/v)) ethanol (60 % (v/v)) and DMSO (~100 % (v/v)). This suggests that the antibody is capable of detecting the drug residues when extracted from the samples using these solvents. The Figure 4.3.13 shows that there is a loss of sensitivity when directly comparing the antibody in 5 and 50 % (w/v) either by titre or in the competitive assay format.

Using these optimised conditions for the assays the detection ranges of the assay were determined. A wide range of free TCB and ABZ concentrations between 3-2,500 ng/mL were prepared in PBS containing 50 % (v/v) methanol and assayed within the ELISA test systems. The antibody showed competition within this range for TCB, however, there was no displacement of the pAb with the free drug ABZ. The ABZ assay development was suspended for this reason and the work was carried forward with TCB alone.

The inter–assay precision was determined for TCB on five different days, analysed in triplicate with varying concentrations and different batches of free TCB. The reproducibility of the assay was proven by the coefficients of variation which are within regulatory limits of 20 %. The inter assay CVs range from 1.96-19.2 % respectively. At the lowest MRL of 100 µg/kg for TCB for bovine species the CV's are calculated as 11.43 % and 2.91 %. The IC_{50} of the assay is 25 ng/mL.

The analytical LOD was calculated by fortifying PBS containing 50 % (v/v) methanol with concentrations of free drug 0.5, 1.5, and 2.0 times the concentration of the lowest established MRL for TCB. Twenty blank samples of PBS and 50 % (v/v) methanol fortified with free

TCB at concentrations of 50,100,150 and 200 ng/mL were analysed and the LOD was determined as 25 ng/mL.

The TCB pAb showed high cross reactivity with TCB-SO and TCB-SO_2 of 100 and 97 % respectively. The reproducibility of the assay was again proven by inter-assay variation and the CV %, these ranged between 1.5-5.5 % and 0.12-6.13 % for TCB-SO and for 1- 7.13 % for TCB-SO_2. The CV's at the MRL of 100 µg/mL were 5.5 and 6.97 % for TCB-SO for TCB-SO_2 respectively. These findings for TCB and its metabolites indicate that a robust multi-analyte reproducible sensitive assay was developed from an avian IgY and may be employed for the detection of free TCB in meat and meat products.

Polyclonal antibodies have definite advantages over monoclonal as they can be generated much more rapidly, less expensively and with less technical skill than their recombinant counterparts. They are also more stable with regards to conformation as they can withstand high salt concentrations and a much broader pH range. However, recombinant antibodies do have their advantages, the principal of these being their homogenicity and consistency and the fact that they can be generated as a constant and renewable resource. In contrast pAb obtained with the same antigen from different animals may have different avidity and the quantity of the antibody is limited to the choice of host. Although pAbs may have better specificity as they are produced by a large number of B cell clones each generating antibodies to a specific epitope, the concentration and purity levels are higher for monoclonal and recombinant antibodies (Lipman, 1998). The main advantage for the generation of recombinant fragments is the ability to improve the avidity, affinity and sensitivity of the antibody resulting in an antibody with a much greater potential for detecting small drug residues at very low levels, much lower than their MRL. Therefore the decision of construct of an scFv library was made.

The mRNA extracted from the immunised chicken was used to synthesize cDNA that was then utilizes as the starting material for the amplification of the variable chains. Following purification, these chains were combined to form the scFv gene fragment. This was digested along with the pComb3xSS vector with the restriction enzyme *Sfi*1 and ligated together. Commercial XL1-Blue *E.coli* cells were used in the library transformation to enhance the chances of the ligated plasmid being transformed into the host cell. The library size achieved was high. The transformed library was panned against both the TCB-HRP and ABZ-HRP

conjugates. The elution methods used were enzymatic (trypsin) and competitive elution using the free TCB and ABZ. Varying the conjugate concentration throughout the panning process allows for more selective and sensitive scFv fragments to be selected. Eluting by the competitive method also allows for the selection of highly specific antibodies. The outputs achieved were high (table 4.5.1) thus indicating that enrichment was occurring throughout the process. Colonies were selected for screening from rounds three, four and five against the conjugates TCB-BTG and ABZ-BSA. Monoclonal phage Elisa's were performed and some positive colonies that were distinguishable from the back ground and negative control of an absorbance of 0.2 were selected for further characterisation. A titre was performed on all of the 15 positive clones. However, there was no signal when the antibody was detected with TMB. This was repeated and there appeared to be no specificity towards the conjugates or the carrier proteins.

To further investigate the clones TCB E4 and ABZ D6 that exhibited a high absorbance in the initial monoclonal ELISA expression studies were carried out as in section 2.11.2 yet no positive clones were obtained. Su *et al.* carried out studies on the optimisation of expression of an scFv in a number of different *E.coli* strains. Their scFv was constructed similarly to the TCB/ABZ scFv as it was fused to the linked His6 tag (0.66kDa), HA tag (0.99kDa), and pIII protein (22.44kDa) at the carboxyl terminal. The translational stop codon (UAG) was placed at the junction between the antibody and gIII protein sequences. When they expressed their scFv in the amber codon (UAG) suppressor strains ER2537, TG1, and XL1-Blue the stop codon suppressor strains do not recognize the stop codon on the transcript and allow the scFv-pIII fusion protein to be produced. This scFv-pIII fusion protein is insoluble and tends to accumulate in the host cells periplasmic compartment. In these studies it was shown that XL1-Blue was the optimal strain for scFv expression along with the aid of glycerol at an optimum concentration of 0.05 %. (Su *et al.,* 2003). As this strain was already used and there was no expression, plasmid preps were prepared and transformed into the soluble strain TOP 10F´. This strain has the F trait that is a self-transmissible, low-copy plasmid used for the generation of single-stranded DNA when infected with M13 phage; that contains a resistance marker to allow maintenance and will often carry the lacI and lacZΔM15 genotypes.

However, no expression was observed. According to Bukhtiyarova *et al.,* one of the most common reasons for poor protein expression of heterologous genes is codon usage bias between the expression host and the donor cDNA (avian). The effect of codon

incompatibility is especially pronounced in situations when several so-called rare codons happen to occur contiguously and close to the amino terminus of the cloned gene. It is generally believed that such an event can cause ribosome pausing or frameshifting and, possibly, lead to un-coupling transcription from translation and premature termination of the product (Bukhtiyarova *et al.,* 2004).

To possibly resolve this problem of expression, plasmid prep's were prepared and transformed into the rosetta cells strain. These cells were selected because they are designed to enhance the expression of eukaryotic proteins that contain the 6 rare codons, AUA, AGG, AGA, CUA, CCC, GGA d in *E. coli*. They also provide universal translation when compared to other *E.coli*. These cells are also derived from a lacZY mutant of BL21 gene that is under the control of a T7 promoter to enable precise control of expression levels using IPTG.
Again all colonies selected from the Rosetta strain for both TCB and ABZ contained the gene of interest. These were scaled up to 5 mL culture and induced. There was no expression again. These outcomes suggest that the scFv is folded in such a way that maybe the antigen sites are inaccessible. It could also be forming aggregates thus reducing its binding capacity. Another problem that can occur is that target molecules immobilized on a solid phase (immune-tube) by passive adsorption during bio-panning may have epitopes that are hindered due to the target molecules themselves or blocking proteins. Since a part of the target molecules on the solid phase are denatured by adsorption only a part of the target molecules adsorbed on the solid phase are effective for phage binding (Zhuang *et al.,* 2001).

Chapter 5

Development of rapid methods for the detection of Triclabendazole

5.0 Introduction.

This chapter gives a detailed account of the development of three methods for the detection of triclabendazole (TCB) in milk samples. The research encompassed the development of ELISA, BIAcore™-based displacement assays, and lateral flow immunoassays (LFIA) for the detection of TCB in milk. Working assays with desired performance characteristics (i.e. sensitivity and reproducibility) were validated for both ELISA and BIAcore™. This chapter also describes the fabrication of a novel LFIA using a TCB-GFP conjugate that allows for the rapid visualisation and quantification of TCB in milk samples.

5.1 Surface Plasmon resonance.

Surface Plasmon resonance (SPR) has facilitated the development of a wide range of applications for the monitoring of bio-molecular interactions in 'real-time'. Consequently this has lead to a number of advancements in various research areas such as drug screening, antibody development, cell signalling pathways and characterisation of the affinities, kinetics and thermodynamics of kinase proteins (Boozer *et al*., 2006). Surface Plasmon resonance is a charge density wave occurring at the interface between a metal and a dielectric (Liedberd *et al.,* 1995). When incident light is coupled with the metal interface at angles greater than the critical angle, the refracted light exhibits a sharp decrease in reflectivity owing to the resonant transfer of energy from the incident light to a surface Plasmon. The incident light wavelength, at which this resonance occurs, is dependent on the refractive index in the immediate vicinity of the metal surface. The binding of bio-molecules to this surface changes the refractive index resulting in a shift in SPR. The change in the SPR signal correlates to the binding of the molecules on the surface in 'real-time' (see schematic in Figure 5.1.1) (Liedberd *et al.,* 1995). This phenomenon is utilised in many biosensor formats, the most prominent of these being the Biacore™, which has led to extensive protein-protein interaction studies between immobilised bio-molecules and solution-phase analytes (Boozer *et al.,* 2006).

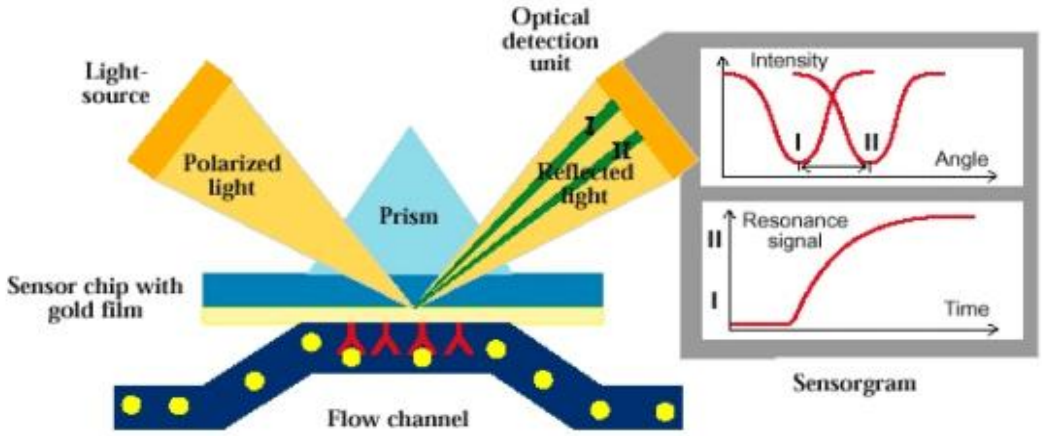

Figure 5.1.1**:** Schematic of the SPR phenomena used by the BIAcore™ system. SPR occurs when surface plasmon waves are excited at a metal/liquid interface. Light is directed at, and reflected from, the side of the surface not in contact with sample and SPR causes a reduction in the reflected light intensity at a specific combination of angle and wavelength. When analyte is passed over the chip surface it binds to the receptor resulting in a change in the mass of the surface layer, leading to a shift in the resonance angle (from I to II in the diagram). This change in signal is proportional to the amount of ligand bound to the receptor, which enables detection of a ligand and determination of the ligand–receptor interaction affinities and kinetics (Diagram taken from Thillaivinayagalingam *et al.*, 2010).

The use of SPR to study the interactions between antigens and antibodies offer many advantages over current techniques such as ELISA or fluorescence. Firstly, the detection of the analyte is 'label-free' and direct as the measurements are based on refractive index changes. Secondly, measurements can be performed in 'real-time' to allow collection of both kinetic and thermodynamic data. Lastly, SPR is an adaptable technique that enables the detection of a multitude of analytes over a broad range of molecular weights and binding affinities (Mc Donnell, 2001).

Using SPR, the Biacore™ system was launched in 1990 by Pharmacia Biosensor (Liedberg *et al.*, 1995). This system generally uses a thin gold surface that is chemically altered with carboxylated dextran to allow covalent coupling of the ligand, whilst eliminating non-specific binding (Lofa and Jonsson, 1990). The Biacore™ also has an integrated liquid handling system to transport samples and reagents to the adsorption and detection spot, combining this with SPR it enables the quantification of protein-ligand interactions (Sjolander and

Urbanicsky, 1991). The binding and dissociation of the analyte to the immobilised ligand is followed in 'real-time' and presented in a sensogram of response versus time. (Stenberg *et al.,* 1991).

Szabo *et al*. determined a basic experimental protocol for examining the binding interaction between two molecules using BIAcore™. This may be condensed down to a four step process that briefly includes: 1) immobilization or capture of one of the binding partners; 2) injection of the second binding partner and the recording of the 'real-time' reaction occurring; 3) studies are then performed using varying concentrations of the second binding partner, and 4) appropriate kinetic models and rate constants are determined dependant on each binding pair (Szabo *et al.,* 1995). This model is utilised across the board for a range of applications already highlighted and is also widely used for the development of assays for the detection of proteins, veterinary residues and controlled and banned substances within a range of matrices.

Although both ELISA and Biacore™ systems are recognized as quick and sensitive techniques for the detection of small drug residues, proteins and cancer markers, there is a significant increase in the development of other low-cost, rapid, qualitative diagnostic methods such as the lateral flow immuno-assay (LFIA). This method is a popular tool for diagnostics and identification of various analytes, and is widely applied to the medical arena, life science research, water and food monitoring, and illicit drug detection (Qian and Bau, 2004; Geertruida *et al.,* 2009).

5.2 Lateral Flow Immuno-assays (LFIAs).

LFIAs are one of the simplest, non-instrument and user-friendly format that produces results in a short time. They have vast commercial potential even though they are regarded as semi-quantitative or qualitative (Li *et al*., 2009). LFIAs essentially, are prefabricated strips of carrier material that contain a dry reagent that is activated when a fluid sample is applied. The format of a LFIA is analogous to the ELISA system. However, LFIA may be used on site by untrained personnel, for example, for the detection of high levels of progesterone in an agricultural environment (Posthuma-trumpie *et al*., 2009).

The principle of an LFIA is the movement of a sample, usually an extract containing the analyte of interest, along a porous membrane that consists of immobilized test and control ligands at pre-determined locations on the strip (Figure 5.2.1). The membrane is generally thin and fragile and is attached to a plastic layer for cutting and handling. LFIAs may be constructed from a number of materials including nylon, polyethersulfone and polyethylene (Posthuma-Trumpie *et al.*, 2009). However, a more popular choice is the use of nitrocellulose (Lonnberg *et al.*, 2001; Zhu *et al.*, 2002; Van dam *et al.*, 2004). The type of membrane is an important factor in LFIA development, as it affects the assay sensitivity, specificity and consistency (Xiang, 2009). Membranes have different pore sizes and capillary rates, which are the most significant factors that can affect the transport of the material up the strip (Henderson and Stewart, 2002). The capillary rate is the speed at which the sample moves along the strip and, thus, a membrane with a higher capillary rate requires a shorter time to complete the assay and vice-versa (Wen *et al.*, 2005).

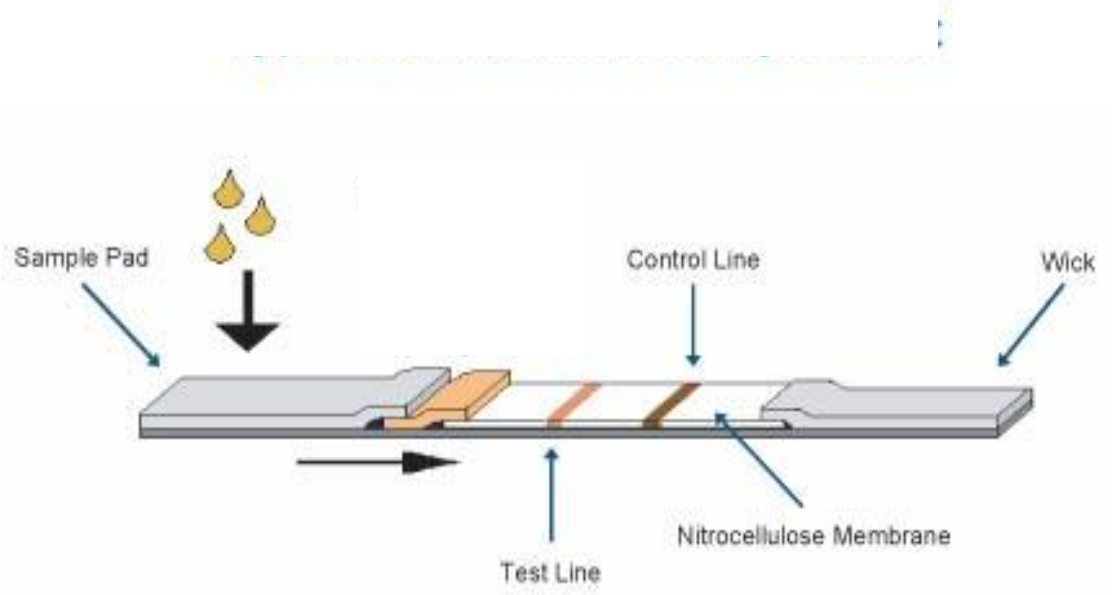

Figure 5.2.1: Schematic diagram of a LFIA device. The sample containing the analyte of interest is applied to the sample pad which may contain previously dried proteins, detergents and buffer salts. The test line consists of antibody, protein or hapten-conjugate immobilised onto the nitrocellulose strip. The control line may be made up of an anti-species enzyme-labelled antibody to indicate the completion of the assay. The absorbent pad consists of material e.g. cellulose filters that are used to wick the fluid through the membrane (Diagram taken from Gessler *et al.,* 2007).

The LFIA is designed to detect the presence or absence of an unwanted compound or analyte. Antibodies to the analyte are used as recognition elements for the assays. Varying formats are used for LFIA depending on the analyte of interest. When the analyte is a small molecule such as a hapten, which has only one epitope, the assay configuration is limited to the competitive format. However, if the analyte is a larger molecule, with more than one available epitope, the sandwich configuration is applied. The competitive assay format has two variations; i) the specific antibody is sprayed onto a test line and a mixture of sample analyte and labelled analyte is applied. Therefore, both analytes compete for binding to the test line (Laitinin, 1996; Ho and Wauchoppe, 2002; Wen *et al.*, 2005), and ii) the hapten-conjugate is sprayed onto the strip, and a mixture of the antibody and sample analyte is added to the conjugate pad (O'Keefe, 2003). In the situation where a labelled antibody is not available a secondary antibody may be applied, followed by the addition of substrate for the visualisation of the presence/absence of the analyte. The sandwich LFIA is created by preparing the test line with an analyte-specific antibody. The conjugate release pad contains a

second, labelled, anti-analyte antibody. The analyte in the sample will bind to the second antibody during the movement of the liquid up the membrane.

In this research the competitive approach was used for all assay formats due to the small size of the TCB hapten.

5.3 Results

5.3.1 Further development of an ELISA assay for the detection of TCB in spiked milk samples.

In chapter four, the anti-TCB pAb was used for the development of an ELISA assay for the detection of TCB in spiked buffer samples with a LOD of 25 ng/mL. The following section details the further development of the assay using TCB extracted from spiked organic milk.

Organic milk was spiked with TCB concentrations ranging between 1 μg/mL and 7 pg/mL. The TCB residues were extracted from the milk using two different methods, a) direct centrifugation (section 2.13.2); and b) a modified QuEChERs method (section 2.13.1) (Keegan *et al*., 2010). Briefly, QuEChERs was a method developed to streamline pesticide residue extraction providing analytical chemists with a quick, easy, cheap, effective, rugged and safe (QuEChERs) procedure (Wilkowska and Bizuik, 2011). The method includes a single phase extraction of sample with acetonitrile followed by liquid-liquid partitioning formed by the addition of anhydrous $MgSO_4$ and NaCl. The removal of residual water and clean-up are performed simultaneously by using a rapid procedure, called dispersive solid phase extraction (dSPE), whereby $MgSO_4$ and a primary amine sorbent (PSA) are mixed with the acetonitrile extract. The dSPE combined with the PSA effectively removes any polar matrix components such as organic acids, polar pigments and sugars (Anastassiades *et al.*, 2003).

Following the extraction of TCB from the milk using the QuEChERs and direct centrifugation methods, the residues were diluted and mixed with a 1/2,560 dilution of the anti-TCB pAb, and used in a competitive ELISA, as described in section 2.13.3. Both methods were directly compared, as shown in Figure 5.3.1.

The QuEChERs method was selected for future further extraction of TCB from organic milk samples as the graph indicates that this method yields a slightly more robust/effective extraction process. Following this the reproducibility and the precision of the assay were determined. Intra-day assays were carried out three times (three replicates per assay) on the same day using the same batch of extracted residue sample, anti-TCB antibody and diluents. Inter-day assays were performed using the same extracted residue sample analysed repeatedly over a period of days. The assay precision is represented by the percentage coefficient of variation (% CV) which is the standard deviation expressed as a percentage of the mean. Figure 5.3.2 represents the anti-TCB intra-day assay and table 5.3.1. contains the inter-assay data including percentage CV's, back calculated concentrations and percentage recoveries. The assay was evaluated using BIAevaluation 4.1 software and the data was fitted using a 4 parameter fit equation.

The LOB of the assay was determined by measuring the mean response of twenty blank samples that were extracted, evaporated and reconstituted and mixed with a 1/2,560 dilution of anti-TCB pAb. These samples gave an average absorbance of 1.47 and a standard deviation of 0.18 at an optical density of 450 nm. Three times the standard deviation was subtracted from the average absorbance, and the value was extrapolated from the calibration curve to give a LOD of approximately 609 pg/mL.

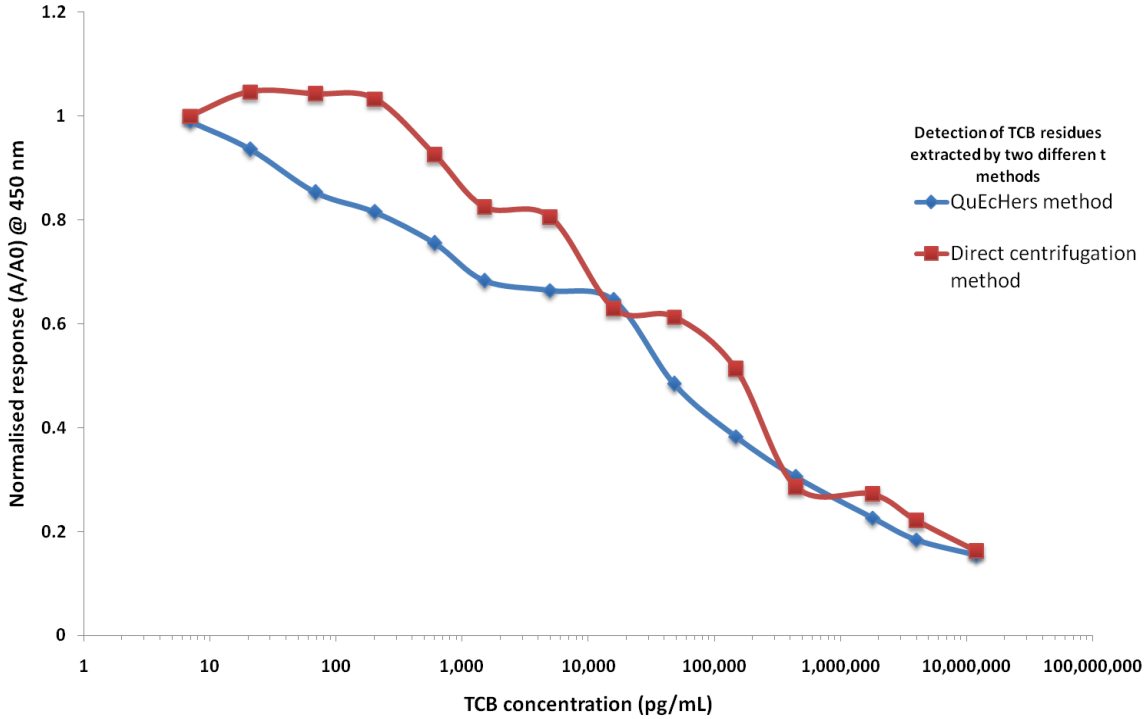

Figure 5.3.1**:** Comparison of methods for the extraction of TCB from spiked milk samples. Plates were coated with 1,500 ng/ml of TCB-BTG overnight and subsequently blocked with 5 % (w/v) BSA in PBS. Anti-TCB pAb (1/2,560) was mixed with reconstituted extracted TCB samples at concentrations ranging from 12,000-7 pg/mL and incubated for 30 min at RT. The detection antibody was HRP-labelled anti-chicken IgY.

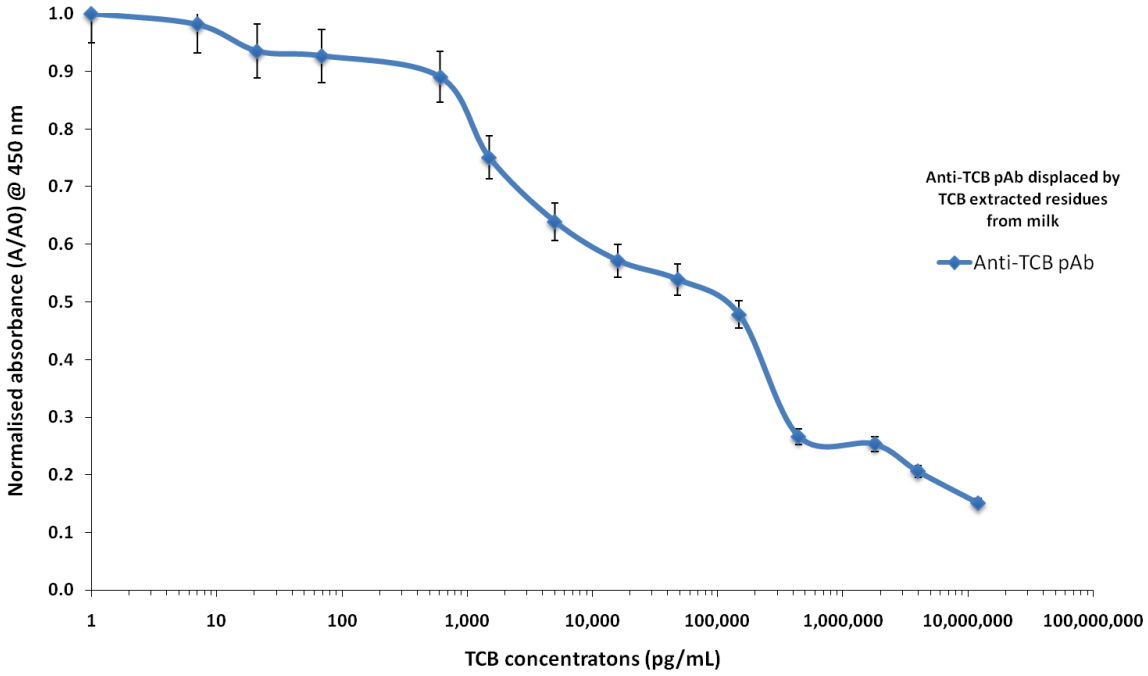

Figure 5.3.2: Inter-day assay curve for the determination of TCB in spiked milk samples. Plates were coated with a 1,500 ng/ml of TCB-BTG and subsequently blocked with 5 % (w/v) BSA in PBS. Anti-TCB pAb (1/2,560) was mixed with TCB reconstituted extracted samples with concentrations ranging from 12,000-7 pg/mL. The detection antibody was HRP-labelled anti-chicken IgY. The plotted results represent the mean response ± 1SD of three replicates.

Table 5.3.1**:** Tabulated values for TCB concentration (pg/ml) of 'spiked' milk samples, back calculated values, percentage coefficient of variation (% CV) and percentage recoveries (% recoveries) obtained for the anti-TCB pAb antibody inter-day assay in 'spiked' milk. Fifteen extracted TCB standards ranging between 12,000,000-7pg/mL were analysed in triplicate on three separate days.

TCB concentration spiked into organic milk (pg/mL)	Back calculated TCB (pg/mL)	Percentage recoveries	% CV
12,000,000	11,042,433	108	3
4,000,000	4,0906,250	97	5
1,800,000	1,733,562	103	12
444,000	436,158	101	12
148,000	140,501	105	1
48,000	44,502	108	17
16,000	14,224	112	14
5,000	5,378	93	2
1,500	1155	129	12
609	653	94	12
203	197	103	12
63	57	110	14
21	19.8	106	8
7	7.67	92	6

5.4 BIAcore™ assay development for the detection of TCB.

Following the successful anti-TCB ELISA development, a BIAcore™ competitive assay was generated for the automated rapid detection of TCB in spiked milk samples.

5.4.1 Pre-concentration studies for the immobilisation of TCB-BTG to a CM5 dextran chip surface.

Pre-concentration studies allow for the determination of the optimal buffer conditions for maximum binding of the ligand to the surface of an un-modified CM5 dextran chip. Briefly, under low ionic strength and a pH lower than the isoelectric point of the conjugated hapten, the protein will become electrostatically attracted to the surface matrix. The highest pH at which the protein is attracted to the surface is the chosen one. Experimentally, the conjugate

TCB-BTG was diluted in a 10 mM sodium acetate buffer with the pH ranging from 3.8. to 5.0. The pre-concentration study was carried out as in section 2.13.4, and is presented in Figure 5.4.1. The optimal pH chosen for the immobilisation of TCB-BTG to the chip was 4.0.

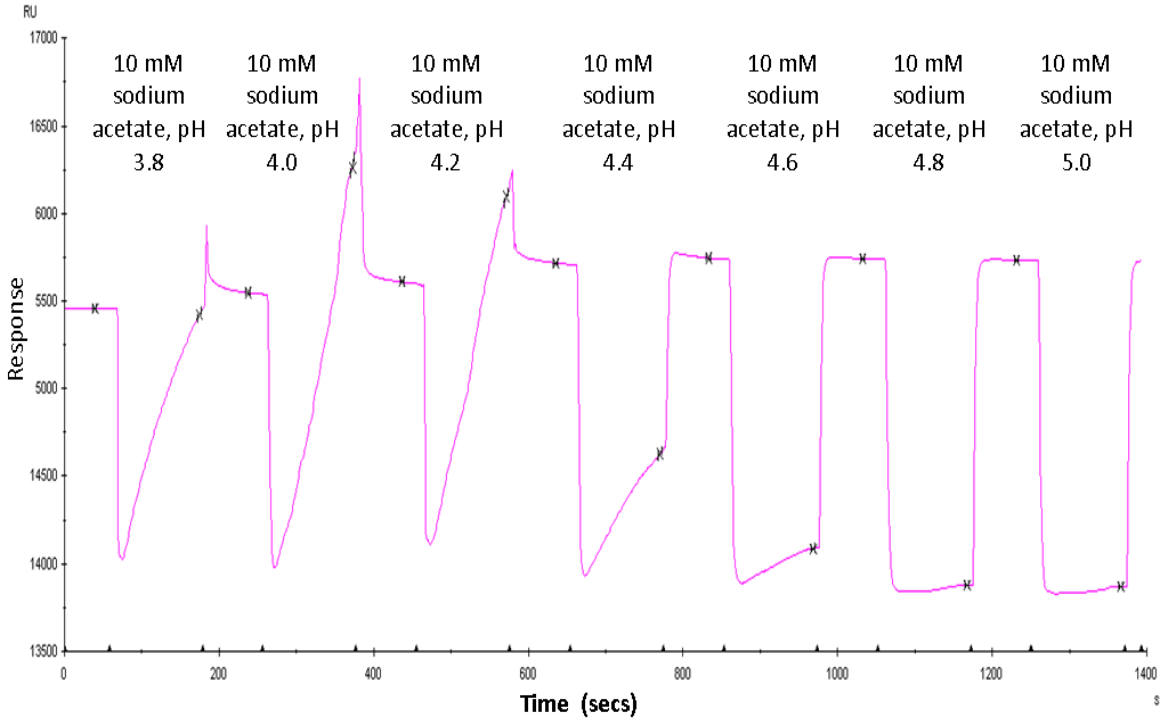

Figure 5.4.1: Pre-concentration studies for the immobilisation of TCB-BTG to the CM5 dextran chip surface. TCB-BTG was diluted to a concentration of 50 µg/mL in 10 mM sodium acetate solutions of pH 3.8, 4.0, 4.2, 4.4, 4.6, 4.8 and 5.0 and passed over the surface of a CM 5 dextran chip at a flow rate of 10 µL/min. The optimal pH for the immobilisation of the TCB-BTG CM5 dextran surface was pH 4.0, as the highest level of apparent binding was observed.

5.4.2 Immobilisation of conjugate TCB-BTG to the dextran chip surface.

The most frequently reported and widespread technique to bind proteins to a BIAcore™ chip is amine coupling. This direct coupling method allows for the binding of the protein via primary amine groups present in lysine residues, or at the n-terminus of the protein, to the chip surface (Thillaivinayagaingham *et al*., 2010). Immobilisation of the protein is carried out in three major steps. The first step is to activate the carboxymethyl groups with N-hydroxysuccinimide (NHS), thus creating a highly reactive succinimide ester which reacts with amine and other nucleophilic groups on proteins. The second, or coupling step, is to inject the protein in pre-concentration buffer, thereby achieving high protein concentrations

which drive the coupling reaction. The third and final stage is the blocking step, which blocks any remaining activated carboxymethyl groups by passing over the chip, high concentrations of ethanolamine. The ethanolamine aids in the elution of any non-covalently bound material (Biacore™ sensor handbook, BR-1005-71). The immobilisation of TCB-BTG to the chip is described in section 2.13.4 and is shown in Figure 5.4.2. Approximately 12,500 RU was immobilised on the dextran surface of flow cell number 2.

Following the successful immobilisation of the conjugate to the chip surface, regeneration studies were performed to establish the least harsh conditions to remove any bound antibody or sample and return the chip surface back to the baseline. Table 5.4.1 shows a list of regeneration solutions and their concentrations. The experiment was carried out as described in section 2.13.6 (Results not shown). The regeneration solution selected was 200 mM NaOH, at a flow rate of 10 μL/min.

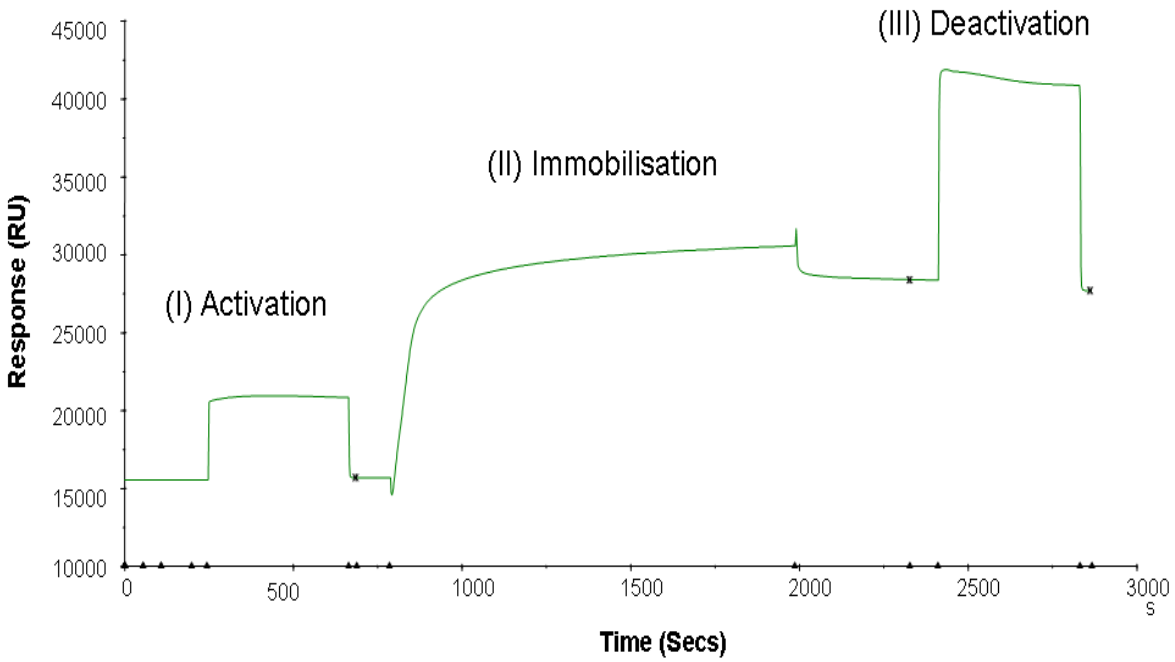

Figure 5.4.2: Immobilisation of the TCB-BTG conjugate to the surface of the CM5 dextran chip. HBS, pH 7.4, is initially passed over the CM5 chip surface. (I) the surface is the activated with a mixture of 0.05 M NHS/ 0.2 M EDC which results in an increase in SPR signal due to the bulk refractive index change; (II) a 50 µg/ml TCB-BTG-conjugate solution, diluted in sodium acetate buffer, pH 4.0, was passed over the activated surface and (III) NHS-esters are deactivated by an injection of 1 M ethanolamine, pH 8.5, over the surface.

Table 5.4.1: Regeneration solutions and their corresponding concentrations. Each buffer was passed over the chip surface at flow rates of 5, 10 and 20 µL/min.

Solution	Concentration (mM)
NaOH	10
	20
	50
	100
	200
HCl	5
	10
	20
Glycine, pH 2.0.	10
Glycine, pH 2.5.	10
Glycine, pH 3.0.	10
NaOH with 0.5 %(v/v) Acetonitrile	10
	50
	100
	200

5.4.3 Regeneration of the TCB-BTG CM5 chip surface

Following the successful immobilisation of the conjugate to the surface and the selection of a regeneration solution for the removal of bound material, an experiment was designed (section 2.13.7) to establish the optimal flow rate, contact time and anti-TCB antibody dilution to be used. The highest response (RU) obtained was 150 RU, using a 500 µg/mL anti-TCB antibody concentration and flow rate of 40 µl/min for 5 min (results not shown). Subsequently a 500 µg/mL anti-TCB antibody dilution was mixed with 1 µg/mL of free TCB, in HBS buffer, and passed over the immobilised chip using the optimised conditions. However, the antibody failed to be displaced by the addition of free TCB. TCB-BTG was immobilised to a new flow cell as the harsh conditions employed during the regeneration scouting may have degraded the modified chip surface. After re-immobilisation a

significantly low level of antibody binding and displacement by the addition of free TCB was observed. Following other immobilisation studies with different TCB conjugates a gluteraldehyde-linked TCB chip was donated by Teagasc, Ashtown (immobilisation chemistry currently IP protected). This chip was used for all further assay development.

5.4.4 Development of a BIAcore™ inhibition assay for the detection of TCB in spiked PBS.

Prior to the development of a BIAcore™ assay for the detection of TCB in spiked milk samples an antibody titre was performed and a working concentration of 40 µg/mL was determined (results not shown). For the generation of standard curves in buffer, samples were prepared by spiking PBS containing 50 % (v/v) methanol with concentrations of free TCB ranging from 1 µg/mL to 7 pg/mL. Samples were mixed with 40 µg/mL of anti-TCB pAb and passed over the TCB-glutaraldehyde-immobilised chip surface at a flow rate of 10 µl/min for 4 min. The surface was regenerated with 200 mM NaOH (section 2.13.8). Both intra- and inter-day assays (Figure 5.4.3) were carried out to determine the reproducibility and precision of the assay. The resulting data was plotted using BIAevaluation software (version 4.1) and four-parameter equations fitted to each data set for analysis. Table 5.4.2 shows the TCB concentration (pg/ml), back calculated values, % CV and percentage recoveries (% recovery) for the spiked buffer inter-day BIAcore™ assay.

The LOB of the assay was determined by measuring the mean response of twenty blank samples (137.5 RU) (40 µg/mL dilution of anti-TCB pAb and PBS containing 50 % (v/v) methanol in PBS) and subtracting three standard deviations (3 x 8.33), to give a response reading of 112.75 RU. This value was extrapolated from the calibration curve to give a LOD of 4 ng/mL.

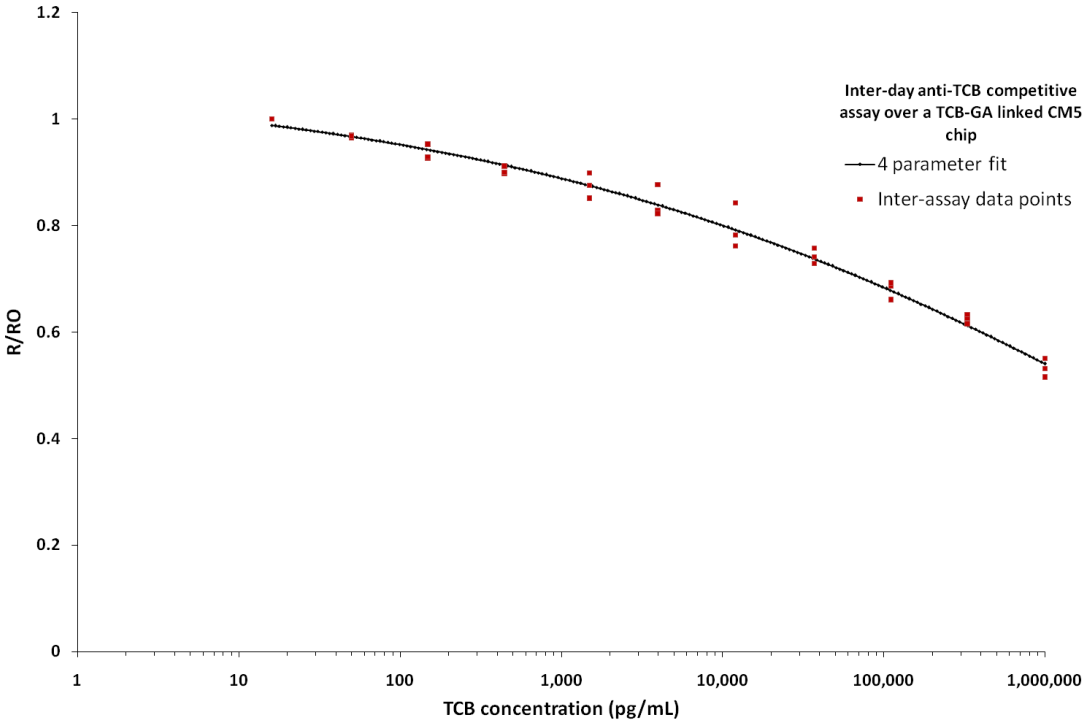

Figure 5.4.3: BIAcore™ inter-day assay for the detection of TCB residues extracted from spiked HBS. Eleven concentrations of TCB (1,000,000-7 pg/mL) were spiked into PBS containing 50 % (v/v) methanol, mixed with 40 µg/mL of anti-TCB pAb and passed across the TCB-glutaraldehyde-immobilised chip surface. A blank sample of PBS containing 50 % (v/v) methanol was also run for subtractive analysis. A 4-parameter equation calibration curve of the mean RU values was plotted against the TCB concentrations (pg/mL) on the x-axis (logarithmic scale) using Biaevaluation software 4.1.

Table 5.4.2: Tabulated values for TCB concentration (pg/ml) of 'spiked' PBS samples, back calculated values, percentage coefficient of variation (% CV) and percentage recoveries (% recoveries) obtained for the anti-TCB pAb antibody inter-day assay in 'spiked' PBS. Eleven extracted TCB standards were diluted in buffer ranging between 1,000,000-7 pg/mL and analysed in triplicate on three separate days.

TCB concentration spiked into organic milk (pg/mL)	Back calculated TCB (pg/mL)	Percentage recoveries %	% CV
1,000,000	1,202,171	120	3
333,000	254,737	76	1
111,000	107,828	97	2
37,000	25,278	68	1
12,000	15,829	128	2
4,000	4,089	99	1
1,500	1,659	121	1
456	502	110	2
150	181	119	2
50	70	140	1
16	11.5	120	2

5.4.5 Development of a BIAcore™ assay for the detection of TCB in spiked milk samples.

Following the successful generation of a spiked buffer assay for the detection of TCB, the method was further developed using spiked organic milk samples. The QuEChERs approach was again utilised for the extraction of TCB from spiked organic milk and the residues prepared as described in section 2.13.1. A BIAcore™ inhibition assay was performed, as described in section 2.13.5. The resulting data was plotted using BIAevaluation software (version 4.1) and four-parameter equations fitted to each data set for analysis (Figure 5.4.4). Table 5.4.3 represents the TCB concentration (pg/ml), back calculated values, % CV and percentage recoveries (% recoveries) for the spiked milk inter-day assay. The LOD of the assay was calculated, as previously described in section 5.4.4, and extrapolated from the curve to give a value of 203 pg/mL.

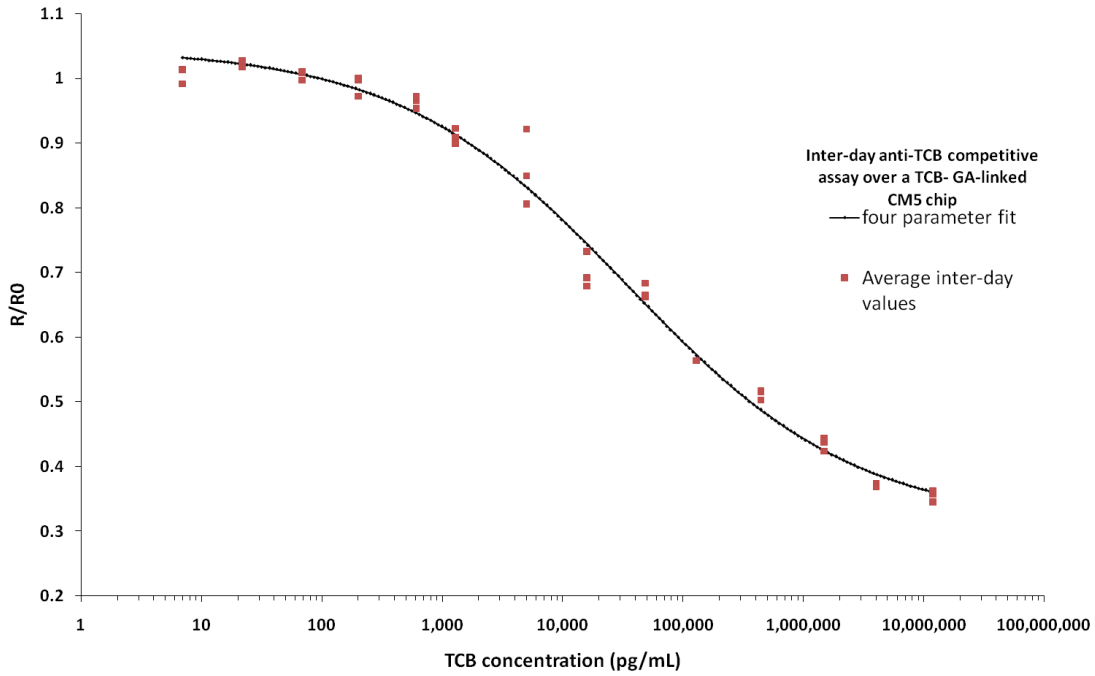

Figure 5.4.4: BIAcore™ inter-day assay for the detection of TCB residues extracted from spiked organic milk samples. Eleven concentrations of TCB were spiked into milk (12,000,000-7 pg/mL), extracted via the QuEChERs method and mixed with 40 µg/mL of anti-TCB pAb and passed across the TCB-glutaraldehyde-immobilised chip surface. A blank sample of un-spiked milk was also run for subtraction analysis. A 4-parameter equation calibration curve of the mean RU values was plotted against the TCB concentrations (pg/mL) on a logarithmic x-axis using Biaevaluation software 4.1.

Table 5.4.3: Tabulated values for TCB concentration (pg/ml) of 'spiked' milk samples, back calculated values, percentage coefficient of variation (% CV) and percentage recoveries (% recoveries) obtained for the anti-TCB pAb antibody inter-day assay in 'spiked' milk. Fourteen extracted TCB standards, 12,000,000-7pg/mL, were analysed in triplicate on three separate days.

TCB concentration in spiked milk (pg/mL)	Back Calculated TCB in spiked milk (pg/mL)	Percentage recovery (%)	% CV
12,000,000	11,042,473	109	2
4,000,000	4,096,250	98	9
1,800,000	1,733,562	104	9
444,000	693,516	64	3
148,000	140,501	105	14
48,000	44502	108	11
16,000	155534	103	1
5,000	5378	93	10
1,500	1577	95	9
609	853	71	10
203	175	106	12
69	57	121	10
21	22	95	6
7	7.7	91	3

5.5 Development of a LFIA for the detection of TCB in spiked milk samples.

ELISA and BIAcore™ assays have proven to be highly sensitive assay platforms for the detection of anti-parasitic drugs. However, because of the long incubation (ELISA), contact and run-time (BIAcore™) a LFIA-based method for the rapid detection of TCB was developed. Preliminary assays were performed to provisionally determine if the TCB metabolite could be detected using the LFIA format.

A competitive assay format was employed whereby the conjugate, TCB-BTG, was coated as the test line on the nitrocellulose strips. Samples were prepared which contained a mixture of free TCB and anti-TCB pAb. In the LFIA assay the free TCB will bind to the anti-TCB pAb

added to the samples and this in turn will prevent this antibody binding to the test line on the strip. The greater the amount of free TCB in the sample the smaller the amount of antibody that can bind to the TCB-BTG conjugate on the test line. i.e. there is an inverse relationship of concentration and intensity of signal at the test line. Any antibody binding to the test line will be detected using a HRP-labelled anti-chicken antibody following reaction of the HRB label with the substrate, TMB. Following the successful optimisation and development of the proof-of-concept for the LFIA, TCB was conjugated to Green Fluorescent Protein (GFP) for the development of a novel LFIA for the rapid visualisation and quantitation of the TCB residues in milk samples.

5.5.1 LFIA optimisation

Preliminary experiments were performed to optimise several components of the assay which included antigen coating/spotting and sample running buffer (section 2.13.10). Table 5.5.1 illustrates the combinations of coating and running buffers used for the optimisation of the LFIA (results not shown). It was established from these experiments that the most favourable conditions for the assay was combination D. A 50 mM sodium phosphate, pH 7.5, buffer was used for the washing of the LFIA strip between the application of the sample and HRP-labelled anti-chicken antibody.

Throughout this LFIA development the control used was an anti-MPO avian scFv that was previously generated and purified 'in-house'. The detection antibody was a HRP-labelled anti-chicken antibody (1/1,000 dilution) and the corresponding substrate was TMB, unless otherwise stated. Once the optimal coating, running, and wash buffers were established an initial experiment was carried out to determine the optimum concentration for the coating of TCB-BTG to the nitrocellulose strips (results not shown). Following this, an experiment was performed to determine the optimum antibody concentration to be used throughout the assay development as described in section 2.15.1. However, as shown in Figure 5.5.1, the control line was not as pronounced in comparison to the test line, therefore, further experiments were required to determine the optimal concentration of anti-MPO for the control line (2.15.2). Ultimately the optimal concentration of anti-MPO scFv that gave a corresponding clear line was 72 µg/mL (Figure 5.5.2). Further optimisation was carried out using different concentrations of anti-TCB pAb with 100 µg/mL chosen for further development of the LFIA.

Table 5.5.1: Buffer combinations for the development of the TCB LFIA,

Combination	Coating Buffer	Sample/Running buffer
A	Borate + (0.1 % (w/v) BSA, 0.05 % (v/v) Tween, 0.01 % (v/v) Triton X)	Borate + (0.05 % (v/v) Tween)
B	Borate + (0.1 % (w/v) BSA, 0.05 % (v/v) Tween, 0.01 % (v/v) Triton X)	50mM sodium phosphate, pH 7.5, + (0.05 % (v/v) Tween)
C	Borate + (0.1 % (w/v) BSA, 0.05 % (v/v) Tween, 0.01 % (v/v) Triton X)	50mM sodium phosphate, pH 7.5.
D	50mM sodium phosphate, pH 7.5. + (1 % (w/v) trehalose)	50mM sodium phosphate, pH 7.5, + (0.05 % (v/v) Tween)
E	50mM sodium phosphate, pH 7.5. + (1 % (w/v) trehalose)	50mM sodium phosphate, pH 7.5.

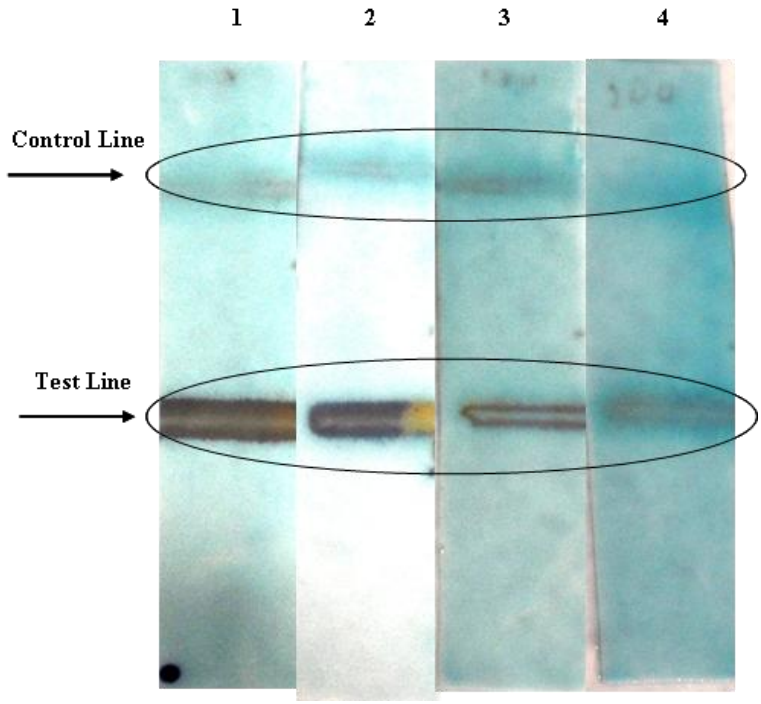

Figure 5.5.1: Developed nitrocellulose membrane strips from the LFIA antibody optimisation experiment. Test lines were sprayed on the nitrocellulose strips (1-4) using the conjugate TCB-BTG at a concentration of 1,500 ng/mL in coating buffer. A control line, consisting of 50 µg/mL chicken anti-MPO scFv in coating buffer, was also sprayed on each strip. Antibody dilutions were prepared in running buffer at concentrations ranging from 10-100 µg/mL and applied to their corresponding strips. A HRP-labelled anti-chicken antibody was used for detection. Test lines: Strip 1: 100 µg/mL of anti-TCB IgY; Strip 2: 50 µg/mL of anti-TCB IgY; Strip 3: 20 µg/mL of anti-TCB IgY; and Strip 4: 10 µg/mL of anti-TCB IgY. Control line: Strips 1-4: 50 µg/mL of anti-MPO scFv.

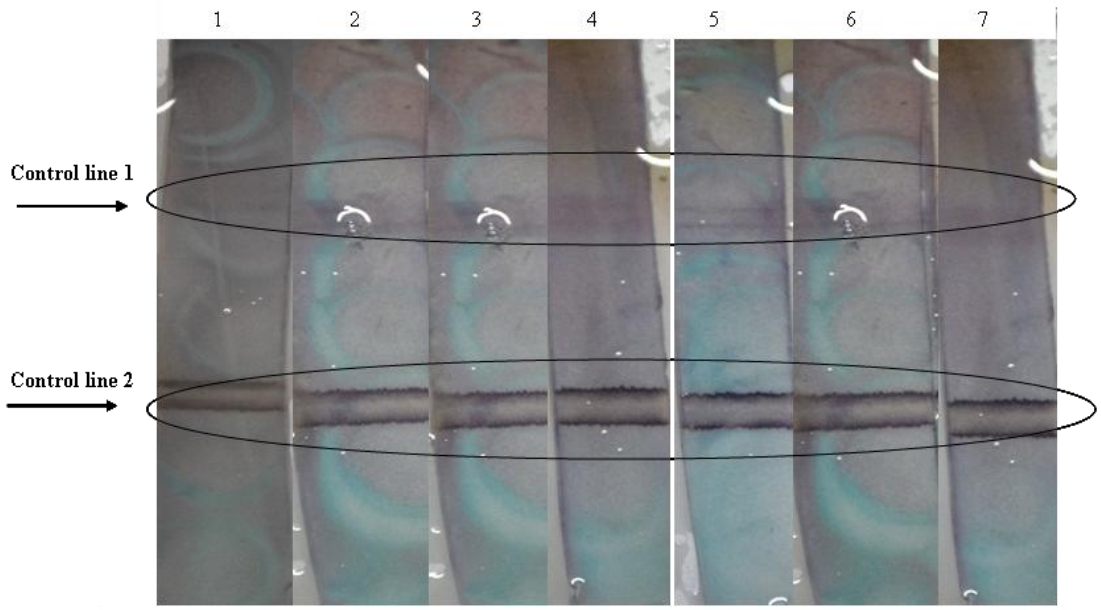

Figure 5.5.2: Developed nitrocellulose membrane strips from the LFIA control line antibody coating optimisation experiment. Two control lines were sprayed on the nitrocellulose strips (1-7) using different concentrations of chicken anti-MPO scFv ranging from 600-2 µg/mL in coating buffer. A HRP-labelled anti-chicken IgY antibody was used for detection. Control line 1: Strip 1: 1 µg/mL of anti-MPO; Strip 2: 2 µg/mL of anti-MPO; Strip 3: 4 µg/mL of anti-MPO; Strip 4: 8 µg/mL of anti-MPO; Strip 5: 16 µg/mL of anti-MPO; Strip 6: 24 µg/mL of anti-MPO; Strip 7: 36 µg/mL of anti-MPO. Control line 2: Strip 1: 72 µg/mL of anti-MPO; Strip 2: 142 µg/mL of anti-MPO; Strip 3: 200 µg/mL of anti-MPO; Strip 4: 300 µg/mL of anti-MPO; Strip 5: 400 µg/mL of anti-MPO; Strip 6: 500 µg/mL of anti-MPO and Strip 7: 600 µg/mL of anti-MPO scFv.

5.5.2 Development of a competitive LFIA for the detection of TCB.

Following the successful optimisation of the concentrations and dilutions required for the control and test lines, the assay was further developed for the detection of TCB in running buffer and finally, following extraction, from spiked milk samples. Nitrocellulose strips and samples were prepared, and the assay performed, as described in section 2.15.3. It was established that TCB can be detected format down to 100 ng/mL of what (Figure 5.5.3), using a competitive LFIA Following this confirmation, organic milk (Glenisk) was spiked TCB and the residues were extracted, as per the modified QuEChERs method described in section 2.13.1. The samples were prepared and the LFIA performed, as in section 2.15.3. An inverse relationship was observed between the band strength and the concentration of TCB, where a clear pattern was evident between the addition of a sample containing a high concentration of

TCB and the band intensity. A distinct band was observed for the ‘negative control’ strip, where the sample contains no analyte, further validating the sensitivity and specificity of the LFIA. This assay has the capability to quantitatively detect TCB residues between 50 and 100ng/mL, which is below the EU recommended MRL of 100 ng/mL (Figure 5.5.4 and 5.5.5).

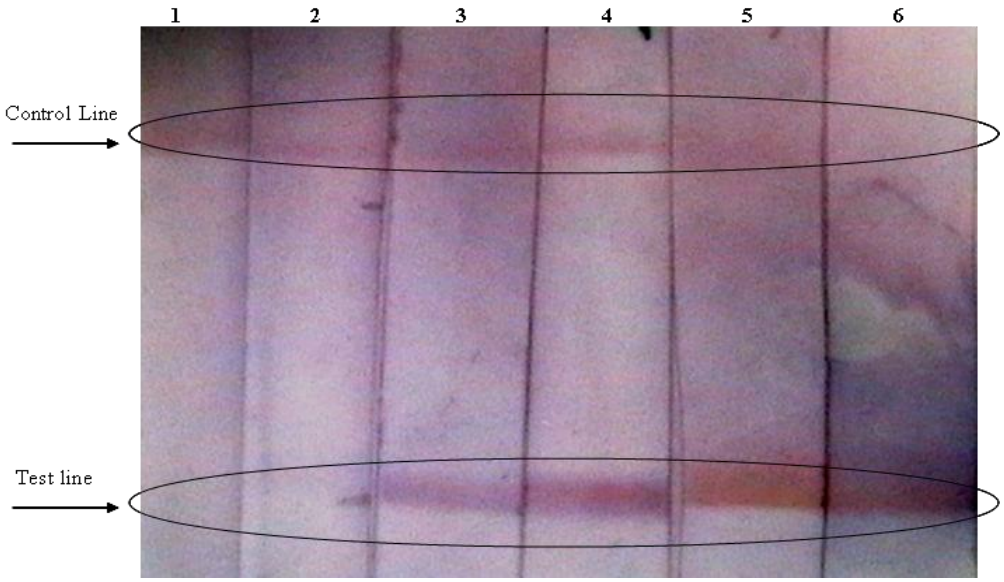

Figure 5.5.3: Developed nitrocellulose membrane strips from the LFIA competitive assay for the detection of TCB in spiked buffer. Test lines were sprayed on the nitrocellulose strips (1-6) with a 1,500 ng/mL concentration of the conjugate TCB-BTG in coating buffer. A control line, consisting of 72 µg/mL chicken anti-MPO scFv in coating buffer, was also sprayed on each strip. All strips were blocked with 50 mM sodium phosphate buffer, pH 7.5, containing 5 % (w/v) milk marvel. PBS containing 50 % (v/v) methanol was spiked with concentrations of TCB ranging from 25-500 ng/mL, mixed with 100 µg/mL of the chicken anti-TCB IgY and applied to the bottom of the strip where the sample flowed up via capillary action. A HRP-labelled anti-chicken IgY was used for detection. Test lines: Strip 1: sample containing 500 µg/mL of free TCB; Strip 2: sample containing 250 µg/mL of free TCB; Strip 3: sample containing 100 ng/mL of free TCB; Strip 4: Sample containing 50 ng/mL of free TCB; Strip 4: Sample containing 25 ng/mL of free TCB; Strip 6: Negative control (sample containing no TCB). Control line 1-6: 72 µg/mL of the anti-MPO scFv.

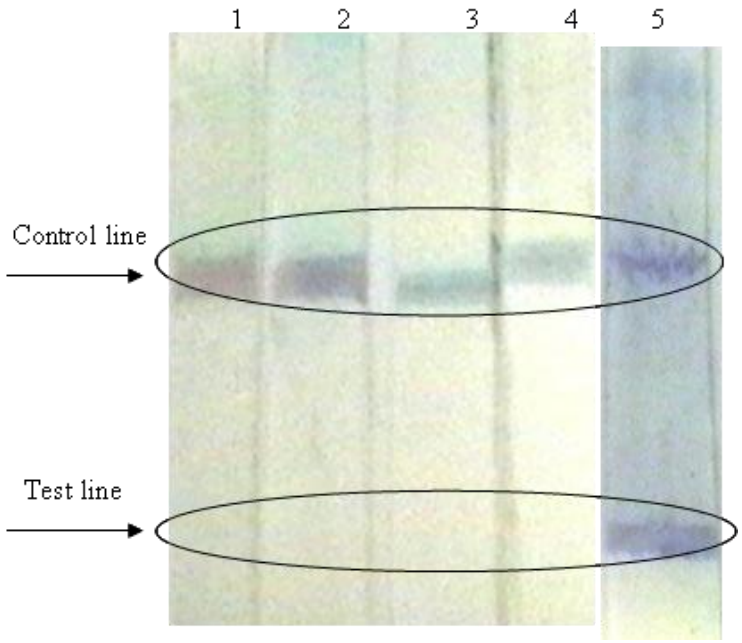

Figure 5.5.4: Developed nitrocellulose membrane strips from the LFIA competitive assay for the detection of TCB in spiked milk. Nitrocellulose strips (1-6) were sprayed at the test line with a 1,500 ng/mL concentration of the conjugate TCB-BTG in coating buffer. Each strip was also sprayed with a control line consisting of 72 µg/mL anti-MPO scFv in coating buffer. All strips were blocked with 50 mM sodium phosphate buffer, pH 7.5, containing 5 % (w/v) milk marvel. Milk samples were spiked with concentrations of TCB ranging from 100-1000 ng/mL and extracted as per the QuEChERs method. The resulting residues were mixed with 100 µg/mL of the chicken anti-TCB pAb IgY and applied to the bottom of the strip where the sample flowed up via capillary action. A HRP-labelled anti-chicken IgY was used for detection. Test lines: Strip 1: sample containing 1 µg/mL of free TCB; Strip 2: sample containing 500 ng/mL of free TCB; Strip 3: sample containing 250 ng/mL of free TCB; Strip 4: sample containing 100 ng/mL of free TCB; Strip 5; Negative control (sample containing no TCB). Control line: Strips 1-5: 72 µg/mL of the anti-MPO scFv.

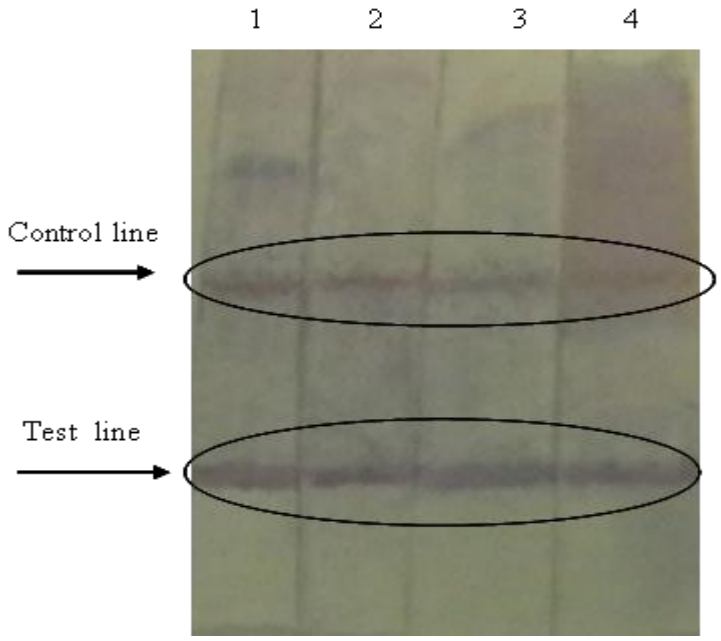

Figure 5.5.5: Developed nitrocellulose membrane strips from the LFIA competitive assay for the detection of TCB in spiked milk. Test lines were sprayed on the nitrocellulose strips (1-5) with a 1,500 ng/mL concentration of the conjugate TCB-BTG. A control line, consisting of 72 µg/mL chicken anti-MPO scFv in coating buffer, was also sprayed on each strip. All strips were blocked with 50 mM sodium phosphate buffer, pH 7.5, containing 5 % (w/v) milk marvel. Milk samples were spiked with concentrations of TCB ranging from 5-50 ng/mL and extracted as per the QuEChERs method. The resulting residues were mixed with a 100 µg/mL of the chicken anti-TCB pAb IgY and applied to the bottom of the strip where the sample flowed up via capillary action. A HRP-labelled anti-chicken IgY was used for detection. Test lines: Strip 1: sample containing 50 ng/mL of free TCB; Strip 2: sample containing 25 ng/mL of free TCB; Strip 3: sample containing 5 ng/mL of free TCB; Strip 4: Negative control (sample containing no TCB). Control line: Strips 1-5: 72 µg/mL of the anti-MPO scFv.

5.6 Development of a LFIA incorporating the conjugate TCB-BTG.

Following the successful development of a LFIA for the detection of TCB, a novel LFIA was generated which employed a conjugated green fluorescent protein (GFP) to TCB for the visualisation of TCB in milk samples. This conjugation was carried out by first reducing the GFP with tris-[2-carboxyethyl]-phosphine hydrochloride (TCEP) followed by the covalent conjugation of the TCB-GFP with the heterobifunctional cross-linker sulfo-GMBS. Next the

LFIA was developed integrating the GFP conjugate for the rapid detection of TCB in extracted milk samples.

5.6.1 Conjugation of GFP to the hapten TCB.

TCEP was used for the reduction of disulphide bonds in GFP for the conjugation of TCB via sulfo-GMBS chemistry. Briefly, TCEP is a highly effective agent for the reduction of disulfide bonds in proteins, peptides and other disulfide bond-containing molecules over a wide pH range (Reugg and Rudinger, 1977). It is a moderately stable compound and does not undergo rapid oxidation which often occurs with other reagents such as β-mercapthoethanol or dithiothreitol (DTT). The reduction of the GFP by TCEP is described in section 2.15.4.

Following successful reduction of GFP, the conjugation was carried out via sulfo-GMBS chemistry. Sulfo-GMBS is a heterobifunctional cross linker that contains N-hydroxysuccinamide (NHS) ester and maleimide groups that facilitate covalent conjugation of amine and sulfydryl-containing molecules. NHS esters react with amines at pH 7.9, to form amide bonds while the maleimide reacts with the sulfydryl groups at pH 6.5-7.5, to form stable thioether bonds. Sulfo-GMBS is used in a two step scheme for the conjugation of hapten-carrier protein conjugates. Firstly the amine-containing protein (GFP) is reacted with a several-fold molar excess of the cross linker (Kitagawa *et al.*, 1981). This is followed by the removal of excess reagent by de-salting or dialysis and, finally, the sulfydryl-containing molecule (TCB) is added to the reaction to react with the maleimide groups already attached to the first protein (GFP) (Section 2.15.5).

5.6.2 GFP-TCB LFIA development

Following the conjugation of GFP to TCB a LFIA was developed. In this format there was competition between the unlabelled TCB and the TCB labelled with GFP in solution for binding to the immobilised anti-TCB pAb IgY. The test lines (approximately 1.5 cm from the bottom of the strip), on the nitrocellulose paper were coated with 100 µg/mL dilution of anti-TCB pAb in 50mM sodium phosphate, pH 7.5, + (1 % (w/v) trehalose) coating buffer. The control line used throughout this study was an anti-GFP mAb, supplied by Sigma-Aldrich. The control lines (approximately 3.5 cm from the bottom of the strip) on the nitrocellulose paper were coated with 10 µg/mL of an anti-GFP mAb in 50mM sodium phosphate, pH 7.5, + (1 % (w/v) trehalose) coating buffer. Running buffer consisting of 50mM sodium

phosphate, pH 7.5, + 0.05 % (v/v) Tween was spiked with concentration of TCB ranging between 5-500 ng/mL and added to a 1/5 dilution of the conjugated TCB-GFP in a ratio of 1:3. Samples were also prepared by spiking milk with concentrations of TCB ranging between 5-500 ng/mL which were subsequently extracted via the QuEChERs method. The resulting TCB residues were diluted with a 1/5 dilution of the conjugate TCB-GFP in a 1:3 ratio prior to application to the strip (section 2.15.6). The strips were visualised using a Typhoon 8600 variable mode fluorescence imager at excitation and emission wavelengths of 488 nm and 507 nm, respectively (Figure 5.6.1). This figure shows that the response is negatively correlated to the analyte concentration i.e. the more TCB present, less fluorescence is observed and where there is little or no analyte present a higher signal is detected, this proof of concept assay indicates that this LFIA has the capability to detect TCB in milk extracts between 50 and 100 ng/mL.

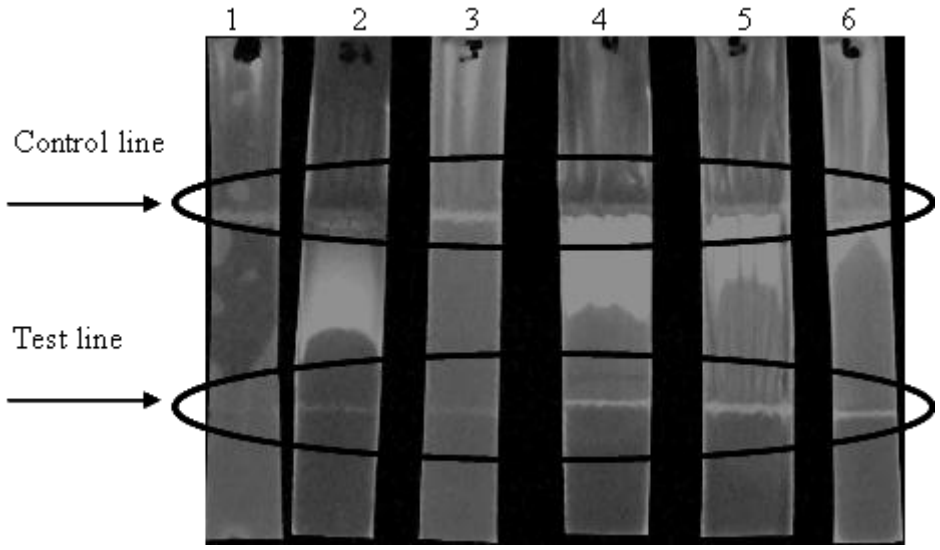

Figure 5.6.1: Novel lateral flow assay for the detection of TCB using conjugated TCB-GFP. Test lines were sprayed on the nitrocellulose strips (1-5) with 100 µg/mL of chicken anti-TCB pAb in coating buffer. A control line, consisting of 10 µg/mL anti-GFP mAb in coating buffer, was also sprayed on each strip. Milk samples were spiked with concentrations of TCB ranging from 5-500 ng/mL, and extracted via the QuEChERs method. The resulting TCB residues were mixed with a 1/5 dilution of the conjugate TCB-GFP prior to application to the strip. Test lines: Strip 1: sample containing 500 ng/mL of free TCB; Strip 2: sample containing 100 ng/mL of free TCB; Strip 3: sample containing 50 ng/mL of free TCB; Strip 4: sample containing 25 ng/mL of free TCB; Strip 5: 5 ng/mL of free TCB; Strip 6: Negative control (sample containing no TCB). Control line: Strips 1-6: 10 µg/mL of anti-GFP mAb.

5.7 Discussion

This chapter gives a detailed description of the development of three separate methods (ELISA, BIAcore™ and LFIA) for the detection of TCB in spiked milk samples.

During the further development of the ELISA assay, two different methods were used for the extraction of TCB from spiked milk samples, direct centrifugation and a modified QuEChERs method. As shown in Figure 5.3.1 the QuEChERs method was revealed to be the more robust extraction procedure of the two. This was the extraction process used throughout the course of this research. The residues remaining subsequent to extraction and evaporation were re-constituted and assayed to determine the within (intra) and between day (inter) precision. The % CV at the low end of the curve for both 7 and 21 pg/mL was 6 %. This is similar for the high end of the assay where the % CV's are 3 and 5 % for 12,000,000 and 4,000,000 pg/mL respectively. There is a higher level of imprecision for the linear portion of the curve with % CV's as high as 17 % at a concentration of 48,000 pg/mL. According to legislation this % CV is still acceptable for assays detecting veterinary residues and may be expected for inter assays as more variability is being introduced. The % recoveries for this assay were between 92-129 %. According to EU legislation acceptable limits of single recovery should normally be in the range 60–140 %. Recoveries outside of this range however, usually require re-analysis of the batch between and within assay. http://ec.europa.eu/food/plant/resources/qualcontrolen.pdf. As previously discussed the LOD of the ELISA assay was determined to be 609 pg/mL, 164 times below the recommended MRL of 100 ng/mL for TCB.

Following the ELISA assay development, a BIAcore™ assay was generated for the automated detection of TCB. The method was initially developed using PBS containing 50 % (v/v) methanol spiked with varying concentrations of TCB, following the success of this procedure this platform was also used for the detection of TCB in spiked milk samples.

Originally BIAcore™ dextran CM5 chips were immobilised with ~50 µg/mL of TCB-BTG in sodium acetate buffer pH 4.0, giving an approximate RU of 12,500. A high concentration of approximately 500 µg/mL of antibody was required to give a low response of approximately 100 RU. Passing varying concentrations of free TCB over the flow cell failed to displace the antibody, therefore it was deemed that the chip had become degraded. The chip was re-immobilised with TCB-BTG and again with TCB-HRP at a number of different

concentrations. These chips appeared to be unstable after numerous regenerations with 200 mM NaOH. Upon receipt of the donated TCB gluteraldhyde linked chip, buffer recalibration curves were generated and inter and intra-assays performed. In buffer, the anti-TCB pAb had a LOD of 4 ng/mL. The precision was low across the full range of the assay with % CV's ranging between 0.44-3.02 %. Recoveries varied throughout the assay reaching as high as 140 % at a concentration of 50 pg/mL. However, as already discussed this is still an acceptable % recovery for veterinary drug residues from milk and meat matrices. For the extracted residues from spiked milk samples the LOD was 203 pg/mL. The % CV's for this assay were significantly higher in comparison to that of the buffer assay. The low end % CV's for 21 and 7 pg/mL were 6 and 3 % respectively, however, when observing the linear range of the assay these were notably higher and reached a % CV of 15 % at 148,000 pg/mL. This indicates that there was substantial variation introduced during the inter day assays. The % recoveries of the assay ranged between 71 and 121 %.

A number of BIAcore™ assays have been developed for the detection of benzimidazoles extracted from real meat and milk samples. Keegan *et al.* generated a BIAcore™ screening assay for the detection of the parent molecules ABZ, FBZ, FLU, MBZ and OXI and their corresponding metabolites from incurred milk samples. This work was carried out using a sheep pAb raised against methyl 5 (6)-[(carboxypentyl)-thiol]-2-benzimidazole carbamate protein conjugate and had a LOD of 2.7 ng/mL and a detection capability of 5 ng/mL for all eleven benzimidazoles reported (Keegan *et al.*, 2009). In a different study they describe the development of two BIAcore™ assays for eleven 11 benzimidazole carbamate (BZT) and four amino-benzimidazole veterinary drug residues in liver tissue. The BZT assay was reported to have an LOD of 32 ng/mL and a detection capability of 50 ng/mL (Keegan *et al.*, 2011). There have been no publications to support the generation of a BIAcore™ assay for the detection of TCB in any matrix.

In this research the development of a LFIA using an enzyme labelled antibody for the detection of TCB in milk samples was described. The detection capability of the assay was approximately 75 ng/ml which is below the recommended MRL of 100 ng/ml. In membrane based assays enzyme labels are often used as the colour produced by the substrate conversion can be detected visually, however, this can be difficult to quantify (Bonenberger and Doumanas, 2006). Therefore, a fluorescent LFIA was developed employing a TCB-GFP conjugate which was prepared via sulfo-GMBS chemistry. Coating the anti-TCB pAb onto

the nitro-cellulose surface allowed for the capture of the TCB-GFP. Mixing samples containing TCB with the TCB-GFP conjugate and applying this to the strip allowed for the competition between the conjugated and free TCB. The more TCB in the sample the less binding sites available for the TCB-GFP on the strip surface, therefore the less florescence emitted and vice versa. Using a Typhoon 8600 variable mode fluorescence imager we were able to quantify that the LFIA assay has a detection capability of 75 ng/mL (Figure 5.6.1).

There is no research currently published for the development of an LFIA for the detection of TCB. However, Smidova *et al*. reported the development of an LFIA for the detection of TBZ in fruit juice. Using a carbon black labelled secondary anti-mouse antibody they were able to detect TBZ down to 0.001 mg/kg, below the current MRL which is currently set at 5 mg/kg (Smidova *et al.,* 2009). Additionally, colloidal gold labels are currently a popular choice for researchers due to their emission of vivid colour caused by localised SPR and excellent chemical stability (Liu *et al.*, 2011; Guo *et al.,* 2009). This approach has been successful for the detection of many chemical analytes such as the sulphonamides, clenbuteral, kanamycin, and toxins such as Aflatoxin B1, aflatoxin B2 and ochratoxin (Ngom *et al.*, 2010). Other labelling techniques are also being explored, such as fluorescent latex beads, liposome containing fluorescent of bioluminescent dyes, magnetic particles and raman active tags (Posthuma-Trumpie *et al.*, 2009; Song and Knotts, 2008). This novel TCB-GFP LFIA is not without its drawbacks, even though it is a rapid method for the quantification of TCB in acetonitrile extracted samples the complex and expensive fluorescence imagery required for its detection will limit the market for such testing systems.

Chapter six

Overall conclusions

6.1 Overall conclusions

The aim of the work described in this research was the generation of polyclonal and recombinant antibodies for the detection of benzimidazoles. Antibodies selected were characterized by ELISA and SDS PAGE and implemented into the development of ELISA, BIAcore™ and LFIA assays.

Chapter three describes the generation of an anti-TBZ Fab using RNA isolated from an anti-TBZ antibody secreting hybridoma cell line. The antibody library was constructed using methods and primers based on the studies by Barbas *et al.* (2001). Anti-TBZ Fab clones were isolated by phage display and colonies secreting the anti-TBZ Fab (thirteem) were screened and selected via ELISA. Clones E6, D2 and F7 demonstrated an affinity to TBZ and were expressed in large-scale, purified using IMAC and the purity verified by SDS-PAGE. The clone E6 was characterised and chosen for all further studies. A competitive assay was developed for the detection of TBZ in buffer containing 5% (v/v) methanol. Intra- and inter-day studies evaluated the assays reproducibility and precision. The limit of detection of the anti-TBZ assay was approximately 2.5 ng/mL, forty times below the recommended MRL for TBZ.

The second phase of the research in chapter three encompassed the modification of the pComb3xTT vector. The vector was designed to alter the valency of the antibody thus increasing functional affinity therefore increasing the sensitivity of the assay to which the antibody is applied. The dHLX gene was amplified from the pAk500 vector and inserted into the pComb vector system using specifically designed primers and the restriction enzymes *BspE*1 and *BsiW*1. This vector allowed for dimerization to occur, assembly *in-vivo* in *E.coli*, and the secretion of the dimerized antibody fragment into the periplasmic space. HPLC results indicate that dimerisation occurred when fusing the Fab fragment to the double helix motif thus resulting in an enhanced avidity.

Chapter four describes the generation of an avian polyclonal antibody raised against the conjugate TCB-BTG. A leghorn New Zealand chicken was immunized with the conjugates TCB-BTG and ABZ-HRP. Initial serum titres showed a significant response to both conjugates. Anti-chicken IgY was purified from the yolks of the eggs obtained during the immunization period and the purity was verified by SDS PAGE. Experiments were carried out to determine the optimal conditions for coating the optimisation studies were performed

to ascertain the most favourable conditions for the conjugate to the ELISA plate surface and the selection of an antibody dilution for the detection of TCB and ABZ. Cross reactivity studies and initial competitive assays were performed for both TCB and ABZ. However, the pAb could not be displaced by ABZ indicating that the antibody was not ABZ specific. Assay development was carried forward for TCB only. The affect of solvents on the antibodys performance was established and a competitive assay was developed for the detection of TCB in PBS containing 50 % (v/v) methanol. The limit of detection of the assay was determined as 25 ng/mL, four times lower than the recommended MRL for TCB.

Another aspect of the research described in chapter four was the construction of an avian scFv library from RNA extracted from the previously immunized chicken. A large library size was obtained and bio-panned using both competitive and enzymatic elution techniques. Colonies selected were grown in 5 mL cultures and the lysates screened for binding activity. No binding occurred and the clones were subjected to a colony pick PCR where the 750 bp confirmed the presence and the successful ligation of the gene fragment into the pComb vector. The vector containing the gene fragment was transformed into other *E.coli* strains to aid the soluble expression of the scFv. However, when colonies were selected, grown in 10 mL cultures, no binding between the lysate and the TCB/ABZ conjugates occurred. These outcomes suggest that the scFv is folded in such a way that maybe the antigen sites are inaccessible or that it may be forming aggregates thus reducing its binding capacity.

The studies in chapter five describe the further development of an ELISA for the detection of TCB in spiked milk samples. Milk was spiked with concentrations of free TCB ranging from 12,000,000-7 pg/mL, and extracted using a modified QuEChERS method. The resulting residues were reconstituted in PBS containing 50 % (v/v) methanol and analysed by ELISA. The limit of detection of this assay was approximately 609 pg/mL. This is significantly lower than the limit of detection of the assay in which TCB in spiked buffer samples was detected, and is also 164 times lower than the recommened MRL.

Following the success of the ELISA assay for the detection of extracted TCB residues from milk, a Biacore™™ assay was developed. The conjugate TCB-BTG was immobilized to a CM5 dextran chip via amine coupling. However, initial studies showed that the anti-TCB pAb could not be displaced from the surface by passing high levels of TCB over the flow cell. Other attempts at chip generation proved unsuccessful and a gluteraldehyde linked TCB chip was donated by by Dr Martin Danaher of Teagasc, Ashtown. A Biacore™ inhibition

curve was generated for the detection of TCB in PBS containing 50 % (v/v) methanol with a limit of detection of 4 ng/mL. Following this, milk was spiked with TCB ranging from 12,000,000-7 pg/mL and extracted via the QuEChERS method. An inhibition assay was generated, and the precision and reproducibility was evaluated by performing intra and inter-day studies. The limit of detection of the assay was 209 pg/mL.

Two LFIAs were developed, one that was analogous to a competitive ELISA and one incorporating a conjugated TCB-GFP molecule. The former LFIA was developed by coating anti-TCB pAb to the nitrocellulose surface, applying samples that contain various concentrations of TCB to strip, and detecting the presence of TCB with a HRP labeled anti-chicken IgY. This proof of concept assay allowed for the detection of TCB im spiked milk samples between 50-100 ng/ml and may be used as a confirmatory assay for the presence of TCB in samples. Following the success of this assay development, TCB was conjugated to GFP via Sulfo-GMBS chemistry. Nitrocellulose was again coated with the anti-TCB pAb. Mixing samples containing TCB with the TCB-GFP conjugate and applying this to the strip allowed for the competition between the conjugated and free TCB. The amount of TCB was measured via fluorescence and the detection capability of this assay was between 50-100 ng/mL.

Chapter 7

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7.1 Bibliography

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EU Directives:

EMEA/CVMP/865/03 2004: Committee for medicinal products for veterinary use Albendazole.

EMEA/CVMP/868, 2004: Committee for medicinal products for veterinary use: Thiabendazole.

EMEA/CVMP/320386, 2004. Committee for medicinal products for veterinary use Triclabendazole.

Chapter 8

Appendix

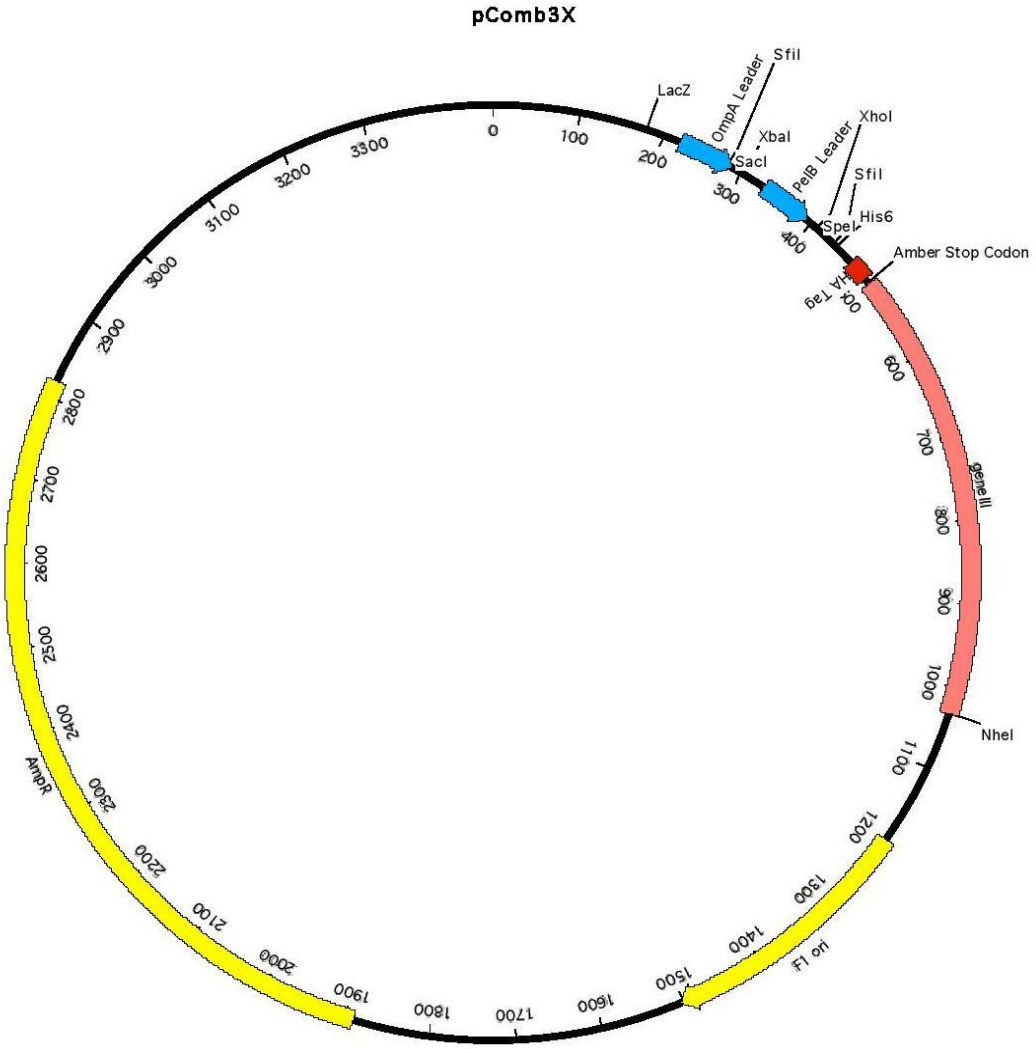

Figure 8.1: Vector map of the plasmid pComb3X used in this research for the phage display of recombinant antibody fragments (Barbas *et al*., 2001).

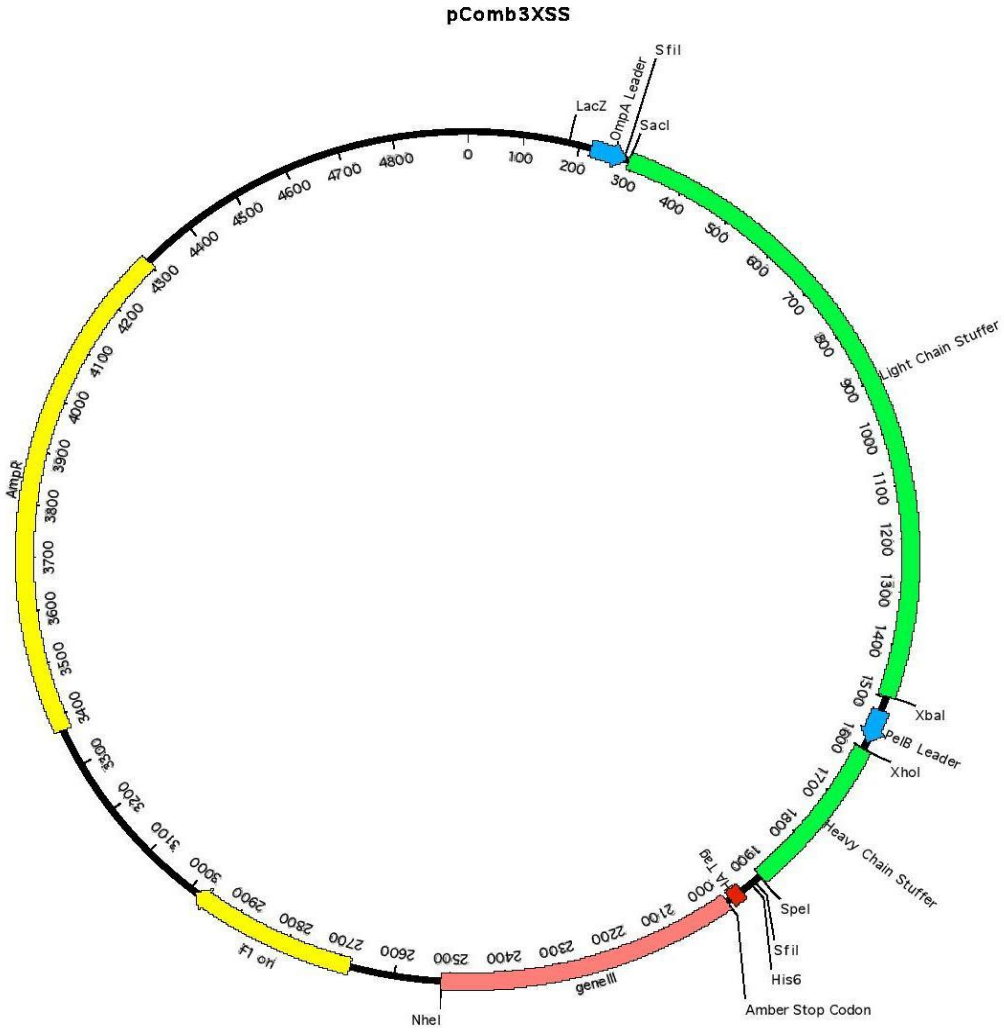

Figure 8.2: Vector map of the plasmid pComb3XSS used in this research for the phage display of recombinant antibody fragments (Barbas *et al*., 2001).

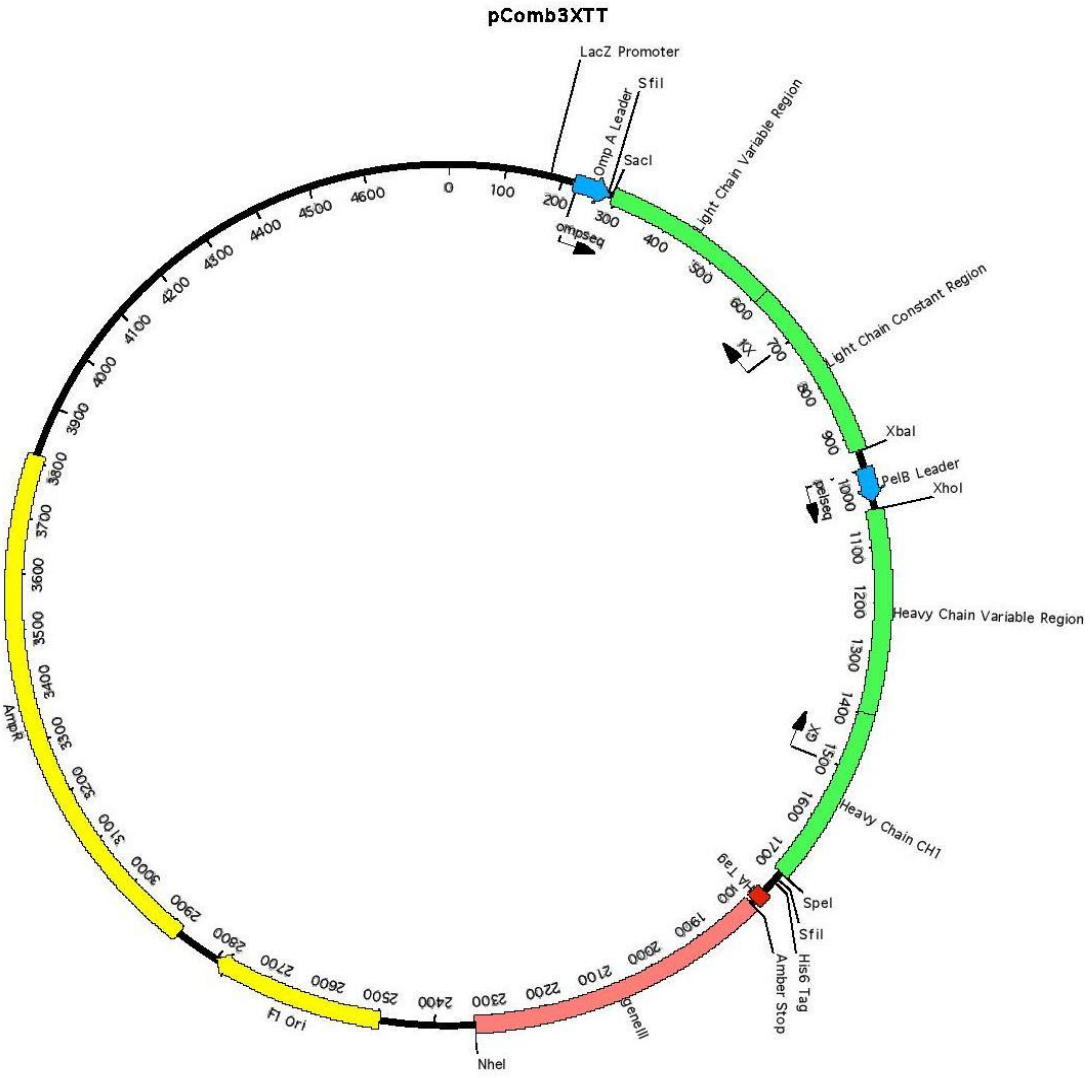

Figure 8.3: Vector map of the plasmid pComb3XTT used in this research for the phage display of recombinant antibody fragments (Barbas *et al*., 2001).

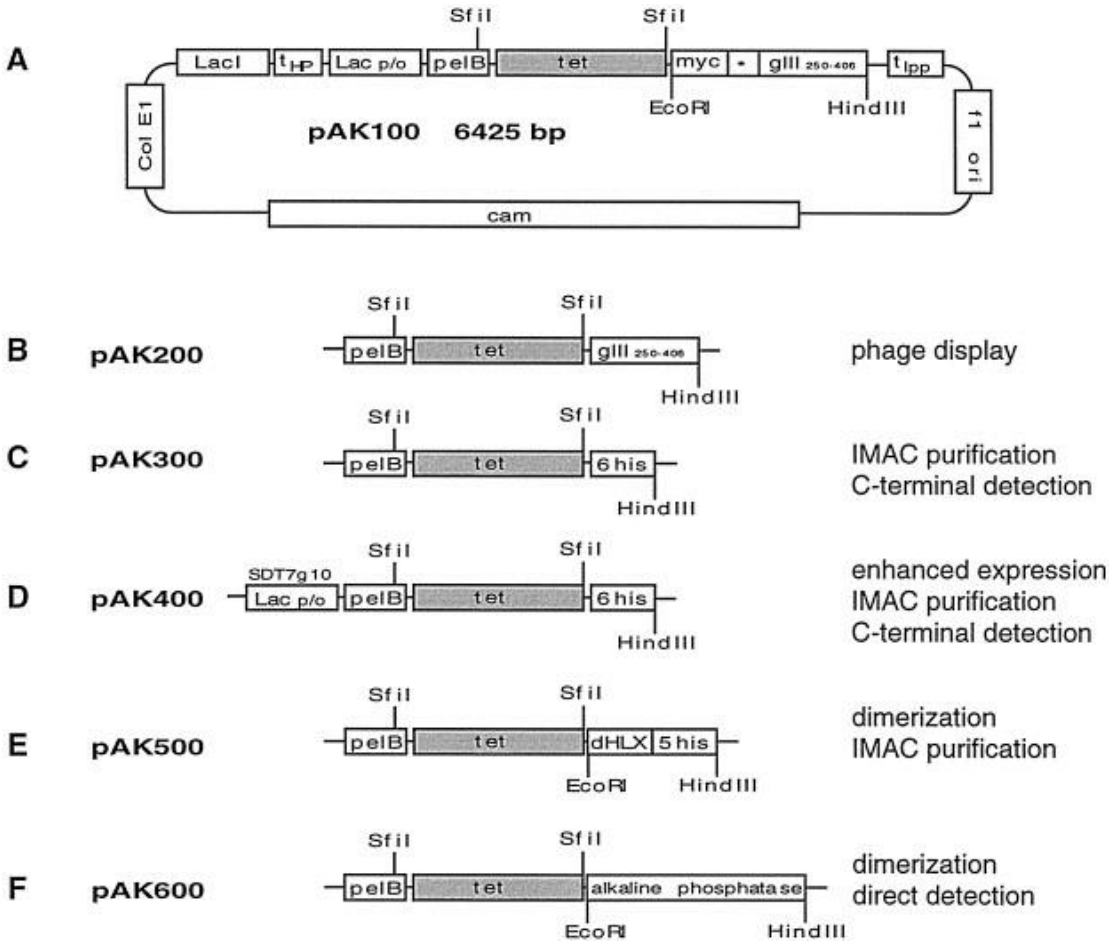

Figure 8.4: Vector maps of the pAK plasmid series. The pAK 500 (E) was used for the isolation of the DHLX gene fragment for the modification of the pComb vector as described in chapter 3.6 (Krebber *et al*., 1996).