Rapid Methods for the Detection of Anti-Protozoan Drug Residues.

A thesis submitted for the degree of Ph.D.

By

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Declaration

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

Signed: _____ (Candidate) ID No.: _____ Date: _____

This thesis is dedicated to my parents, Cathy and Tony, for their endless help and support, their constant encouragement and unconditional love.

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Abbreviations

Ab	Antibody
Abs	Absorbance
Ag	Antigen
AP	Alkaline phosphatase
BIA	Biomolecular interaction analysis
bp	Base pairs
BSA	Bovine serum albumin
BTG	Bovine Thyroglobulin
Cam	Chloramphenicol
CCα	Decision Limit
CCβ	Detection capability
cDNA	Complementary DNA
cfu	Colony forming units
CDR	Complementary determining region
CH_1	Constant heavy chain 1
CH_2	Constant heavy chain 2
CH_3	Constant heavy chain 3
CL	Constant light chain
CM	Carboxymethylated
conc.	Concentration
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	Diclazuril
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidyl triphosphates
EC	European Commisson
E.coli	*Escherichia coli*
EDC	N-ethyl-N'-(dimethylamiopropyl) carbodiimide
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Medicines Agency

EU	European Union
Fab	Antibody binding fragment
Fc	Constant region of an antibody molecule
FCA	Freund's Complete Adjuvant
Fv	Variable binding region of an antibody
HBS	HEPES buffered saline
HFG	Halofuginone
HRP	Horse radish peroxidise
HSA	Human Serum Albumin
IgG	Immunoglobulin class G
IMAC	Immobilised metal affinity chromatography
Kan	Kanamycin
Log	Logarithmic
mRNA	Messenger RNA
MW	Molecular weight
n	Refractive index
NEB	New England Biolabs
NHS	N-hydroxysuccinimide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline/Tween
PEG	Polyethylene glycol
pH	Log of the hydrogen ion concentration
RI	Refractive index
RT	Room temperature
RU	Response units
scFv	Single chain Fv antibody derivative
SDS	Sodium dodecyl sulphate
SOE	Splice by overlap extension
SPR	Surface plasmon resonance
Tet	Tetracycline
TLZ	Toltrazuril
TMB	Tetramethylbenzidine dihydrochloride

UV	Ultraviolet
V_H	Variable region of heavy chain of antibody
V_L	Variable region of light chain of antibody

Units

µg	microgram
kDa	(kilo) Daltons
µL	microlitre
µM	micromoles
^oC	degrees Celcius
cm	centimetres
g	grams
h	hours
kg	kilogram
L	litre
m	metre
M	molar
mg	milligram
min	minute
mL	millilitre
mm	millimetres
nM	nanomolar
pg	picograms
rpm	revolutions per minute
RU	response units
sec	seconds
v/v	volume per unit volume
w/v	weight per unit volume

Publications and Outputs

Publications

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, *Anal. Biochem.*, **410 (1)**, 27-33.

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Darcy, E., Leonard, P., **Fitzgerald, J.**, and O'Kennedy, R. (2010), Purification of antibodies using affinity chromatography. Accepted as an experimental design chapter in *Protein Chromatography: Methods and Protocols*, part of the *Methods in Molecular Biology* book series. Edited By D.Walls and S. Loughran, Humana Press Inc., Totowa, NJ, USA.

Presentations

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Conference Publications

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Stapleton, S., Dunne, L., **Fitzgerald, J.,** Bruneau, J., and O'Kennedy, R., (2008), Detection of Aflatoxins using Recombinant Antibody Fragments in Biosensor-based Assays. Poster presented at: Europtrode IX, Dublin, Ireland, and 30th March -2nd April.

Abstract

This research describes the development of immunoassays for the detection of residues of three anti-protozoan drugs (coccidiostats), namely, halofuginone, toltrazuril and diclazuril, used in the treatment of *Eimeria spp.* infections in cattle, pigs, chickens and turkeys.

An avian-derived halofuginone-specific scFv library was constructed, from which the best scFv isolated did not possess the required analytical sensitivity. To circumvent this, genetic engineering via light-chain shuffling of the optimal light: heavy chain pairings of the scFv library were investigated. Use of this light-chain shuffled library and rigorous screening, led to the isolation of a scFv with a 185-fold greater sensitivity than the original. This scFv was incorporated into both enzyme-linked immunosorbent assay (ELISA) and Biacore assay formats that were applied successfully for the detection of halofuginone in 'spiked' egg samples.

Immune phage display libraries were constructed and screened for diclazuril using a number of diclazuril-conjugates. A functional diclazuril-specific scFv could not be isolated, due to complications which arose during library amplification. Subsequently, an anti-diclazuril polyclonal antibody was sourced externally, characterised and used in the development of a high sensitivity lateral-flow immunoassay for diclazuril detection in eggs.

Rabbit polyclonal antibodies to the primary metabolite of toltrazuril, 'ponazuril', were produced, characterised and were subsequently used to generate ELISA, lateral-flow and Biacore-based assays. The performance of each of these assay formats for the detection of toltrazuril in 'spiked' egg samples was directly compared.

Finally, multi-coccidiostat detection in a lateral-flow dipstick format, for the simultaneous determination of halofuginone, diclazuril and toltrazuril in eggs, using antibody-coated coloured latex particles as the detection element, was successfully optimised.

Chapter 1

Introduction

1.1 Coccidiosis: prevalence and life-cycle of *Eimeria species*

Protozoa are unicellular eukaryotes which feed heterotrophically. They constitute their own "kingdom" according to taxonomic classification. Coccidian parasites are protozoa in the subphylum *Apicomplexa* (**Figure 1.0**). Coccidiosis is a disease caused by intracellular parasites of species in the genera, *Eimeria, Isospora, Caryospora, Cryptosporidium, Atoxoplasma, Sarcocystis* and *Toxoplasma* (Douglas *et al.*, 1995). **Figure 1.0** illustrates the taxonomic positions of the genera of the seven coccidian species mentioned above. Coccidian species are generally small micro-organisms ranging from 2 μm to 1 mm in diameter, primarily present in livestock (Douglas *et al.*, 1995). They cause diseases in several species of animals, which in turn has severe economic implications.

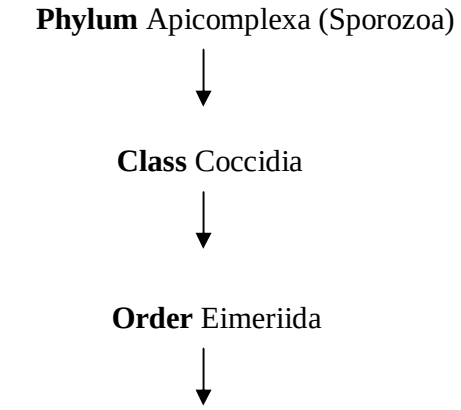

Figure 1.0 *Taxonomic positions of the genera of Eimeria spp. Class Coccidia are the largest group of Apicomplexan protozoa. The Order Eimeriidia comprises numerous genera in which there are many protozoa of medical and veterinary importance (Barta, 1989).*

In the phylum Apicomplexa, most protist possess a unique organelle called an apicoplast and an 'apical complex' structure, which is involved in the entry of the host cell. The invasion of host cells and parasite multiplication generally relies on this 'apical complex' set of structures (Barta, 1989). **Figure 1.1** illustrates the structure of an Apicomplexan parasite. The apical complex labelled in **Figure 1.1** is made up of the conoid which is a hollow structure of hundreds of fibres arranged in a spiral, a band of microtubules called polar rings, and subpellicular microtubules, which are involved in the stabilising cell boundary complex or pellicle (Scholtyseck *et al.*, 1970).

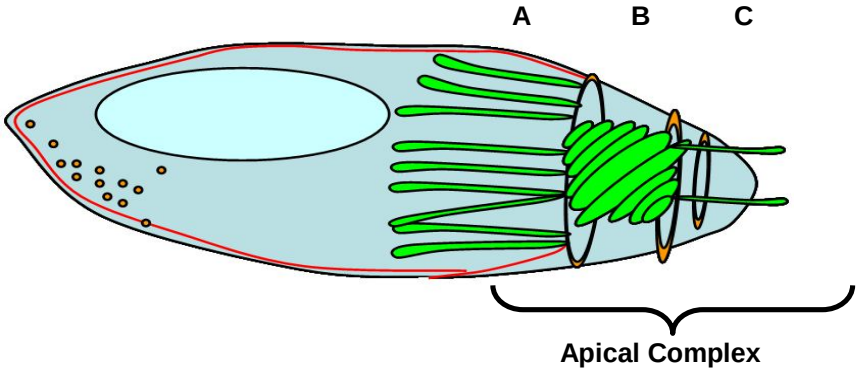

Figure 1.1 *Diagram of an Apicomplexan parasite showing the apical complex. The central part of the apical complex is the conoid (B), which is made of hundreds of fibres arranged in a spiral. The apical complex also consists of polar rings (C) and subpellicular microtubules (A). This structure acts like a drill bit during host cell invasion. The apical complex can retract and extend, and also secrete enzymes that aid in attachment and penetration of the host cell. Illustration adapted from Hu et al.* (2006).

In previous studies it was suggested that if all animals were examined, 45,000 species of *Eimeria* would be identified (Douglas *et al.*, 1995). The life-cycles of *Eimeria* species occur both exogeneously and endogenously and generally include three different phases, namely; sporogony, merogony and gametony. The exogenous stage (gametony), retaining the oocysts, is where infection occurs (**Figure 1.2**). These oocysts are encased in a thick-walled shell, making them impervious to even the harshest environmental conditions. Coccidia are generally transmitted via ingestion of contaminated food or water, primarily from faeces of infected animals. Parasitised

animals shed environmentally resistant oocysts in their faeces which undergo sporogony exogenously (Jeurissen *et al.*, 1996). Sporulation produces four sporocysts with two sporozoites each. Only oocysts containing fully formed sporozoites can infect animals. After ingestion, the oocysts encyst in the small intestine releasing infective sporozoites. The sporozoites subsequently infect epithelial cells and undergo asexual proliferation (formation of schizonts). After two to five schizont generations, the sexual proliferation stage, or the formation of male and female gametes, occurs. Following this, fertilisation occurs, producing zygotes and then finally oocysts. This entire cycle takes about 4-6 days depending on the species of animal (Jeurissen *et al.*, 1996).

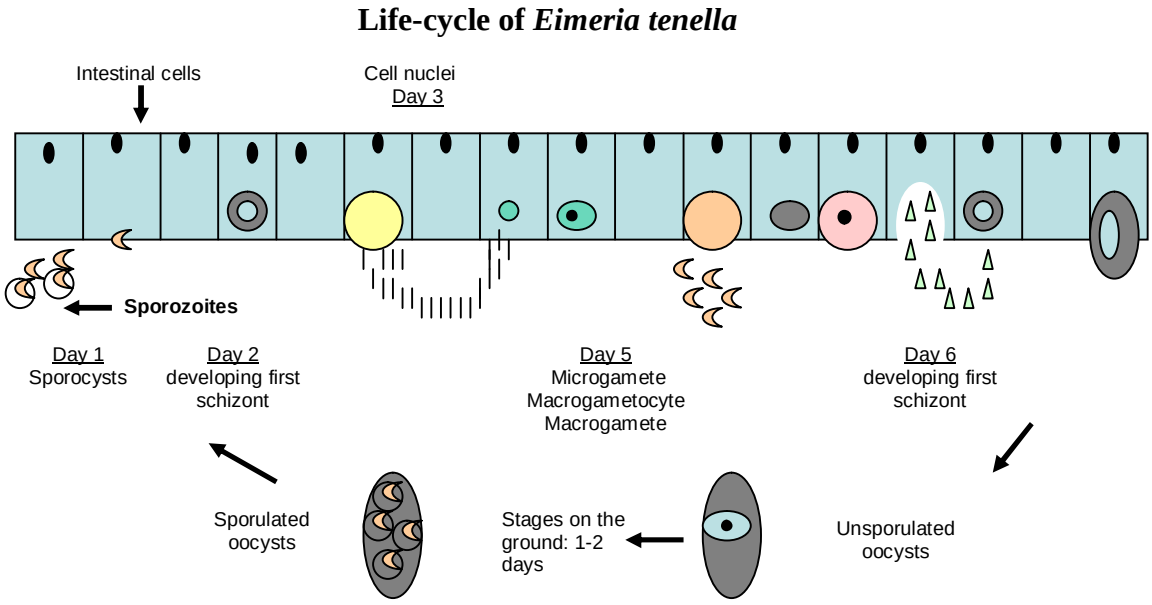

Figure 1.2 *The life-cycle of Eimeria tenella, typical of the genus Eimeria, adapted from Jeurissen et al. (1996). Eimeria have a complex life-cycle that begins with oocysts in the faeces being ingested. Sporozoites that are released search out particular regions of the gut and invade the epithelial cells lining the intestine. The sporozoites are then replicated, which leads to another stage of the cycle, called merozoites (Day 5). These develop into micro- and macrogametes which fuse to form a zygote. The zygote develops into an oocyst and at this stage is released in the faeces.*

Animals which are bred in intensive environments, usually for commercial purposes, are most susceptible to coccidiosis. All *Eimeria* species parasitise the epithelial cells

of the intestinal lining, causing varying degrees of physiological harm to the host. This can range from effects such as weight loss, dehydration, inflammation, bloody diarrhoea and endothelial lesions. *Eimeria* can also affect the host systemically causing effects such as blood loss, shock syndrome and even death (Vermeulen *et al.*, 2001). The pathological effects of infection vary with each species. *E. tenella* can develop deep in the intestinal mucosa and cause gross lesions and major damage, whereas *E. praecox* is known to reduce weight gain and feed conversion efficiency without any cases of morbidity being reported (Morris and Gasser, 2006). Diagnosis of intestinal *Eimeria* is traditionally made using faecal flotation and a direct smear of freshly excreted material. Detecting or enumerating oocysts excreted, and measurement of oocyst/sporocyst dimensions, will determine the extent of infection in the intestines of animals (Morris and Gasser, 2006). Immunity is species-specific, since even after exposure to one type of *Eimeria*, the animal is still susceptible to infection from other species of *Eimeria*. Seven different species of *Eimeria (E. acervulina, E. brunette, E. maxima, E. mitis, E. necartrix, E. praecox and E. tenella)* are known to infect chickens (Vermeulen *et al.*, 2001) and fifteen different species are known to infect cattle (Cox, 1998), each of which has its own pathogenicity related characteristics.

Coccidiosis has a major economic impact on the poultry industry worldwide, with an estimated cost of approximately US $3.0 billion per year in financial losses (Dalloul and Lillehoj, 2006). This is as a result of reduced production efficiency caused by retarded growth rates, a drop in egg production, mortality, and the costs of veterinary treatment and prophylactic chemotherapeutic methods.

The control of coccidiosis has relied mainly on vaccines and preventative methods, such as the use of anti-coccidial drugs (coccidiostats). However, the use of anti-coccidial drugs has also led to the development of drug-resistant strains. To limit the selection of drug-resistant strains, rotation programs are employed in which different coccidiostats are alternately used either within a single flock, or in a series of consecutive flocks (Vermeulen *et al.*, 2001). It was estimated in 2006 that over 25,500 tonnes of coccidiostats were used in broiler production alone in the EU (Advisory Committee on Novel Foods and Processes, 2007).

Coccidiostats are currently regulated as feed additives under Annex 1 in EC Regulation 1831/2003, and, at present, 11 different coccidiostats have been granted 28 different authorisations for use in different livestock species (European

Parliament-COD, 2002). In general, they are used as prophylactic drugs and are not necessarily used to treat coccidiosis. In 2008, a decision was required from the EU member states on the proposed 'phasing out' of coccidiostats for use in animals by January 1st, 2012. However, it was concluded that the use of coccidiostats for the prevention of coccidiosis is essential, as alternative feed additives, such as vaccines or herbal remedies, do not offer the same advantages as coccidiostats (European Parliament- COD, 2002). The authorization and safety evaluation of coccidiostats approved for use in animals in the European Union is set and monitored by several agencies including the European Agency for the Evaluation of Medicinal Products (EMEA), the European Food Safety Authority (EFSA) and also by the Joint Food and Agriculture Organisation/ World Health organisation (FAO/WHO) Expert Committee on Food Additives. Human health risks associated with anti-protozoan drug residues are primarily confined to sensitive individuals such as pregnant females, infants and the elderly. Teratogenic and embryotoxic effects were observed in rats treated with toltrazuril (EMEA, 2000). Gastrointestinal inflammation and necrotic lesions were observed (at recommended dosages) in calves treated with halofuginone, and at higher dosages, deaths were observed. Toxicity effects were also observed in dogs, mice and rats when treated with high concentrations of diclazuril (EMEA, 1996). To ensure that no threat is posed by the incorporation of coccidiostats in animal feed, maximum residue limits (MRLs) have been set for all feed additives by the EU. However, a study carried out in 2005 demonstrated that 36 % of 320 egg samples tested positive for the presence of coccidiostat residues (Mortier *et al.,* 2005b). This indicates that the current methods for the monitoring of these residues are not sufficient to ensure food safety.

Analytical detection methods are characterised by parameters, such as selectivity, specificity, accuracy, precision, repeatability, analytical range, and detection sensitivities described by the limits of detection (LOD) and quantification (LOQ) (Blanchflower *et al.,* 1994). Alternatively, two new parameters have recently been introduced by the EU for residue detection, namely the decision limit (CCα) and the detection capability (CCβ). These are analytical parameters that must be defined to provide a better characterization of the LOD and LOQ for a validated analytical method intended for regulatory use within the EU. The decision limit is defined as the limit at and above which it can be concluded with an error probability (1 %) that a

sample is noncompliant, while the detection capability is the smallest content of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β (5 %). Validated residue detection assays are required to provide results for these parameters (Blanchflower *et al.*, 1994). The lack of rapid and inexpensive validated methods for the detection of anti-protozoan residues has made national screening programs somewhat difficult.

This research describes the development of several specific antibodies to the coccidiostats halofuginone, diclazuril and toltrazuril, and their subsequent incorporation into immunoassays to allow the rapid and cost effective detection of these residues in a variety of foodstuffs.

1.2 Target drugs

1.2.1 Halofuginone

Halofuginone (HFG) is a halogenated analogue of the naturally occurring quinazolinone alkaloid, febrifugine. HFG is licensed for use in broilers and turkeys and is marketed by Intervet either in the hydrobromide form as Stenerol ® (Intervet, Mechelen, Belgium) or the lactate salt form as Halocur ® (Intervet, Mechelen, Belgium). The molecular weight of HFG is 414.68 Da. The mode of action of HFG is unknown. The chemical structure of HFG is shown in **Figure 1.3**. HFG ingestion was recognised as having adverse effects in the cardiovascular and central nervous systems in rat and mice models (EC No. 124/2009). Hence, the European Medicines Agency (EMEA) established HFG as the marker residue and has set a maximum residue limit (MRL) of 10 and 30 μg/kg for bovine muscle and liver, respectively. According to a pharmacokinetics study of halofuginone by Stecklair *et al.*, in 2001, halofuginone was the main substance detected in tissue and plasma, in several animals administered with the drug, where no halofuginone metabolites were detected. The EU has specific legislation in place under Commission Regulation (EC) No. 2430/1999 which permits HFG for use in laying hens but no residues may be found in eggs. In February 2009, under EU legislation, a commission decision regarding "unavoidable contaminating residues'' of coccidiostats was amended (2009/8/EC). This introduced MRLs for HFG in certain foodstuffs that were not previously required (Dubois *et al.*, 2004). The MRLs for HFG for food of animal origin from animal species other than chickens for fattening, turkeys and bovine (except dairy cattle) were set, as outlined in **Table 1.0**;

Table 1.0 *Maximum residue limits set by the EU for halofuginone for animal species*

Tissue	Maximum Residue Limit (µg/kg)
Liver	30
Kidney	30
Milk	1
Eggs	6

To ensure that food is free from any HFG residues and safe for human consumption, monitoring schemes usually adopt a two-tier testing system consisting of screening and confirmatory tests (Hagren *et al.*, 2005). Screening tests can be typically perceived as rapid, low-cost and high throughput tests, which classify a substantial number of samples as compliant or potentially non-compliant, whereas, confirmatory methods, tend to be specific but are also slow, high-cost and of low volume, and are utilised to re-analyse the potentially non-compliant samples (Hagren *et al.*, 2005). Currently, there are several detection systems available for the monitoring of HFG residues in foodstuffs. These are primarily based around methods such as chromatography (Mortier *et al.*, 2003; Dubois *et al.*, 2004) mass spectrophotometry (Yakkundi *et al.*, 2002) and immunosassay technology (Hagren *et al.*, 2005; Huet *et al.*, 2005).

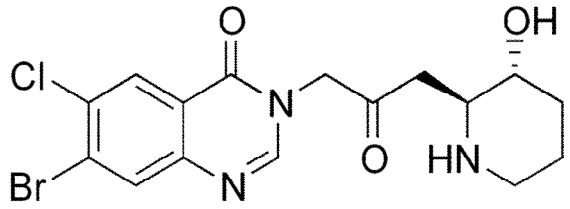

Figure 1.3 *Chemical structure of halofuginone (trans-7-bromo-6-chloro-3-(3-hydroxy-2-piperidyl)-acetonyl)-4-(3H)-quinazolinone).*

1.2.2 Diclazuril

Diclazuril (DIC) (4-chlorophenyl [2, 6-dichloro-4-(4, 5-dihydro-3H-3, 5-dioxo-1, 2, 4-triazin-2-yl) phenyl] acetonitrile) is a benzeneacetonitrile anti-coccidial drug primarily used for broiler chickens. The molecular weight of DIC is 407.64 Da. It is marketed commercially as 'Clinacox™', a 0.5% (w/v) premix by Janssen® (Germany), and is recommended for use in poultry feed at a concentration of 1 mg/kg with a withdrawal period of 5 days prior to slaughter. The chemical structure of DIC is shown in **Figure 1.4.**

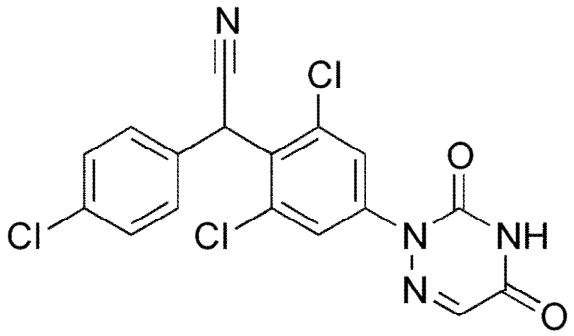

Figure 1.4 *Chemical structure of diclazuril (4-chlorophenyl) [2, 6-dichloro-4-(4, 5-dihydro-3H-3, 5-dioxo-1, 2, 4-triazin-2-yl) phenyl] acetonitrile).*

DIC has been shown to be effective against intestinal *Eimeria* spp. in avian coccidiosis, intestinal and hepatic coccidiosis in rabbits, toxoplasmosis in mice (Mortier *et al.*, 2005a) and *Eimeria* spp. in turkeys (Vanparijs *et al.*, 1991). The activity of DIC is directed only against specific endogen stages of *Eimeria* species. Thus, DIC is active against second generation schizonts of *E. acervulina*, *E. mitis* and *E. necatrix*, against gamonts and late schizonts of *E. brunetti*, zygotes of *E. maxima*, and first and second generation schizonts and sexual stages of *E. tenella*. While sporulation is delayed by DIC (Maes *et al.*, 1988), the actual mode of action of DIC is still unknown.

DIC is licensed by the Joint FAO/WHO Expert Committee on Food Additives, the EMEA and the EFSA (Olejnik *et al.*, 2009). The MRLs set by these agencies are shown in **Table 1.1**.

Table 1.1 *Maximum residue limits set by the EU for diclazuril for both turkeys and chickens for fattening, and for chickens reared for laying.*

Tissue	Maximum Residue Limit (µg/kg)
Liver	1.5
Kidney	1.0
Muscle	0.5
Skin/Fat	0.5

The EU has also recently put in place MRLs for DIC in non-target feeds caused by unavoidable cross contamination. These are listed in **Table 1.2.**

Table 1.2 *Maximum residue limits for diclazuril in non-target feedstuffs under Annex 1 to directive 2002/32/EC (amended in February 2009 under Commission Directive 2009/8/EC).*

Non-target food	Maximum residue limit (µg/kg)
Laying birds	10
Rabbits for fattening or during withdrawal feeding.	10
Other species excluding laying chickens (<16wks old) or chickens/turkeys for fattening (<12wks old)	30

Current methods for the detection of DIC include liquid chromatography-mass spectrophotometry/MS with detection limits (CCβ) of 0.6 µg/kg in poultry meat (Mortier *et al.,* 2005a) and 1 ng/mL in animal plasma (Croubels *et al.,* 2002), liquid chromatography with UV detection, with detection limits down to 0.1 mg/kg, and gas chromatography-MS with a detection limit of 20 µg/kg (Blanchflower *et al.,* 1994). It appears that no methods are published in current relevant literature reporting immunoassay screening of diclazuril residues.

1.2.3 Toltrazuril

Toltrazuril (TLZ) (1-methyl-3-[3-methyl-4-[4-(trifluoromethylsulfanyl) phenoxy] phenyl]-1, 3, 5-triazinane-2, 4, 6-trione) is a triazinetrione derivative widely used in

chickens, turkeys, pigs and cattle for the prevention of coccidiosis. The molecular weight of TLZ is 425.38 Da. The chemical structure of TLZ is shown in **Figure 1.5**.

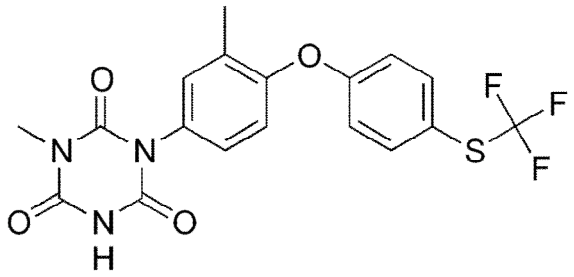

Figure 1.5 *Chemical structure of toltrazuril (1-methyl-3-[3-methyl-4-[4-(trifluoromethylsulfanyl) phenoxy] phenyl]-1, 3, 5-triazinane-2, 4, 6-trione).*

TLZ administration affects all intracellular developmental stages of *Eimeria* spp., including those of schizogony and gametogony (Steinfelder *et al.,* 2005). It was reported that chickens treated with TLZ and infected with *E. tenella* showed a level of immunity to re-infection in comparison with control models. This was observed when the titre of antibodies, specific to *E. tenella,* was higher in TLZ-treated chickens than in those treated with a polyether ionophore (Greif, 2000). This is thought to be due to the destruction of intracellular stages that stay in the host cells and act as antigens. TLZ is marketed commercially as Baycox® by Bayer Healthcare. Following oral administration, it is oxidised to toltrazuril sulfone and toltrazuril sulfoxide. Toltrazuril sulfone (Ponazuril - **Figure 1.6**), the major metabolite, is listed as the marker residue for TLZ detection by the EMEA. The molecular weight of ponazuril is 489.5 Da. The EMEA observed that ponazuril represented almost 100% of residues remaining in edible tissues after a 16-day withdrawal period in chickens (EMEA, 1998).

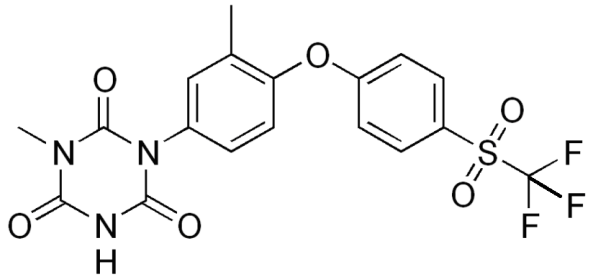

Figure 1.6 *Chemical structure of ponazuril (1, 3, 5-Triazine-2, 4, 6(1H, 3H, 5H)-trione, 1-methyl-3-[3-methyl-4-[4-[(trifluoromethyl) sulfonyl] phenoxy] phenyl]-(9C).*

Previous methods for the detection of ponazuril include the use of a high-performance liquid chromatography diode array method for residues in plasma, which has a detection capability of 10 ng/mL (Dirikolu *et al.*, 2009a). Using GC/MS, a capability of also detecting down to 10 ng/mL was reported (Tobin *et al.*, 1997). The lowest detection capability was observed at 2 µg/kg in kidney using a HPLC method based on fluorescence detection (EMEA, 2005). It is understood that only one antibody has previously been reported for the detection of ponazuril. This was a polyclonal antibody which exhibited an IC_{50} of 18 µg/kg, in a competitive ELISA format (Connolly *et al.*, 2002).

The current MRLs for ponazuril, as set by the EMEA, are outlined in **Table 1.3**. TLZ was not included in the amended Annex 1 to directive 2002/32/EC under Commission Directive 2009/8/EC for cross-contamination of unintended foodstuffs.

Table 1.3 *Maximum residue limits for toltrazuril sulfone (ponazuril) in foodstuff, as set by the EMEA.*

Foodstuff		Maximum residue limit (µg/kg)
Chicken/Turkey – Muscle		100
	- Skin/Fat	200
	- Liver	600
	- Kidney	400
Porcine	- Skin	150
Bovine/Porcine	- Muscle	100
	- Skin	150
	- Liver	500
	- Kidney	250

It is understood there is currently no immunoassay available for the detection of TLZ or ponazuril in foodstuff. In order to develop immunoassays to detect these residues below the current EU MRLs, recombinant antibody technology can be employed to developed antibodies with tailored affinities and specificities.

1.3 Introduction to Antibodies

Antibodies are a group of glycoproteins generated by the immune system in response to any foreign particles (antigens) that enter the body. The body's defence system generates antibodies upon exposure to potentially hazardous pathogens which eliminate and destroy them. The molecules are also referred to as immunoglobulins (Ig) because of their globular structure. All Ig consist of a basic four-polypeptide chain structure of two identical heavy chains (each approximately 440 amino acids long) and two identical light chains (each approximately 220 amino acids long), which are held together by disulphide bonds. The heavy and light chains also contain intrachain disulphide bonds, which stabilise their folding into 110-amino acid domains. Sequence analysis has revealed that antibodies have one of two types of light chain, kappa (κ) or lambda (λ), and their ratio varies with species (mouse 95% κ, humans 60% κ) (Benjamini *et al.*, 2000). There are five distinct classes of antibody designated by their heavy chain, IgA, IgM, IgD, IgE and IgG. The structure of an antibody varies depending on its isotype. However, antibody structure is generally represented by the immunoglobulin G (IgG) molecule. Recombinant antibody technology can be used to develop different antibody formats such as scFv, Fab, scAb, and dimeric antibodies. Their structural formats are shown in **Figure 1.7**. An IgG is composed of four polypeptide chains, consisting of two identical heavy (H) and two identical light (L) chains, held together by interchain disulphide bonds. Heavy chains have a molecular weight of approximately 50 kDa and consist of one variable region and three constant domains. These constant domains determine the antibody class. Light chains consist of one variable region and one constant domain and have a molecular weight of approximately 25 kDa.

While specificity is derived from variable regions, the constant regions are involved in effector functions of the Ig (e.g. binding to phagocytes, activation of complement). It is both this specificity and biological activity that makes their role crucial for the immune system. The variable regions, which are located at the N-terminal portions of both the heavy and light chains, form the antigen-binding fragment (Fab). Comparison of these variable (VL and VH) gene sequences showed the greatest amount of variability in regions referred to as the hypervariable regions (Kabat *et al.*, 1971). These regions, which participate in antigen binding, are also referred to as complementarity-determining regions (CDRs), as they form regions complementary in structure to antigen epitopes. It is the variation in the amino acid sequence of the

CDRs that allows for the generation of a repertoire of antibodies that exhibit great diversity, each specific to a particular antigen. The CDRH3, which displays the greatest variation in terms of length and sequence, is considered (in conjunction with the CDRL3) to make the most significant contributions to affinity and specificity (Barbas *et al.*, 2004). The remainder of the variable heavy and light domains, referred to as framework regions (FRs), exhibit far less variation, and fold into relatively rigid beta strands that maintain the overall Ig structure.

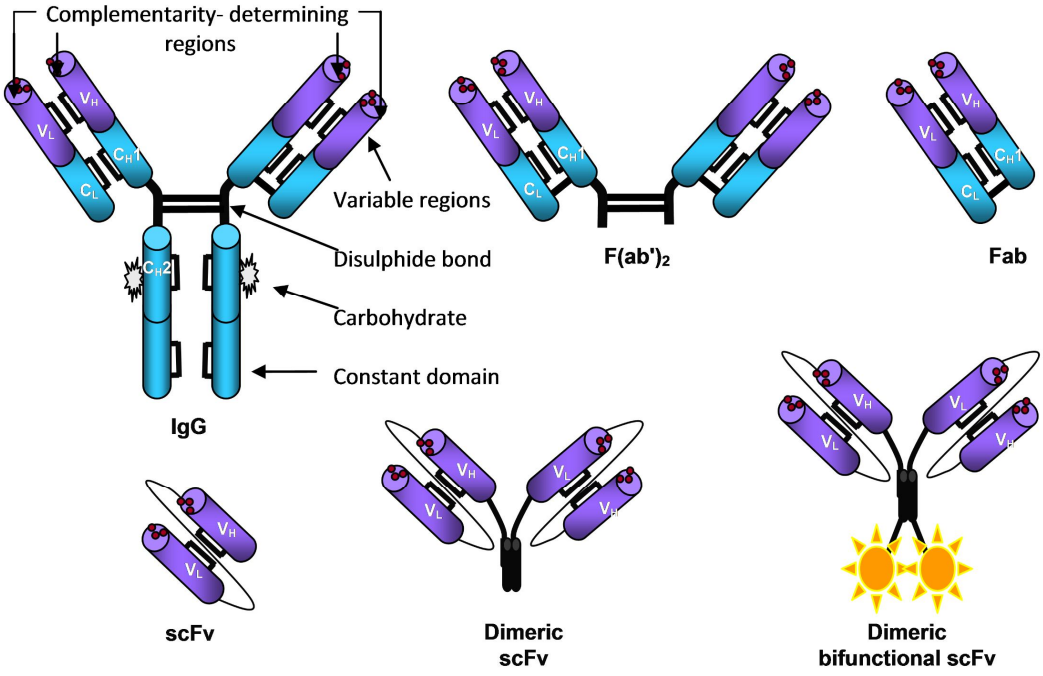

Figure 1.7 *Diagrammatic representation of different antibody 'formats' IgG, F(ab')$_2$, Fab, scFv, dimeric scFv and the dimeric bifunctional scFv. The amino terminal end of the antibody is characterised by variability (V) of the sequences in both the heavy (H) and light (L) chains, which are known as the VH and VL regions, respectively. The CDR regions confer the specificity of the antibody to a particular antigen and this is known as the antigen binding site.*

The ability of antibodies to detect and distinguish between infinite numbers of antigens has focused increased attention on immunoassay technology as an alternative to 'standard' analytical techniques. While immunoassays have been proven in their sensitivity, specificity, reproducibility and robustness (Fitzpatrick *et al.*, 2000), the

quality of the assay is entirely dependent upon the availability of high quality antibodies for detection. Previous assays for detecting anti-protozoan drug residues have predominantly used mass spectrophotometry or liquid chromatography for analysis (Ding *et al.,* 2005; Mortier *et al.,* 2005a; Qi *et al.,* 2007 and Dirikolu *et al.,* 2009b). However, in many instances, for drug residue screening, an immunological procedure is preferred due to the low cost and simplicity associated with such methods. Antibody engineering has transformed the field of immunoassays, allowing the generation of an immortal repertoire of antibody fragments with a defined affinity and specificity towards target molecules. It also offers the possibility of altering the antibody's affinity and specificity, if desired (Plückthun and Pack, 1997).

Recombinant antibody technology allows the generation of antibody fragments from several species including human, rabbit, mouse, chicken and sheep in both prokaryotic and eukaryotic expression systems (Daly *et al.,* 2000). Recombinant antibodies can be sensitive and specific for their target molecules, and can be produced relatively easily and cheaply. They can be generated by directly cloning the heavy and light chain variable domains from a monoclonal antibody-producing hybridoma cell or by amplifying the variable genes from the cDNA from the lymphocytes of an immunised source. The challenge in producing recombinant antibodies arises in the selection procedure, where specific antibody fragments are isolated and enriched from a large repertoire of non-functional and non-specific antibody fragments. In the immune system, a diverse collection of antibody binding sites is created by the combinatorial assembly of germline-encoded segments. This produces a repertoire of naive B-cell lymphocytes which, when activated, express a unique antibody binding site on their surface (Hoogenboom, 2005). Exposure to antigen promotes somatic hypermutations in the variable genes, allowing subsequent selection of mutations that improve the affinity of the antibody for the antigen. Antibodies can also be isolated from recombinant antibody libraries in the laboratory, using methods for selection that 'mimic' this *in vivo* process. Many of these selection platforms share four key steps with the process for antibody generation in the immune system: first, the generation (or cloning) of genotypic diversity; second, the coupling of genotype to phenotype; third, the application of selective pressure; and fourth, amplification (Hoogenboom, 2005). Various display mechanisms, including phage, yeast and ribosomal display, have been developed for the isolation of specific

antibody fragments, which provide an efficient method to select antibodies based on the antigen-binding behaviour of individual clones. These systems enable coupling of genotype to phenotype, allowing simultaneous isolation of the desired antibody fragment and the genetic sequence encoding it.

1.4 Phage Display Technology

Phage display is currently the most widespread method used for the display and selection of large collections of antibodies, and for the engineering of selected antibodies (Hoogenboom, 2002). The principle of phage display was first introduced in 1985 by George Smith (Smith, 1985). Phage display is a molecular diversity technique that employs non-lytic filamentous bacteriophage fd/M13 to display ligands on the surface of their phage coat. *E. coli* containing the F conjugative plasmid are a natural host for replication of this phage family. Initially, complete phage vectors or bacteriophage, which carried all the genetic information required for the phage life-cycle, were used as the display vector. Now, small plasmid vector or phagemids, which contain the appropriate packaging signal and cloning sites, have become a more popular type of vector for display. Phagemids have high transformation efficiencies, making them ideal for generating large antibody repertoires. When using phage to display antibody fragments, phagemids usually consist of the DNA encoding a scFv, fused to the gene encoding a phage coat protein (pIII or pVIII) (See **Figure 1.8**).

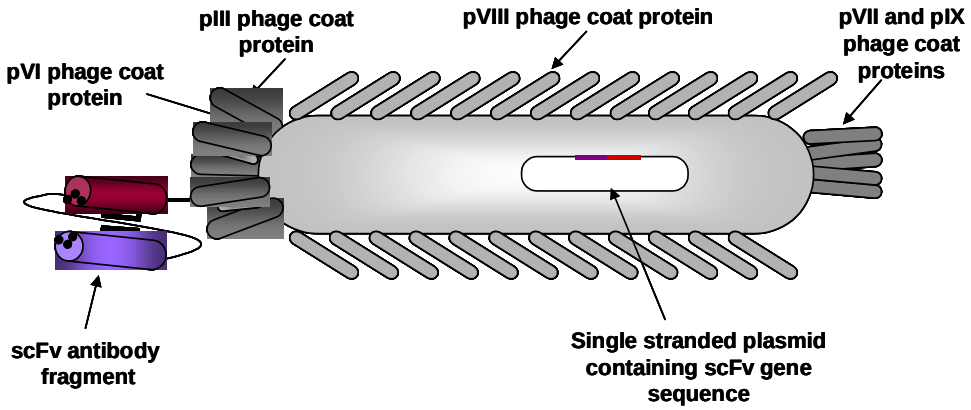

Figure 1.8 *Filamentous phage structure, PIII, PVIII, PVII and PIX are phage coat proteins. Exogenous fragments are usually expressed or 'displayed' on PIII or PVIII.*

Other vector features include an antibiotic resistance marker, an origin of replication, a promoter region and an affinity tag to aid in purification. Helper phage, such as M13K07 or VCSM13 are also required to supply all necessary structural proteins for correct packaging of the phage particle. The lacZ promoter is the most common promoter used to control expression. Expression may be suppressed by the addition of the catabolic repressor, glucose, or induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG). Upon induction, the scFv-coat fusion β-isopropyl-protein is incorporated into new phage particles that are assembled in the bacterium and displayed on their surface, while the genetic information encoding the scFv remains within the phage particle (Barbas *et al.,* 2004) (**Figure 1.8**).

Phage display has been successfully employed to isolate antibody fragments specific for small molecules (Sheedy *et al.,* 2007). Small molecules alone do not have the molecular mass required to elicit an immune response and hence, must be conjugated to larger carrier proteins (Fodey *et al.,* 2007a). Various carrier proteins, including bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH) and thyroglobulin (THY), are commonly used for this purpose. Regardless of the type of molecule, a carrier must be highly immunogenic, have the required solubility properties, be non-toxic *in vivo* and possess suitable functional groups for coupling to the hapten (Hermanson, 2005). Conjugation is a crucial step in antibody production, as the specificity of the resultant antibodies is dependent on the hapten used to produce them. The choice of method is governed by the functional groups available on both the hapten and the carrier and the orientation of the hapten desired, for presentation to the immune system. However, a major problem associated with the use of conjugates for antibody production is that antibodies will be produced against the hapten, the carrier and the linker molecule. To reduce the production of non-specific antibodies and direct the production of antibodies to the hapten rather than the carrier, the number of haptens conjugated to each carrier molecule can be optimized. The use of conjugation procedures employing EDC (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride), does not introduce a bridging molecule between the hapten and carrier, thus, eliminating the potential for antibodies being generated against the coupling reagent (Hermanson, 2005).

1.5 Biopanning of phage display libraries

Biopanning is an affinity selection technique which relies on subjecting a library of antibodies on filamentous phage to bind to a specific target (immobilized on a solid support or in solution). This process enriches analyte-specific clones, by incubation with the target molecule in consecutive rounds, with increasing stringency. Unbound phage are washed away and specific phage are eluted by changing the binding conditions, either by pH elution, enzymatic cleavage or incubation with excess antigen. Specific phage are then amplified in *E. coli*. This selection process is outlined in **Figure 1.9**. In practice, several rounds of selection (approximately 3-5) are necessary, as the binding of non-specific phage limits the enrichment that can be achieved in one single cycle (Azzazy and Highsmith, 2002).

Using the pComb vector series, specific phage can then be solubly expressed by infection into a non-supressor *E.coli* strain such as Top10F, which allows the scFv to be secreted independently of the phage particle. Screening of the enriched library is then performed by analysing single colonies in a monoclonal ELISA to determine the specificity of each isolated clone.

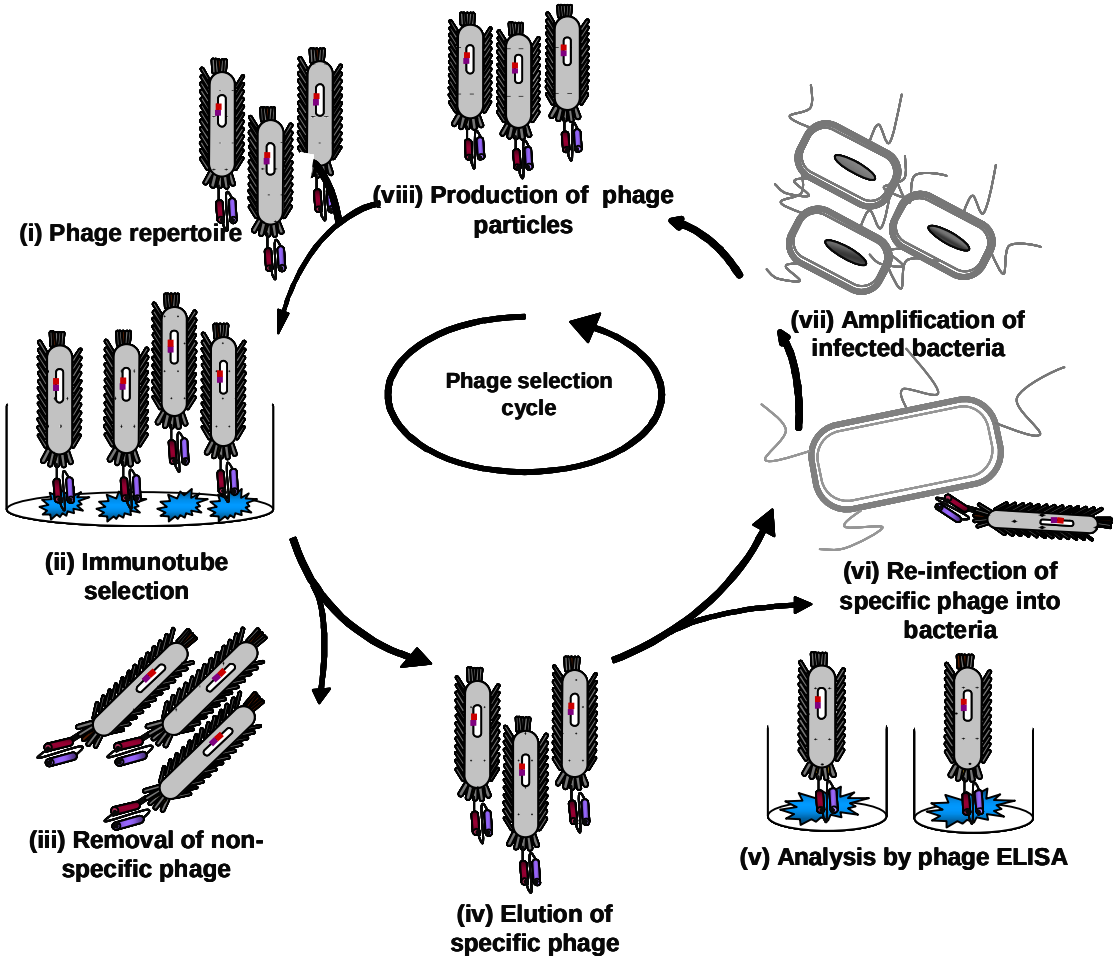

Figure 1.9 *Bio-panning of a phage display library. The library is subjected to several different steps; (i) the phage library is constructed (ii) binding of phage to the target antigen (in this case antigen immobilized on a solid support in the form of an immunotube), (iii) washing to remove unbound phage, (iv) dissociation to recover antigen-specific phage, (v) analysis of specific phage by ELISA, (vi) Re-infection of phage into bacteria, (vii) amplification of the antigen-specific phage by infection of E. coli and (viii) production of enriched phage repertoire for the specific target.*

Once a specific antibody is isolated, affinity maturation may be employed to enhance the affinity of a low sensitivity antibody. Several different antibody engineering techniques may be employed for this purpose.

1.6 Antibody Engineering and affinity maturation

Antibody engineering for the purpose of affinity maturation is discussed in greater detail in Chapter 3. In brief, understanding the molecular mechanisms of antibody affinity and specifity towards a given antigen is a key goal in antibody engineering. In order to improve or alter antibody functionality, affinity and specificity may be manipulated in a multitude of ways. Mutations in antibody structure may affect the antibody affinity indirectly by influencing the positioning of side chains contacting the antigen. Taking this into account, antibody function may be directed by providing new contact residues (particularly when they are located in or near the centre of the antigen combining site) or by replacing 'repulsive' or low-affinity contact residues for contact residues with more favourable energetics (Hoogenboom, 1997). Error prone PCR (McCullum *et al.,* 2010), site directed mutagenesis (Lou and Marks, 2010), CDR walking mutagenesis (Yang *et al.,* 1995), alanine scanning (Da Silva, *et al.,* 2010), the use of bacterial mutator cells (Low *et al.,* 1996), DNA shuffling (Nakajima *et al.,* 2002) and chain shuffling (Yoshinaga *et al.,* 2008; Hur *et al.,* 2010 and Lou *et al.,* 2010) are some of the numerous methods used for the affinity maturation of antibodies. The method of chain shuffling was originally proposed in 1989 by Huse and colleagues, and involves the re-cloning of the gene for one of the variable domains (*e.g.* VH) into a repertoire of genes for the other chain (*e.g.* VL). The resulting mutant library contains the VH domain gene of the parent antibody and random VL chain genes. It is a rapid approach for generating high affinity antibodies, and, since shuffling approaches mimic somatic hypermutation, they are believed to be more efficient than random or site-directed mutagenesis in producing functional antibodies (Sheedy *et al.,* 2007). A 300-fold improvement in sensitivity has previously been observed for an anti-hapten scFv from a naïve scFv library (Marks *et al.,* 1992). Other than affinity maturation, chain shuffling has also been reported to overcome improper prokaryotic expression of certain scFv (Rojas *et al.,* 2004). Additionally, chain shuffling has been employed to improve the sensitivity of an scFv against another environmental toxin, domoic acid (Finlay *et al.,* 2006).

1.7 Thesis Outline

The main aim of this research was the development of rapid immunoassays for the detection of the food contaminants halofuginone, diclazuril and toltrazuril.

Chapter 3 describes the use of antibody chain shuffling for the affinity improvement of an anti-halofuginone-specific scFv and subsequent incorporation into ELISA and Biacore detection assays. The repertoire of antigen-sensitised heavy chain regions was shuffled with the original wild-type light chain fragment repertoire, resulting in the production of a secondary heavy chain-biased library. This chain shuffling-technique was employed for a bio-panned halofuginone-specific phage library. From this, an affinity matured scFv with 185-fold greater sensitivity than the wild-type was isolated. This scFv was used in the generation of validated ELISA and Biacore assays, in spiked egg samples, for the detection of halofuginone.

Chapter 4 focuses on the development of an immune library for diclazuril, and discusses the numerous problems encountered with this murine library.

Chapter 5 describes the production of anti-toltrazuril polyclonal antibodies using an antigen mimic and a commercial conjugate as immunogens. The identification and purification of anti-toltrazuril antibodies is reported. The antibodies are incorporated into validated ELISA, Biacore, and lateral flow assays, and their performance of each assay is subsequently compared. This chapter also describes the development of a multi-analyte lateral flow assay for the simultaneous detection of halofuginone, diclazuril, and toltrazuril.

Chapter 2

Materials and Methods

2. 1 Equipment

Apparatus	Supplier
Balances (Chyo-Model JK-180 and Mettler Model-PJ300) Orion 3 star-pH meter	Medical Supply Company Ltd., Damastown, Mulhuddart, Dublin 15, Ireland.
Biacore 3000™ Instrument Biacore sensor CM5 chips	Pharmacia Biosensor AB, Uppsala, Sweden.
Biometra $T_{GRADIENT}$ Thermal Cycler Orbital Shaker Millipore Ultra Pure H_2O Milli-Q Academic Sanyo Orbital Incubator	ABG Scientific Ltd., Dublin Industrial Estate, Glasnevin, Dublin 9, Ireland.
Bio-Rad Gene Pulser Xcell SDS Bio-Rad Min-Protean® 3 Cell Trans-Blot® SD Semi-Dry Transfer cell Bio-Rad PowerPac™ Basic DNA Gel Apparatus Bio-Rad (Wide-Mini-Sub® Cell GT)	Alpha Technologies, The Leinster Technology Centre, Blessington Industrial Estate, Blessington, Co. Wicklow, Ireland.
Blood Tube Rotator SB1	Stuart Scientific, Beacon Road, Stone, Staffordshire, ST15 0SA, UK.
Gelaire BSB4 Laminar Flow Cabinet	Gelman Ltd, 71 Broomhill Road, Tallaght

	Industrial Estate, Tallaght, Dublin 24, Ireland.
Hermle Z233M-2 Air-cooled version Microcentrifuge	Hermle Labortechnik GmbH, 25 Siemensstrasse, Wehingen, 78564, Germany.
Homogeniser Ultra Turrax	Janke & Kunkel IKA-Werk Ultra-Turrax, Staufen, 79129, Germany.
Camag Linomat 5 Lateral Flow Instrument New Brunswick Scientific-Excella® Incubator Shaker Type E24 ND-1000 Spectrophotometer (Nanodrop) Tomy Autoclave SX-700E High Pressure Steam Sterilizer Eppendorf 5810r Benchtop centrifuge	Mason Technologies, Greenville Hall, 228 South Circular Road, Dublin 8, Ireland.
IKA® MTS 2/4 Digital Microtitre Shaker Platform Shaker STR6	Lennox, John F. Kennedy Drive, Naas Road, Dublin 12, Ireland.
Tecan Safire^2 Plate reader	Tecan Austria GmbH, Untersbergstrasse, 5082 Grödig, Austria.
ThermostaticWater Bath Model Y6	Grant Instruments (Cambridge) Ltd, 29 Station Road, Shepreth, Royston, Herts., SG8 6PZ, UK.
Beckman Coulter Delsa Nano C (DLS)	Beckman Coulter Diagnostics Limited Lismeehan, O'Callaghans Mills, Co. Clare Ireland.

2.2 Reagents

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

Reagent	Supplier
Acetic acid Hydrochloric acid	Riedel de-Haen AG, Wunstorfer, Strabe 40, D-30926, Hannover, Germany.
Superscript III cDNA synthesis kit	Invitrogen, Biosciences Ltd., 3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Ireland.
Bacteriological Agar Tryptone Powder Yeast Extract	Oxoid, Basingstoke, Hampshire, RG24 8PW, England.
DNA ligase	Boehringer-Mannheim Ltd., East Sussex, BN7 1LG, England.
Trizol	Gibro BRL, Renfew Rd, Paisley, PA49RF, Scotland.
Pre-stain markers	Fermentas/Fisher Scientific Fermentas GmbH Opelstrasse 9, 68789 St. Leon-Rot, Germany
PCR Primers	MWG-Biotech Ltd, Milton Keynes, MK12 5RD, UK.
Restriction enzymes	New England Biolabs, Hitchin, Hertfordshire, SG4 0TY, England.
VCSM13/M13K07 Helper Phage	Stratagene, North Torrey Pines Rd. La Jolla, USA.
Wizard Plus Mini-prep kit PCR prep DNA purification kit Reverse-Transcription kit	Promega Corporation, 2800 Wood Hollow Rd., Madison, WI 53711-5399, USA.

Diclazuril polyclonal Antibody (sheep IgG) Toltrazuril-BSA/BTG conjugate	Randox Laboratories Ltd., 55 Diamond Road, Crumlin, Co. Antrim, BT29 4QY, Northern Ireland.
Perfect Prep gel cleanup kit	Unitech, Unitech House, Magna Drive, Magna Business Park, City West, Dublin 24, Ireland.
Polybead® Carboxylate Microspheres 0.2 µm	Polysciences Europe GmbH, Handelsstrasse 3, D-69214 Eppelhein, Germany.
HiFlow Plus HF135 nitrocellulose membrane	G Scientific Ltd., Dublin Industrial Estate, Glasnevin, Dublin 9, Ireland.
HRP-labeled Anti-HA, Anti-Rabbit and Anti-Goat antibodies. HRP-labeled Anti-M13 antibody. Anti-HA, Anti Rabbit and Anti-Goat antibodies.	Sigma-Aldrich Ireland Limited, Vale Road, Arklow,Wicklow, Ireland. (All other reagents were sourced from Sigma if not previously stated)

2.2.1 Culture formulations and buffer compositions

Media	Formulation
2 x Tryptone and Yeast Extract (TY) medium	Tryptone 16 g/L Yeast Extract 10 g/L NaCl 5 g/L
Luria Bertani Broth (LB) medium	Tryptone 10 g/L Yeast Extract 5 g/L NaCl 5 g/L
Super Broth (SB) medium	MOPS 10 g/L Tryptone 30 g/L Yeast Extract 20 g/L
Super Optimal Broth (SOB) medium	Tryptone 20 g/L Yeast Extract 5 g/L NaCl 0.5 g/L

	KCl 2.5 mM MgCl2 20 mM
Super Optimal Broth with Catabolite repression (SOC) medium	Tryptone 20 g/L Yeast Extract 5 g/L NaCl 0.5 g/L KCl 2.5 mM MgCl2 20 mM Glucose 20 mM pH 7.0
Top Agar	Tryptone 10 g/L Yeast Extract 5 g/L NaCl 10 g/L Bacto agar 7 g/L
1 x 505 media supplement	0.5% (v/v) glycerol 0.05% (v/v) glucose
Solid media was made by adding 15 g/L bacteriological agar to the media formulations above.	

2.2.2 Buffer compositions

Buffer	Composition
Phosphate buffered saline (PBS)	0.15 M NaCl 2.5 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 Reagents are dissolved in 800 mL ultra pure H_2O, pH is adjusted to 7.4, by titration with appropriate acid or base and made up to 1 litre with ultra pure H_2O.
PBS-Tween 20 (0.05%) (PBST)	0.15 M NaCl 2.5 mM KCl 10 mM Na_2HPO_4

	1.8 mM KH_2PO_4 0.05% (v/v) Tween 20
(Milk/BSA)PBST	PBS formulation above Specified % (w/v) BSA/Milk Marvel^{TM} powder pH 7.4
TBS	0.05 M Tris 0.15 M NaCl pH 7.4
Sonication Buffer	1X PBS 0.5M NaCl 20mM Imidazole
Running Buffer	1X PBS 0.5M NaCl 20mM Imidazole 1% (v/v) Tween 20
10X Electrophoresis Buffer	50mM Tris, pH 8.3 196mM Glycine 0.1% (w/v) SDS
Agarose Electrophoresis loading buffer (4x)	25% (w/v) Tris 0.5M, pH 6.8 20% (v/v) Glycerol 14.4mM β- Mercaptoethanol 5% (w/v) SDS 0.1% (w/v) Bromophenol blue
SDS sample loading buffer	25% (v/v) Glycerol 2% (w/v) SDS 14.4 mM β-Mercaptoethanol 0.1% (w/v) Bromophenol blue
Transfer Buffer (Western Blot)	20% (v/v) Methanol 10% (v/v) 10 x Electrophoresis Buffer 70% (v/v) De-ionised water

Sodium Citrate Buffer	150 mM NaCl, 15 mM Sodium Citrate, Use 1M NaOH to adjust the pH to 7.0

2.3 SDS PAGE and Western Blotting

12.5% Separation Gel	1 gel/6mL
1 M Tris-HCl, pH 8.8	1.5 mL
30% (w/v) Acrylamide (Acrylagel)	2.5 mL
2% (w/v) Methylamine Bisacrylamide (Bis-Acrylagel)	1.0 mL
Water	934 µL
10% (w/v) Sodium Dodecyl Sulfate (SDS)	30 µL
10% (w/v) Ammonium Persulfate (APS)	30 µL
Tetramethylethylenediamine (TEMED)	6 µL

4.5% Stacking gel	1 gel/2.5mL
1 M Tris-HCl, pH 6.8	300 µL
30% (w/v) Acrylamide (Acrylagel)	375 µL
2% (w/v) Methylamine Bisacrylamide (Bis-Acrylagel)	150 µL
Water	1.625 mL
10% (w/v) SDS	24 µL
10% (w/v) APS	24 µL
TEMED	2 µL

2.3.1 Coomassie blue staining for SDS-PAGE

Coomassie blue staining solution ((0.2%, w/v) Coomassie blue R250 in 30:10:60 (v/v/v) methanol: acetic acid: water), was prepared and gels were stained for 30 minutes. Gels were destained in 50 mL of destaining solution (30:10:60 (v/v/v) methanol: acetic acid: water) overnight at 4°C. The destain solution was changed 2-3 times, until the protein bands were clearly visible.

2.4 Bacterial cells for cloning and expression

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

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

2.4.1 Maintenance of bacterial stocks

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

2.5 General Lab techniques*

**These methods are generic and any modification made will be cited in the corresponding sections.*

2.5.1 Agarose gel electrophoresis for DNA characterisation

Agarose gel electrophoresis is a highly effective method for separating, identifying and purifying DNA fragments. Agarose gels are prepared containing a concentration of agarose appropriate for the size of DNA fragments to be resolved. The DNA is stained with the fluorescent dye, ethidium bromide, and can then be visualised under U.V light.

DNA was analysed by resolution on agarose gels (containing 0.5 μg/mL ethidium bromide), in a horizontal gel apparatus. Gels were prepared by dissolving agarose (typically 0.7-1.2% (v/v)) in 1 x Tris-Acetate-EDTA (TAE) buffer by heating. Ethidium bromide was added to the cooled gel prior to setting. The gels were placed in the gel apparatus and 1 x TAE was used as the running buffer. A tracker dye was incorporated into the sample (1 μl dye + 5 μl sample) to facilitate loading of samples. Small gels were

frequently run at 100 Volts for 1 hour or until the dye reached the end of the gel while larger gels were frequently run at 40-80 Volts. Gels were then visualised on a UV transilluminator and photographed using a UV image analyser.

2.5.2 SDS-PAGE

The following stock solutions were prepared: 0.5 M Tris-HCl, pH 6.8, 0.4% (w/v) SDS, 10% (w/v) APS and 1.5 M Tris-HCl, pH 8.8 (Tris base, rather than Tris-HCl, should be used for buffer preparation and pH adjustment should be made with HCl). Use of Tris-HCl would result in higher ionic strength, poor migration and diffuse protein bands. The composition of each gel formulation is outlined in *Section 2.3*. SDS denatures the protein and coats it with a negative charge. The amount of SDS bound is relative to the size of the protein, and, therefore, all proteins will have a similar mass-to-charge ratio. This separation method also employs β-mercaptoethanol, a reducing agent. Heat is applied to dissociate proteins into individual polypeptides before they are loaded onto the polyacrylamide gel. Glass plates are held together by using Bio-rad gel holder cassettes. Free radical-induced polymerisation of the resolving gel acrylamide was catalysed by addition of APS and the accelerator TEMED. Once the gel had polymerised, the stacking gel was added directly onto the resolving gel. A plastic comb was placed in this gel to create the wells for sample application. When the stacking gel had fully polymerised, the plates were then placed in an electrophoresis chamber, the comb was removed and the chamber and wells filled with electrophoresis buffer. The pore size of the gel, dependent on the amount of acrylamide and degree of crosslinking, facilitates protein separation on the basis of size. Excess sample buffer is essential to maintain reduction of protein sulfhydryls and to prevent intramolecular disulfide bond formation through oxidised cysteines. Therefore, samples were mixed 4:1 with SDS sample buffer and heated for 5 minutes at 95°C. A Fermentas pre-stained colourburst marker (Product number: SM1811) was used with each gel. A sample volume of 20 µL was added to the respective wells on the gel. The gel was run at 30 mA until the sample had reached the bottom of the gel. The separated protein was then visualized by staining with Coomassie blue for 1-2 hours followed by the addition of destain for 2-3 hours, or the gels were used for subsequent Western blotting, as described in *Section 2.5.3*.

2.5.3 Western Blotting

Proteins are eluted from a polyacrylamide gel onto a nitrocellulose membrane by electrophoretic transfer. This technique is used for the qualitative detection or characterisation of a specific protein. The transfer buffer used is essentially SDS running buffer with methanol included. The presence of methanol in the transfer buffer serves two main purposes. Firstly it promotes dissociation of SDS from the protein and secondly it dramatically improves adsorption of proteins onto membranes in the presence of SDS, although these effects may vary with proteins (Pettegrew *et al.*, 2009). Following separation of the proteins by SDS-PAGE, as described in *Section 2.5.2*, the polyacrylamide gel, filter paper and nitrocellulose membrane were soaked in transfer buffer for approximately 15 minutes. The proteins were then transferred to the membrane by electrophoresis using a Trans-Blot SD semi-dry cell (Bio-Rad) at 15V for 20 minutes. The membrane was blocked with PBS containing 5% (w/v) Milk Marvel (Premier Foods Ltd., UK) overnight at 4ºC. The membrane was washed three times with PBST for 10 minutes each time. Ten mLs of the appropriate antibody, at the required dilution, was prepared in PBS containing 2% (w/v) Milk Marvel and added to the nitrocellulose for 1.5 hours, while shaking at room temperature. The membrane was then washed thoroughly as before and a secondary antibody (if required), prepared in PBS containing 2% (w/v) Milk Marvel, was added and incubated as before. Once the blot was washed, TMB peroxidase substrate is added which forms an insoluble blue precipitate when acted upon by the HRP-labelled antibody. This allows the protein bands to be clearly visualised.

2.5.4 Quantification of proteins/nucleic acids on the Nanodrop 1000 (Thermo Scientific)

The Nanodrop spectrophotometer is set to the correct parameters depending on the samples to be quantified. Proteins are measured at an absorbance of 280nm and DNA is measured at 260nm. The Nanodrop is initialized by adding water, and blanked by addition of the sample buffer. The sample to be quantified (1.6 µL) is pipetted onto the measurement pedestal of the spectrophotometer and a measurement is then taken. This is repeated in duplicate to

ensure accuracy. The sample is then simply wiped clean from the pedestal and another sample can be measured.

2.6 Generation of the chicken anti-halofuginone scFv library-immunisation schedule and antibody serum titre for the production of anti-halofuginone antibodies

A Leghorn chicken was initially immunised sub-cutaneously with a mixture of equal parts of halofuginone-HSA (HFG-HSA) conjugate and Freund's complete adjuvant. The final concentration of the initial immunisation was 200 µg/mL. The first boost (day 14) was then administered using 100 µg/mL of HFG-HSA in PBS, mixed in a 1:1 ratio with Freund's incomplete adjuvant, in a final volume of 1 mL. The final 4 boosts that followed (days 36, 52 and 66) all contained 100 µg/mL of HFG-HSA and were administered in the same manner as the first boost using incomplete adjuvant. Finally, the chicken was sacrificed (day 71), and the spleen and bone marrow removed. Nunc Maxisorb^{TM} immunoplates were coated with 100 µL per well of the HFG-BTG at a concentration of 1 µg/mL and incubated overnight at 4ºC. Excess conjugate was discarded. The plates were then blocked with PBS containing 5% (w/v) Milk Marvel for 1 hour at 37ºC. Serial dilutions of each of the chicken anti-serum in PBS containing 1% (w/v) Milk Marvel were added to the wells and allowed to bind at 37ºC for 1 hour. The plates were washed 3 times with PBST and 3 times with PBS. This was followed by the addition of a 1 in 2,000 dilution of HRP-labelled rabbit anti-chicken antibody (Sigma) in PBS containing 1% (w/v) Marvel. The plate was incubated for 1 hour at 37°C, washed as before, and TMB substrate was added (100 µL/well). Following incubation for 30 minutes at 37ºC, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450nm on a Tecan Safire2^{TM} platereader.

2.6.1 Extraction and isolation of total RNA from chicken spleen

Prior to the extraction procedure, several preparatory steps are taken. Oakridge tubes are cleaned with Virkon® to ensure that no contamination is present. They are then washed with 'Rnase-free' water (Sigma), sprayed with Rnase zap® (Sigma) and left overnight to ensure no contaminating Rnases remain. The homogenizer to be used is incubated in Precept^{TM}, washed thoroughly with 'Rnase-free' water, autoclaved and then baked in an

oven at 60°C overnight. Ethanol (75% (v/v) in 'Rnase-free' water) was prepared in 'Rnase-free' tubes and stored at -20°C. A laminar flow hood was de-contaminated by cleaning with 70% (v/v Ethanol in 'Rnase-free' water) IMS and Rnase Zap. Materials and reagents to be used during the extraction procedure are also decontaminated. The chicken was sacrificed by cervical dislocation. The spleen was removed, placed in a 'Rnase-free' tube and homogenized in 30 mL of TriZol® reagent (Sigma) using a sterile homogenizer. The tubes were centrifuged in an Eppendorf 5810r centrifuge at 1575 g for 10 minutes at 4°C. The supernatant was removed into 50 mL Oakridge tubes containing 6 mL of chloroform. This was mixed well by shaking and incubated at room temperature for 15 minutes. The samples were then centrifuged at 17,500 g for 20 minutes at 4°C. After this centrifugation step, three visible layers are produced. The upper aqueous layer is carefully removed (ensuring that none of the organic layer of protein is transferred from the interphase) and added to a large Oakridge tube with 15 mL of isopropanol for precipitation of RNA. This is mixed and left at room temperature for 10 minutes. It is then centrifuged at 17,500 g for 20 minutes at 4°C. The supernatant is carefully removed and discarded ensuring that the pellet is not disrupted. The RNA pellet is washed by adding 30 mL of 75% (v/v) ethanol. This is centrifuged again at 17,500 g for 10 minutes at 4°C and the supernatant is again discarded. The pellet was air dried in the laminar flow cabinet and then gently resuspended in 500 µL of molecular grade water (Sigma). The RNA was subsequently quantified on the Nanodrop and cDNA synthesis is performed.

2.6.2 cDNA synthesis

Total RNA was transcribed to cDNA by reverse transcriptase PCR. The cDNA will subsequently serve as a template for the amplification of the variable heavy and variable light chain gene fragments. This reaction was performed using a Superscript III first strand cDNA synthesis kit (Invitrogen). This polymerase is superior to other superscripts due to its reduced Rnase H activity and full enzymatic activity at 50°C. The reaction components were as follows;

Mixture 1	Volume	Mixture 2	Volume
Total RNA (100ug)	22 µL	10x RT Buffer	40 µL
Oligo dT	20 µL	25mM $MgCl_2$	80µL
10mM dntp's	20 µL	0.1M DTT	40 µL
Water	138 µL	Rnase out	20 µL
		Superscript III	20 µL

Mixture 1 was split into eight 25 µL aliquots and incubated at 65°C for 5 minutes (Biometra $T_{Gradient}$ PCR machine). This was then left on ice for 1 minute. An equal volume of mixture 2 was added to each 25 µL sample of mixture 1 and incubated at 50°C for 5 minutes. The reaction was continued at 85°C for 5 minutes. Five µL of RnaseH was then added to the mixture and subsequently incubated at 37°C for 20 minutes. Finally, the eight separate aliquots were combined and stored at -20°C. The amount of cDNA present was then determined by quantification on the Nanodrop.

2.6.3 PCR primers for amplification of chicken scFv (pComb3XSS vector)

V_λ Primers

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´

V_H Primers

CSCVHo-F (sense), Short Linker

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

CSCVHo-FL (sense), Long Linker

5´ GGT CAG TCC TCT AGA TCT TCC GGC GGT GGT GGC AGC TCC GGT GGT GGC GGT 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´

Overlap Extension 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 primers listed above were obtained from Eurofins MWG and are compatible with the primers set described by Barbas for the pComb vector system (Barbas *et al*., 2004).

2.6.4 Amplification of antibody variable domain genes using pComb series primers

The standard PCR reaction components and conditions were as follows;

Component	Concentration in 50 µL reaction
cDNA	0.5 µg
dNTP's	0.2 mM
Forward primer	60 pm
Reverse primer	60 pm
$MgCl_2$	4 mM
5X buffer	1 X
Go Taq Polymerase (5units/µL)	1 unit

This mixture is made up to 50 µL with MgH_2O

Go Taq polymerase and its corresponding buffer were used. The PCR amplification of the variable regions from avian cDNA was performed in a Biometra $T_{Gradient}$ PCR machine under the following conditions:

Step 1	94°C for 5 minutes
Step 2	
30 cycles of:	94°C for 15 seconds
	56°C for 15 seconds
	72°C for 90 seconds
Step 3	72°C for 10 minutes

After successfully optimizing the amplification of the variable regions the PCR products

(5 μL of each reaction) were analysed on a 2% (w/v) agarose gel, as described in *Section 2.2.5*. The reaction was scaled up (5X), the PCR products were pooled and ethanol precipitated. The ethanol precipitated DNA was then purified from a 2% (w/v) agarose gel and DNA was quantified by measuring the absorbance at 260nm on the Nanodrop.

2.6.5 Ethanol Precipitation of DNA

One-tenth the volume of 3M sodium acetate, pH 5.2, was added to a solution of DNA. The solution was mixed and two volumes of absolute ethanol were added, mixed and incubated at –20ºC for 2 hours. Following incubation, DNA was pelleted by centrifugation at 20,000 *g* for 15-30 minutes at 4ºC in a microcentrifuge. The supernatant was removed carefully without disrupting the pellet and the tube was centrifuged at 20,000 *g* for 1 minute at 4ºC to remove any residual liquid. The pellet was washed to remove excess contaminants (especially salts and chelators) in 1 mL of 70% (v/v) ethanol and centrifuged at 20,000 *g* for 2 minutes at 4°C. The supernatant was removed carefully and the tube re-centrifuged briefly to remove any remaining liquid. The pellet was air-dried and dissolved in the required amount of molecular grade water.

2.6.5.1 Purification of the variable region gene fragments using the Qiagen clean up

The variable gene fragments were purified by excising the corresponding sized band from the agarose gel (approx. 400bp for V_H and approx. 350bp for V_L chains). The excised gel slices were weighed and binding buffer was added at a ratio of 1 g gel slice: 3 mL of binding buffer. The gel slices were incubated at 50°C until completely melted. An equal volume of isopropanol to the weight of the excised fragment was added and the resulting mixture was applied to the silica membrane of the Qiagen purification column. The column was centrifuged at 21,000 g for 1 minute in a microcentrifuge. The 'flow-through' was discarded and the column was washed with 750 μL of wash buffer. This was allowed to pass through the membrane by centrifugation at 21,000 g for 1 minute. The 'flow-through' was discarded and any residual buffer was removed by centrifuging again for a further 2 minutes. The column was transferred into a sterile 1.5 mL eppendorf tube and the DNA was eluted from the column with the addition of 30 μL MgH_2O at 60°C and centrifugation for 1 minute at 21,000 g. The eluted DNA was then quantified using the Nanodrop.

2.6.6 Splice by overlap extension PCR

In the SOE-PCR the appropriate purified variable heavy and variable light chain gene fragments are mixed in equal ratios to generate the overlap product. The V_H and V_L purified fragments were joined using an SOE-PCR via a glycine-serine linker $(G_4S)_4$ incorporated in the primers, producing a fragment of approximately 800bp.

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

Component	Concentration in 50 µL reaction
5 X buffer	1 X
$MgCl_2$	4 mM
dNTP	0.2 mM
VH	100 ng
VL	100 ng
Forward primer	60 pm
Reverse primer	60 pm
Phusion high fidelity polymerase (5 units/µL)	1 unit

This mixture is made up to 50 µL with molecular grade H_2O

The polymerase used to generate the full length SOE product was Phusion high fidelity polymerase. In Phusion polymerase a unique dsDNA-binding domain is fused to a '*Pyrococcus*-like' proofreading polymerase. This is designed to produce PCR products with high fidelity, accuracy and speed. The PCR amplification of the SOE product was performed in a Biometra $T_{Gradient}$ PCR machine under the following conditions:

Step 1	94°C for 5 minutes
Step 2	
25 cycles of;	94°C for 15 seconds
	56°C for 15 seconds

	72 °C for 2 minutes
Step 3	72 °C for 10 minutes

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

The restriction digest was performed using *Sfi*1 enzyme from NEB alongside the recommended NEB Buffer.

Restriction digest of the purified overlap PCR product:

Component concentration per reaction	**(1X)**
Purified overlap PCR product	10 µg
*Sfi*I (36U per µg of DNA)	360 U
NEB Buffer 2 (10X)	20 µL
BSA (100X)	2 µL

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

Restriction-digest of the purified vector DNA:

Component Concentration per reaction	**(1X)**
Vector DNA (pComb3XSS)	20 µg
*Sfi*I (6U per µg of DNA)	120 U
NEB Buffer 2 (10X)	20 µL
BSA (100X)	2 µL

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

The digests were incubated for 5 hours at 50ºC. The digested PCR fragment, the pComb3XSS vector and pComb3XSS stuffer fragments were purified on a 1% (w/v) agarose gel and 0.6% (w/v) agarose gel, respectively, by electroelution. DNA was quantified by measuring the absorbance on the Nanodrop at 260nm.

2.6.8 Ligation of the digested overlap PCR product with vector DNA

The gel-purified *Sfi*I-restricted scFv insert fragment was cloned into the *Sfi*1-digested pComb3XSS which was facilitated by the use of T4 DNA ligase. The ligase works by catalyzing the formation of phosphodiester bonds between 5^{1} phosphate and 3^{1} hydroxyl groups in duplex DNA.

Component Concentration per reaction	**(20X)**
pComb3XSS (digested and purified)	1.4 µg
Overlap PCR product (digested and purified)	700 ng
T4 DNA ligase buffer	40 µL
T4 DNA Ligase	10 µL

Molecular grade water was added to a total volume of 400 µL

Ligations were incubated at 16ºC overnight. The ligated vector and insert was then ethanol-precipitated, as described in *Section 2.6.6*. The DNA pellet was resuspended in 20 µL of molecular grade water and transformed into *E. coli* XL1-Blue electrocompetent cells (Stratagene) by electroporation.

2.6.8.1 Transformation of *E.coli* XL1-Blue electrocompetent cells with pComb3XSS vector containing light and heavy chain genes and measurement of transformation efficiencies

The ligated DNA and electroporation cuvettes (0.2 cm, Bio-Rad) were incubated on ice for 10 minutes. At the same time the electrocompetent *E.coli* XL1-Blue cells were thawed on ice. Each ligation was added to the cells (300 µL), mixed gently, transferred to a chilled cuvette and incubated on ice for 1 minute. The sample was then electroporated with settings of 25 µF, 1.25 kV and gene pulse controller at 200 Ω (Bio-Rad Gene Pulser Xcell^{TM}). The cuvette was immediately flushed with 1 mL SOC medium, followed by 2mL SOC (x2) at room temperature, and transferred to 50 mL polypropylene tube. The cells were incubated at 37ºC for one hour with shaking at 250 rpm. Following incubation 10 mL of pre-warmed (37ºC) SB media, containing 100 µg/mL carbenicillin, was added and the

culture was incubated for a further hour at 37ºC with shaking. Ten µL of the culture was taken to estimate the titre of transformed bacteria before incubation. PComb3XSS transformants were plated out on SB agar supplemented with 100 µg/mL carbenicillin. Control transformants were plated out on SB agar supplemented with 20 µg/mL of kanamycin. Both sets of plates were allowed to grow overnight at 37°C. PComb3XSS transformed colonies were scraped off the plates and used as library stocks. These stocks were suspended in 15% (v/v) glycerol and stored at –80°C.

2.7 Rescue and subsequent precipitation of scFv-displaying phage

The transformed library stock was inoculated into 400 mL of SB media with 100 µg/mL carbenicillin. The culture was grown at 37°C, while shaking at 250 rpm, until an $O.D_{600}$ of 0.4 - 0.5 was reached. The optimal cell density is in the mid-log phase of growth, $O.D_{600}$ is used because the cellular components of bacteria absorb greater light at wavelengths lower than 600 nm. Helper phage M13K07 (200 µL) was added to the culture, which was then incubated at 37°C stationary for 30 minutes. The culture was then incubated at 30°C for one hour, while shaking at 250 rpm. Kanamycin was added to the culture to a final concentration of 60 µg/mL and the culture was incubated overnight shaking at 30°C and 250 rpm.

An immunotube was then coated with 500 µL of 100 µg/mL halofuginone-BTG and left at 4°C stationary overnight. An overnight culture of XL1-Blue was also prepared. This was sub-cultured the next day by adding 500 µL of the overnight culture to 50 mL of fresh 2xTY and left growing until an $O.D_{600}$ of 0.4- 0.5 was reached. Agar plates were prepared with 100 µg/mL of carbenicillin. The overnight phage culture was then removed from the 30°C incubator, transferred to sterilin tubes and centrifuged for 10 minutes at 3220 g in an Eppendorf 5810r centrifuge. The phage supernatant was transferred to sterile sorval tubes and centrifuged at 15,300 g for 20 minutes. The supernatant was then transferred to clean sterile sorval tubes and PEG and NaCl were added at concentrations of 4% and 3% (w/v), respectively. The sorval tubes were incubated on ice for 1 hour at 4°C to allow for phage precipitation. Following precipitation the tubes were centrifuged at 15,300 g for 20 minutes at 4°C. The phage supernatant was discarded and residual supernatant was removed by inverting the sorval tubes onto dry tissue paper. The phage pellet was then resuspended in

500 μL of PBS and 500 μL of 3% (w/v) BSA. This suspension was the phage input. Ten microlitres of this phage library input was taken for library titre estimation.

2.7.1 Enrichment of phage library via biopanning against immobilised antigens

The antigen solution was poured off the immunotube and 3% (w/v) BSA solution was added filling the tube entirely, to ensure any immunosorbent sites not occupied by antigen are blocked by the BSA. This was left incubating at RT for 2 hours. The input phage was pre-blocked by adding it to immunotubes just coated with 1% (w/v) BSA and incubating for 1 hour at RT. The pre-blocked phage were then transferred to the halofuginone-coated immunotube and left gently shaking at RT for 2 hours. The phage were discarded and the tube was washed several times with PBST/PBS, which varied depending on the panning stage (see *Table 2.0 below,* for biopanning conditions). The tube was treated with trypsin to elute any bound phage. This was performed by adding 500 μL of trypsin-PBS solution (50 μL of trypsin 10 mg/mL in 450 μL PBS) to the tube; the trypsin was made in Tris-HCl, pH 7.4, with 1 mM $CaCl_2$. The trypsin-treated sample was then labelled the 'output'.

Table 2.0 *Biopanning conditions for the generation of the halofuginone-specific chicken scFv library using varying concentrations of halofuginone-BTG coated on immunotubes. The stringency of each consecutive round of panning was altered by increasing the number of washes with PBS (Phosphate buffered saline) and PBST (Phosphate buffered saline with 0.5% (v/v) Tween 20) and decreasing the coated antigen concentration.*

Biopanning round	Concentration of coated antigen (μg/mL)	Washing frequency
1	100	3 X PBS, 3 X PBST
2	50	5 X PBS, 5 X PBST
3	25	7 X PBS, 7 X PBST
4	10	10 X PBS ,10 X PBST
5	5	15 X PBS, 15 X PBST

2.7.2 Re-infection of *E. coli* XL1-Blue cells with eluted phage

Eluted phage were added to an overnight culture of *E. coli* XL1-Blue (5 mL) and incubated for 15 minutes at room temperature. Pre-warmed SB (6 mL), containing 20 μg/mL carbenicillin, was then added and the culture incubated at 37ºC for 1 hour. Ten μL of this culture was kept aside for estimation of output titre. Following incubation, carbenicillin was added to a final concentration of 30 μg/mL and the culture incubated for 1 hour at 37ºC, while shaking at 250 rpm. M13 helper phage (2 mL at 10^{11}pfu/mL) and pre-warmed SB (39 mL), containing 50 μg/mL of carbenicillin, were added and the culture incubated for approximately 2 hours at 37ºC while shaking at 200 rpm. Kanamycin was then added, to a final concentration of 60 μg/mL, and the culture incubated overnight at 37ºC with shaking at 200 rpm. Following incubation, the culture was centrifuged at 3,220 g in an Eppendorf 5810r centrifuge for 15 minutes at 4ºC. The supernatant was transferred to a sterile sorval tube and the phage concentrated by PEG/NaCl precipitation, as described in *Section 2.7*. Freshly prepared phage were then used in the next round of panning. After the final round of panning, the output was re-infected into the non-suppressor strain of *E.coli* Top10F for soluble scFv expression.

2.7.3 Library titre estimation

For estimation of input and output titre, infected eluted phage (10 μL) was diluted in 1 mL of SB media and plated out (in 10 and 100 μL volumes) on SB agar containing 100 μg/mL carbenicillin. The plates were incubated overnight at 37°C and the total number of transformants calculated.

2.8 Polyclonal phage pool ELISA and colony pick PCR

A Nunc Maxisorb™ plate was coated with 100 μL per well of 1 μg/mL of HFG-transferrin conjugate (and a BTG control) and incubated overnight at 4°C. The excess conjugate was discarded and the plate was subsequently blocked with 200 μL PBS containing 3% (w/v) BSA for 1 hour at 37°C. Phage (from each round of panning), diluted 3 fold in PBS containing 1% (w/v) BSA, was added to the plate in triplicate at 100 μL per well. Negative (M13 helper phage) controls were also added to the plate in triplicate. The plate was incubated for 2 hours at 37°C and then washed 3 times with PBST and 3 times with PBS.

Bound antibodies were detected following the addition of 100 µL of a HRP-labelled anti-M13 secondary antibody in PBS containing 1% (w/v) BSA. The plate was incubated for 1 hour at 37°C, washed 3 times with PBST and 3 times with PBS, and TMB substrate (Sigma) was added (100 µL/well). Following incubation for 30 minutes at 37°C, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450nm on a Tecan Safire2™ platereader.

Twenty single colonies were randomly picked from the 5th round of panning and incorporated into a 'colony-pick' PCR as outlined in Barbas *et al*., (2001), to ensure the vector was harboring the scFv fragment. A sterile tip was used to pick a single colony into the following mixture, which was then placed in a Biometra $T_{GRADIENT}$ PCR machine. The amplified scFv fragments were subsequently analysed via gel electrophoresis on a 1% (w/v) agarose gel.

Component	Concentration in 50 µL reaction
5 X buffer	1 X
$MgCl_2$	4 mM
dNTP	0.2 mM
Forward primer	60 pm
Reverse primer	60 pm
Single Colony	1 colony
Phusion high fidelity polymerase (5 units/µL)	1 unit

The colony pick PCR was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions:

Step 1	94°C for 5 minutes
Step 2	
25 cycles of;	94°C for 15 seconds
	56°C for 15 seconds
	72°C for 2 minutes
Step 3	72°C for 10 minutes

2.9 Monoclonal soluble scFv ELISA for the detection of halofuginone-specific scFv

Individual colonies (192 in total) were picked and grown overnight at 200 rpm and 37°C in single wells containing 100 µL SB media with 100 µg/mL carbenicillin (stock plates). The stock plates were then sub-cultured into fresh 2 x TY media (180 µL) containing 1 x 505 (0.5% (v/v) glycerol, 0.05% (v/v) glucose final concentration), 1 mM $MgSO_4$ and 100 µg/mL carbenicillin. Glycerol was added to the overnight stock plates to a final concentration of 15% (v/v) and then transferred to a -80°C freezer for long-term storage. The sub-cultured plates were incubated at 37°C, while shaking at 200 rpm until cells reached an optical density at 600 nm (OD_{600}nm) of ~0.6. Expression was then induced by addition of IPTG to a final concentration of 1 mM and incubating at 30°C (180 rpm) overnight. Two Nunc Maxisorb™ plates were coated with 100 µL of 1 µg/mL HFG-transferrin conjugate and incubated overnight at 4°C. The excess conjugate was discarded and the plates were blocked with 200 µL per well of PBS containing 3% (w/v) BSA for 1 hour at 37°C. Meanwhile, the overnight plates of expressed clones were removed from the 30°C incubator and subjected to a freeze-thaw protocol for the production of scFv-enriched lysate. The plates were placed at -80°C until frozen and then thawed at 37°C (this step was repeated a total of 3 times). The plates were then centrifuged at 3,220 g for 15 minutes in an Eppendorf 5810r centrifuge, to obtain the scFv-enriched lysate supernatant. The lysate supernatant (100 µL) was added to the corresponding well in each ELISA plate, mixed gently and incubated for 1 hour at 37°C. The plates were washed three times with PBST and three times with PBS, followed by the addition of 100 µL per well of HRP-labelled anti-HA antibody, at a 1/2,000 dilution in PBST containing 1% (w/v) BSA. The plate was incubated for 1 hour at 37°C, washed as previously stated, and 100 µL TMB substrate was added per well. Following incubation for 30 minutes at 37°C, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450 nm on a Tecan Safire2™ platereader.

2.9.1 Competitive ELISA for scFv characterization

Expressed scFv binding immobilised HFG-Tranferrin were subjected to competitive analysis, by incubation with free halofuginone (Halocur™). Nunc Maxisorb™ plates were coated with 1 µg/mL HFG-transferrin and blocked as described in *Section 2.8*. Different

halofuginone concentrations were incubated with an equal volume of each of the specific lysates chosen from monoclonal ELISA analysis. This mixture was added to the ELISA plate for 1 hour at 37°C. Plates were washed 3 times with PBST and 3 times with PBS and 100 µL of 1/2,000 dilution of anti-HA HRP-labelled antibody in PBS-Tween containing 1% (w/v) BSA was added to each well. The plate was incubated for 1 hour at 37°C, washed as before and TMB substrate was added (100 µL/well). Following incubation for 30 minutes at 37°C, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450nm.

2.9.2 Antibody expression and purification

Approximately 10 mL SB media, containing 1% (w/v) glucose and 100 µg/mL carbenicillin was inoculated with 100 µL of transformed scFv stocks and grown overnight at 37°C, while shaking at 250 rpm. The overnight cultures were then used to seed 500mL SB media containing 1 x 505 supplement and 100 µg/mL carbenicillin with a cell density of 1% and grown at 37ºC, while shaking at 220 rpm, until an $O.D_{600}$ nm of ~0.6 was reached. The culture was then induced by the addition of 1 mM IPTG and incubated overnight at 30°C with shaking at 220 rpm. The following morning the culture was centrifuged at 3,220 g for 30 minutes at 4°C and the supernatant was discarded. The cell pellet was thoroughly resuspended in 10 mL of lysis buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 8). This was sonicated on ice three times for 45 seconds (3 second intervals) at an amplitude of 40, using a microtip Vibra Cell™ sonicator. The cell debris was then removed by centrifuging at 15,000 g for 10 minutes at 4°C in an Eppendorf 5810r centrifuge.

2.9.2.1 Purification of anti-HFG scFv antibody fragments by immobilised metal affinity chromatography

The lysate supernatant was filtered through a 0.2 µm filter to remove any residual cell debris. Two mL of nickel resin (Ni-NTA resin slurry-Qiagen) was added to a 20 mL column and equilibrated with lysis buffer (50 mM $Na_2H_2PO_4H_2$), 300 mM NaCl, 10 mM imidazole, pH 8). The filtered lysate was then added to the equilibrated column and the 'flow-through' collected. The column was then washed with 20 mL of 50 mM

$Na_2H_2PO_4H_2O$, 300 mM NaCl, pH 8. Twenty mL of 50 mM $Na_2H_2PO_4H_2O$, 300 mM NaCl, 10 mM imidazole, pH 8, was then used to further remove any loosely bound proteins. Elution of bound scFv was performed using a high concentration of imidazole by adding 5 mL of elution buffer (50 mM $Na_2H_2PO_4H_2O$, 300 mM NaCl, 250 mM imidazole, pH 8) to the column. Antibody-containing fractions were buffer-exchanged against filtered PBS using a 5 kDa cut-off Vivaspin™ column (AGB, VS0611). The buffer-exchanged scFv was then quantified using the Nanodrop. The resultant antibody solution was dispensed in 20 µL volumes and stored at 20°C until required. The performance of the purification process was evaluated using SDS-PAGE and Western-blotting, as described in *Sections 2.5.2 and 2.5.3*.

2.9.3 ELISA characterisation

2.9.3.1 Checkerboard ELISA for the determination of optimal concentrations of immobilised antigen, and dilution of antibody, for the development of a validated ELISA for the detection of halofuginone

Nunc Maxisorb™ plates were coated with varying antigen concentrations (8 µg/mL -1 µg/mL), with each antigen concentration added to a different row on the ELISA plate. Plates were blocked with 3% (w/v) BSA. One hundred µL samples of purified antibody, serially diluted 1:5 in PBST containing 1% (w/v) BSA was added to each well and detected as described in *Section 2.8*.

2.9.3.2 Inter/Intra-day variability studies

The intra-day study was performed to assess the precision and variability within the assay by directly comparing three replicate samples within the same assay. Nunc Maxisorb™ plates were coated with the appropriate concentration of HFG-BTG conjugate and blocked, as described in *Section 2.9.3.1*. A 500 µg/mL stock of Halocur (free halofuginone) was obtained from Janssen (Intervet). Standards of decreasing concentrations of analyte were prepared in PBS. Each standard was added to an equal volume (50 µL) of HFG-specific antibody at the optimal concentration in PBST containing 1% (w/v) BSA. The samples were incubated for 30 minutes at room temperature and added to the microtitre plate in triplicate. The plates were washed 3 times with PBST and 3 times with PBS. Bound antibody was detected following the addition of HRP-labelled anti-HA antibody in PBST

containing 1% (w/v) BSA. The plate was incubated for 1 hour at 37°C, washed as before and TMB substrate was added (100 µL/well). Following incubation for 30 minutes at 37°C, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450nm on a Tecan Safire2™plate reader. The inter-assay study was performed under the same conditions on three separate days to assess the reproducibility of the assay and to determine the overall precision and accuracy of the assay. The co-efficients of variation (CVs) of the assay were calculated as a percentage from the standard deviation between results.

2.9.3.3 Cross-reactivity studies

The HFG-specific scFv was assessed for cross reactivity against five commonly used veterinary drugs, which included, Toltrazuril, Ponazuril, Nicarbazin, Diaveridine, and Ethopabate, which were obtained from Teagasc, Ashtown Food Research Centre, Co. Dublin, Ireland. Calibration curves were prepared in assay buffer, as per *Section 2.9.3.2*, using each of the individual drugs at concentrations from 1 mg/L to 1 ng/L. The IC_{50} values of the calibration curve for each respective drug were then directly compared to that of HFG to determine the degree of cross reactivity.

2.9.3.4 Precision assay and determination of analytical limits

An inhibition ELISA was performed to determine what concentration of halofuginone could be accurately distinguished from blank samples with 95% confidence. To do this, 20 blank replicas (1% (w/v) BSA in PBST), 20 replicas of 80 pg/mL and 20 replicas of 100 pg/mL halofuginone, were incubated with approximately 10 µg/mL anti-HFG scFv. The mixture was added to a HFG-Transferrin coated plate, whereby free and immobilised HFG compete for binding to the scFv. Binding of anti-HFG scFv was detected using a rat anti-HA-HRP-labelled antibody. The plate was incubated for 1 hour at 37°C, washed as before and TMB substrate was added (100 µL/well). Following incubation for 30 minutes at 37°C, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450 nm. The precision assay was used to determine the concentration of halofuginone that can be detected with 95% confidence. This requires the antibody to be able to identify at least 19 out of the 20 positive samples when assayed with 20 positive and 20 negative samples.

It is the lowest concentration of analyte where a response can be reliably distinguished from the background.

2.10 Light chain shuffling and scFv affinity maturation

In order to find the best combination of V_H-V_L pairings and to improve antibody affinity maturation, light chain shuffling was employed. The heavy chain region from the panned library (after pan 5), was amplified from the phage pool (output-Pan 5) by PCR, using the same primers listed in *Section 2.6.4* and the conditions outlined in *Section 2.6.7*. This PCR amplified the whole heavy chain pool from the enriched HFG-panned library. The light chain from the un-panned library pool (PEG-precipitated phage after initial library transformation) was also amplified, as previously described. The SOE-PCR of these new gene constructs was performed under the conditions outlined in *Section 2.6.7*. The SOE-product was produced on a large-scale and purified, as described in *Section 2.6.6.1*. This new light-heavy chain gene assembly was ligated into the pComb3XSS vector, and transformed into XL1-Blue electrocompetent cells (*Sections 2.6.9 and 2.6.9.1)*. The library was then subjected to biopanning again using a HFG-BTG conjugate (conditions are outlined in *Table 2.1*), starting from concentrations of 25 µg/mL down to concentrations as low as 2.5 µg/mL. The starting conjugate concentration was lower than the preceding biopanning selection because the shuffled library was previously enriched with HFG-specific scFv.

Table 2.1 *Biopanning conditions for the light chain-shuffled halofuginone-specific scFv library using varying concentrations of halofuginone-BTG coated on immunotubes. The stringency of each consecutive round of panning increased by adjusting the number of washes and decreasing the coated antigen concentration.*

Biopanning round	Immobilised antigen concentration (µg/mL)	Washing frequency
1	25	3 X PBS, 3 X PBST
2	12.5	5 X PBS, 5 X PBST
3	5	7 X PBS, 7 X PBST
4	2.5	10 X PBS, 10 X PBST

2.10.1 Sequencing of anti-halofuginone scFv

The avian anti-halofuginone scFv were sent to Eurofins MWG for sequencing. The clones were sent as plasmid preparations in 1.5 mL centrifugation tubes supplemented with carbenicillin (100 µg/mL). The heavy and light chain complementarity determining regions (CDRs) of the antibody were identified using the Kabat rules for antibody CDR regions. The programs used for the amino acid sequence determination and alignment of the clones were located in the Expasy website.

Expasy translate tool: http://www.expasy.ch/tools/dna.html

MultiAlin: http://multalin.toulouse.inra.fr/multalin/

2.11 Surface Plasmon Resonance characterization studies

2.11.1 Biacore maintenance

The Biacore instrument was cleaned using a 'super desorb' program at 25°C. This method involves thoroughly cleaning the unit prior to starting an assay. It is imperative that the instrument is properly sanitized to remove any protein residuals, thus ensuring high-quality data and complete confidence in generated results. A maintenance chip was docked and primed 5 times with desorb solution 1 (0.5% (v/v) (SDS)), once with water, 5 times with desorb solution 2 (50 mM glycine, pH 9.5), and, finally, 5 times with water.

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

Biacore binding assays were performed using a Biacore 3000™ (GE Healthcare) instrument using a Series 'S' CM5 sensor chip (GE Healthcare) immobilised with HFG-BTG conjugate. The chip matrix is carboxymethylated dextran covalently attached to a gold surface. The gold on the Biacore sensor chip is covered with a covalently bonded monolayer of alkanethiol molecules. This serves to protect the biological samples from contact with the gold and at the same time provides a means of attachment of a surface matrix (Biacore, Sensor surface handbook, 2005). Data analysis was performed using BiaEvaluation™ software. The running buffer for all Biacore experiments was PBST buffer, pH 7.4, and 0.05% (v/v) Tween 20. The running buffer was filtered (pore size of 0.2 µm) and degassed using a Millipore Filtration Apparatus (Millipore sintered Glass Filtration unit) immediately before use.

2.11.3 Preconcentration studies

In order to optimise the conditions needed for the immobilisation of the halofuginone conjugate on the sensor chip surface, an initial 'preconcentration' step is performed. Pre-concentration occurs from electrostatic binding of protonated amine groups on the biological component, to the negatively-charged carboxyl groups on the chip surface. Preconcentration of a desired molecule on the sensor surface can be facilitated by adjusting the pH below the isoelectric point (pI) of the molecule. Therefore, halofuginone conjugate solutions were prepared in 10 mM sodium acetate at a range of different pHs and these solutions passed over an underivatised chip surface, with the degree of electrostatic binding monitored. HFG-transferrin conjugate was diluted in 10 mM sodium acetate that had been adjusted to pH values of 4.0, 4.2, 4.4, 4.6, and 4.8 using 10% (v/v) acetic acid. The conjugate was diluted to a working concentration of 10 µg/mL, for each respective pH value, and sequentially passed over an un-activated carboxymethylated CM5 sensor chip. The pH at which highest preconcentration of protein on to the underivatised surface was observed, was chosen as the pH for immobilisation.

2.11.4 Immobilisation studies

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

2.11.5 Regeneration studies

To assess the stability of immobilised interactant (in this case the HFG-Transferrin conjugate) surfaces, a known concentration of antibody was passed over the chip surface, and the surface regenerated by passing over various concentrations of NaOH and HCl

ranging from 1-30 mM. This cycle of regeneration was usually completed for several cycles, and the binding signal measured to assess the stability and suitability of the immobilised surface for the development of a sensitive and reproducible assay.

2.11.6 Optimisation of assay parameters for SPR detection of HFG residues in a competitive format

The optimal flowrate and contact time of the anti-HFG scFv for use on the immobilised HFG-transferrin chip, was investigated. Contact times of 3, 6, 9 and 12 minutes, each at flowrates of 10, 20 and 30 μL/minute, were evaluated. This was to determine which parameters gave the highest response change with respect to analyte concentration. To assess the stability of the immobilised conjugate, regeneration scouting was performed. A known concentration of antibody was passed over the chip surface, and the surface regenerated using a panel of different regeneration solutions. The solutions used included 10 mM glycine at pH 2, 2.5 and 3.0, 1 M NaCl, 10 mM HCl, pH 1.3, 50 mM HCl, pH 1.3, 20 mM, NaOH, pH 12, 20 mM NaOH/ 1% (v/v) acetonitrile, pH 12, and 200 mM NaOH, pH 12. The regeneration studies proved that two pulses of NaOH/1% (v/v) acetonitrile for 1 minute was the most effective solution for optimal regeneration. This cycle of regeneration was completed in excess of 50 times, and the binding signal was measured to assess the stability and suitability of the immobilised surface for the development of a sensitive and reproducible assay.

2.11.7 Generation of a calibration curve for HFG

Assay reproducibility may be affected by environmental conditions and lead to significant variation in the analytical results. To determine the intra-day (within the same assay) and inter-day (between assays) variation of results, a calibration curve was generated for HFG. The co-efficients of variation (CV) values between the inter and intra-day batches of analyses were calculated from the calibration curve. For the inter/intra-day studies; egg samples were spiked with HFG at concentrations of 0, 156, 312, 625, 1,250, 2,500, and 5000 ng/mL. The spiked-HFG residues were subsequently recovered from the matrix and resuspended in PBS/ 5% (v/v) MeOH. They were mixed with an equal volume of the HFG-specific scFv at a final concentration of 10.6 μg/mL prior to injection over the sensor–chip

surface. Report points were recorded before and after sample injection. The surface was regenerated after each sample was passed across the chip surface to remove any bound scFv. The inter-assay studies were all performed on separate days; whereas, the intra-day studies were all performed in unique assays on the same day.

2.12 Extraction procedure for HFG residues from eggs

Three extraction methods previously employed for the isolation of halofuginone residues from eggs were performed and directly compared for percentage recoveries. The first method tested used an acetonitrile and hexane extraction, as described by Huet *et al.* (2005). The second method, optimised in the Teagasc Laboratories, Ashtown, utilised an acetonitrile separation, whereby the residues are extracted from the acetonitrile layer by partitioning into cyclohexane. A solid phase extraction process using C18 silica, facilitated the extraction of the residues. The third extraction method, which was subsequently employed for all extractions, was previously described by Thompson *et al*. (2009). This method used an acetonitrile separation. Organic egg was homogenised using a Ultra-Turrax T18 homogeniser at an output of 50 % for 2 minutes, and 5 g was weighed into separate tubes for each corresponding negative and spiked sample. The 'HFG-positive' standards were spiked with varying concentrations of HFG ranging from 100 ng/mL to 1 ng/mL. All samples were allowed to stand for 10 minutes at room temperature. Acetonitrile (10 mL) was added to each tube, vortexed vigorously for 10 seconds and then mixed on a roller, at room temperature, for 30 minutes. All tubes were centrifuged in a Beckmann 5810r centrifuge at 1500 g for 10 minutes at 4°C. The pellets were discarded and the supernatants were evaporated to dryness using a SpeedVac at 50°C. The resulting extracts were reconstituted in PBS/5% (v/v) methanol and then directly compared to HFG standards spiked in PBS/5% (v/v) methanol. The SPR format used to assess the efficiency of the extraction was a competitive assay format. Purified anti-HFG scFv, added to all samples at a fixed dilution, was displaced by free HFG in the samples.

2.13 Generation of a murine-derived diclazuril-specific scFv library

2.13.1. Production of carboxydiclazuril-BTG /carboxydiclazuril-HSA conjugates

Note: Conjugations were performed with the assistance of Dr. Om Prakash Edupuganti, Applied Biochemistry Group, Dublin City University, Ireland.

2.13.1.1 Synthesis of DIC-NHS ester

DIC-COOH (5 mg) was dissolved in 300 µl pyridine. To this, 1.9 mg/mL of NHS and 3.5 mg/mL of DCC were added. This was left to react by stirring at room temperature for 4 hours. The solvent was concentrated by evaporating the samples in the SpeedVac (Fisher Scientific) for 1 hour at 50°C. This was resuspended in 20% (v/v) methanol/ 30% (v/v) DCM. The mixture was subsequently purified by a silica gel column with 20% (v/v) methanol in DCM. *See* **Figure 2.0** below for a detailed schematic of the conjugation procedure.

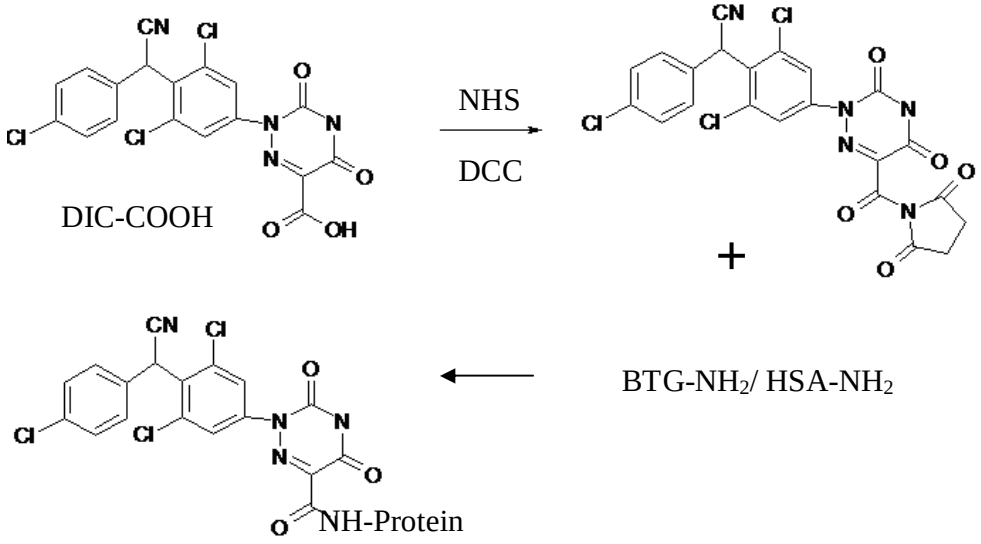

Figure 2.0 *Schematic of the conjugation procedure for the production of carboxydiclazuril-BTG and carboxydiclazuril-HSA proteins.*

2.13.1.2 Purification and de-salting of conjugates by reverse phase column chromatography using C_{18} silica gel 100

Column Equilibration-Flash chromatography

The silica gel was placed in an empty pD10 column, which had a final bed volume of 5 mL after the mobile phase was added. Molecular grade water (6 mL) was added to the column and forced down by the addition of positive air pressure. This process was repeated twice. A 6 mL volume of 50% (v/v) acetonitrile was then added to the column and passed through by the addition of air pressure. This process was then repeated. Methanol (100%) was added to the column under air pressure and this step was then repeated. The column was then ready for the sample to be loaded. Water was added to the column once more before the sample was loaded. The diclazuril succinate ester to be purified was added to the column. No air pressure was added during this step. The diclazuril derivative was passed through the column 4 times under gravity flow without any additional air pressure, to allow maximum interaction time with the column bed for efficient separation. Elution was achieved by addition of acetonitrile to the column under air pressure 5 times. In total a volume of 30 mL was eluted from the column. The column was then washed with methanol and stored for re-use. The 30 mL eluted fraction was then concentrated to a volume of 500 μL using an Amicon Vivaspin 5 kDa MW 'cut-off' column (GE Healthcare).

2.13.1.3 Conjugation of HSA-DIC.

To prepare the diclazuril-HSA conjugate, 8 mg of HSA was added to 4 mg of the succinic ester (outlined in *Section 2.13.1.1*) in potassium phosphate buffer at pH 8.2. The mixture was allowed to react by stirring overnight at room temperature. The conjugate was then buffer exchanged into PBS and concentrated by ultrafiltration (Amicon, Vivaspin). The conjugate mixture was then concentrated further by evaporation in the SpeedVac.

2.13.1.4 Conjugation of BTG-DIC

To prepare the diclazuril-BTG conjugate, 8 mg of BTG was added to 4 mg of the succinic ester (outlined in *Section 2.13.1.1*) in Potassium Phosphate buffer at pH 8.2. The mixture was allowed to react by stirring overnight at room temperature. The conjugate was then

buffer exchanged into PBS and concentrated by ultrafiltration (Amicon, Vivaspin,). The conjugate mixture was then concentrated further by evaporation in the SpeedVac.

2.13.2 Immunisation schedule for the production of anti-diclazuril scFv

A leghorn chicken was initially immunised sub-cutaneously with a mixture of equal parts of diclazuril-HSA (DIC-HSA) conjugate and Freund's complete adjuvant. The final concentration of the initial immunisation was 200 µg/mL. The first boost (day 14) was then administered using 100 µg/mL of DIC-HSA in PBS, mixed in a 1:1 ratio with Freund's incomplete adjuvant, in a final volume of 1mL. The 3 boosts that followed (days 36, 52 and 66) all contained 100 µg/mL of DIC-HSA and were administered in the same manner as the first boost. After all 4 boosts, no response was observed using the DIC-HSA conjugate.

2.13.2.1 Evaluation of the diclazuril-conjugates using a commercial anti-diclazuril polyclonal antibody (Randox)

To assess the integrity of the 'in-house' prepared conjugates, the DIC-BTG/DIC-HSA conjugates received from Queen's University Belfast and the conjugates produced in-house, a direct ELISA was performed. Concentrations of 1 µg/mL of each conjugate was added to a Nunc Maxisorb^{TM} plate and left overnight at 4°C. The conjugate-containing solution was decanted from the plate in the morning and the plate was blocked with 200 µL of 3% (v/v) BSA in PBS for 1 hour at 37°C. Dilutions of the commercial anti-diclazuril antibody from 100 µg/mL (Randox) were prepared in 1% (v/v) BSA/PBS. The plate was washed 3 times with PBST and 3 times with PBS. The antibody dilutions were added to each conjugate-coated plate and left incubating for 1 hour at 37°C. Following washing as described previously, a 1/2,000 dilution (0.5 µg/mL) of anti-rabbit-HRP-labeled antibody (Sigma) was added to the plate. The plate was incubated for 1 hour at 37°C, washed as before, and TMB substrate was added (100 µL/well). Following incubation for 30 minutes at 37ºC, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450nm on a Tecan Safire2^{TM} plate reader.

2.13.3 Immunisation of BALB/c mice with diclazuril-HSA conjugate

BALB/c mice aged 5-8 weeks were initially immunised with a final concentration of 100 µg/mL DIC-HSA, mixed in a 1:1 ratio with Freund's complete adjuvant. A volume of 200 µL was injected into the peritoneal cavity of the BALB/c mice for immunisation. Subsequent injections were performed with 25 µg/mL DIC-HSA mixed in a 1:1 ratio with Freund's incomplete adjuvant. A tail bleed was taken from the mice 7 days after injection and the antibody titre against diclazuril determined. Once a high titre response was reached, the mice were given a final boost into the peritoneal cavity and then sacrificed 7 days later.

2.13.4 Extraction and isolation of total RNA from immunised mice

Prior to the extraction procedure, several preparatory steps are taken. Oakridge tubes are cleaned with Virkon® to ensure no contamination is present. They were then washed with 'Rnase free' water (Sigma), sprayed with RnaseZap® (Sigma) and left overnight to ensure no contaminating Rnases remain. The homogenizer to be used is incubated in Precept™, washed thoroughly with 'Rnase free' water, autoclaved and then baked in a 60°C oven overnight. Ethanol (75% (v/v) in 'Rnase free' water) was prepared in 'Rnase free' tubes and stored at -20°C. A laminar flow hood was de-contaminated by cleaning with 70% (v/v) IMS in 'Rnase free' water and RnaseZap. Materials and reagents to be used during the extraction procedure are also decontaminated. The mice were sacrificed by cervical dislocation. The spleen was removed, placed in an 'Rnase free' tube and homogenized in 10 mL of TriZol reagent (Sigma) using a sterile homogenizer. The tubes were centrifuged in an Eppendorf 5810r centrifuge at 1575 g for 10 minutes at 4°C. The supernatant was removed into 50 mL Oakridge tubes containing 2 mL of chloroform. This was mixed well by shaking and incubated at room temperature for 15 minutes. The samples were then centrifuged at 17,500 g for 20 minutes at 4°C. After this centrifugation step, three visible layers are produced. The upper aqueous layer is carefully removed ensuring that none of the organic layer of protein is transferred from the interphase. This aqueous layer was then added to a large Oakridge tube with 5 mL of isopropanol for precipitation. This is mixed and left at room temperature for 10 minutes. It is then centrifuged at 17,500 g for 20 minutes at 4°C. The supernatant is carefully removed and discarded ensuring that the pellet is not disrupted. The RNA pellet is washed by adding 10 mL of 75% (v/v) ethanol. This is

centrifuged again at 17,500 g for 10 minutes at 4°C in an Eppendorf 5810r centrifuge and the supernatant is again discarded. The pellet is air dried in the laminar flow cabinet and then gently resuspended in 250 µl of molecular grade water (Sigma). The RNA is subsequently quantified on the Nanodrop and cDNA synthesis is performed.

2.13.5 Reverse transcription of total RNA to cDNA

This protocol is performed as outlined in *Section 2.6.2*

2.13.6 cDNA synthesis

This protocol is performed as outlined in *Section 2.6.2*

2.13.7 PCR Primers for the construction of a murine scFv library (pComb3XSS vector).

All primers were synthesized by MWG.

Vκ5' 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′

Vκ 3' Reverse primers

MSCJK12-BL
5′ GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA TTT KAT TTC CAG YTT GGT CCC 3′

MSCJK4-BL
5′ GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA TTT TAT TTC CAA CTT TGT CCC 3′

MSCJK5-BL
5′ GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA TTT CAG CTC CAG CTT GGT CCC 3′

Vλ 5' Sense primer

MSCVL-1
5′ GGG CCC AGG CGG CCG AGC TCG ATG CTG TTG TGA CTC AGG AAT C 3′

V λ 3' Reverse Primer

MSCJL-BL

5′ GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA GCC TAG GAC AGT CAG TTT GG 3′

VH 5' Sense primers

MSCVH1

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTR MAG CTT CAG GAG TC 3′

MSCVH2
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTB CAG CTB CAG CAG TC 3´
MSCVH3
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG CAG CTG AAG SAS TC 3´
MSCVH4
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTC CAR CTG CAA CAR TC 3´
MSCVH5
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTY CAG CTB CAG CAR TC 3´
MSCVH6
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTY CAR CTG CAG CAG TC 3´
MSCVH7
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTC CAC GTG AAG CAG TC 3´
MSCVH8
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AAS STG GTG GAA TC 3´
MSCVH9
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AWG YTG GTG GAG TC 3´
MSCVH10
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG CAG SKG GTG GAG TC 3´
MSCVH11
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG CAM CTG GTG GAG TC 3´
MSCVH12
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AAG CTG ATG GAR TC 3´
MSCVH13
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG CAR CTT GTT GAG TC 3´
MSCVH14
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTR AAG CTT CTC GAG TC 3´
MSCVH15
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AAR STT GAG GAG TC 3´
MSCVH16
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTT ACT CTR AAA GWG TST G 3´
MSCVH17
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTC CAA CTV CAG CAR CC 3´
MSCVH18
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AAC TTG GAA GTG TC 3´
MSCVH19
5´ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AAG GTC ATC GAG TC 3´

VH 3'Reverse primers

MSCG1ab-B

5´ CCT GGC CGG CCT GGC CAC TAG TGA CAG ATG GGG STG TYG TTT TGG C 3´

MSCG3-B

5´ CCT GGC CGG CCT GGC CAC TAG TGA CAG ATG GGG CTG TTG TTG T 3´
MSCM-B
5´ CCT GGC CGG CCT GGC CAC TAG TGA CAT TTG GGA AGG ACT GAC TCT C 3´

Overlap extension primers

RSC-F (sense)

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

RSC-B (reverse)
5´ GAG GAG GAG GAG GAG GAG CCT GGC CGG CCT GGC CAC TAG TG 3´

2.13.8 Amplification of antibody variable domain genes using pComb series primers

The standard PCR reaction components and conditions were as follows;

Component	Concentration in 50 µL reaction
cDNA	0.5 µg
dNTP's	0.2 mM
Forward primer	60 pm
Reverse primer	60 pm
$MgCl_2$	4 mM
5X buffer	1 X
Go Taq Polymerase (5units/µL)	1 unit

This mixture is made up to 50 µL with MgH_2O

The polymerase used was Go Taq polymerase (Polymerase) and the corresponding buffer. The PCR amplification of the variable regions from murine cDNA was performed in a Biometra $T_{Gradient}$ PCR machine under the following conditions:

Step 1	94°C for 5 minutes
Step 2	
30 cycles of;	94°C for 15 seconds
	56 °C for 30 seconds
	72 °C for 90 seconds
Step 3	72 °C for 10 minutes

After successfully optimizing the amplification of the variable regions, five microlitres of each reaction was analysed on a 2 % (w/v) agarose gel, as described in *Section 2.5.1*. The reaction was scaled up (10X) and the PCR products were pooled and ethanol-precipitated. The PCR products were purified from a 2 % (w/v) agarose gel, and DNA was quantified by measuring the absorbance at 260 nm on the Nanodrop.

2.13.8.1 Ethanol-precipitation and purification of PCR fragments

PCR fragments were ethanol-precipitated and purified via a gel clean-up kit as described in *Sections 2.6.6* and *2.6.6.1*.

2.13.9 Splice by overlap extension PCR

In the SOE-PCR, the appropriate purified variable heavy and variable light chain (V_H and V_L) gene fragments are mixed in equal ratios to generate the overlap product. The V_H and V_L purified fragments were joined using an SOE-PCR via a glycine-serine linker $(G_4S)_4$, producing a fragment of approximately 800 bp.

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

Component	Concentration in 50µL reaction
5 X buffer	1 X
$MgCl_2$	4 mM
dNTP	0.2 mM
VH	10 ng
VL	10 ng
Forward primer	60 pm
Reverse primer	60 pm
Phusion high fidelity polymerase (5 units/µL)	1 unit

This mixture is made up to 50 µL with MgH_2O

The PCR amplification of the SOE product was performed in a Biometra $T_{Gradient}$ PCR machine under the following conditions:

Step 1	94°C for 5 minutes
Step 2	
20 cycles of;	94°C for 15 seconds
	56 °C for 15 seconds
	72 °C for 2 minutes
Step 3	72 °C for 10 minutes

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

Restriction digest of the purified overlap PCR product:

Component Concentration per reaction	(1X)
Purified overlap PCR product	10 µg
*Sfi*I (36U per µg of DNA)	360 U
NEB Buffer 2 (10X)	20 µL
BSA (100X)	2 µL

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

Restriction-digest of the purified vector DNA:

Component Concentration per reaction	(1X)
Vector DNA (pComb3XSS)	20 µg
*Sfi*I (6U per µg of DNA)	120 U
NEB Buffer 2 (10X)	20 µL
BSA (100X)	2 µL

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

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

2.13.11 Ligation of the digested overlap PCR product with vector DNA

The gel-purified *Sfi*I restricted scFv insert fragment was cloned into the digested pComb3XSS facilitated by the use of T4 DNA ligase.

Component Concentration per reaction	(20X)
pComb3XSS (digested and purified)	1.4 µg
Overlap PCR product (digested and purified)	700 ng
T4 DNA ligase buffer	40 µL
T4 DNA Ligase	10 µL

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

Ligations were incubated at 16ºC overnight. The ligation was then ethanol-precipitated, as described in *Section 2.6.6.* The DNA pellet was resuspended in 20 µL of molecular grade water and transformed into *E. coli* XL1-Blue electrocompetent cells (Stratagene) by electroporation.

2.13.12 Transformation of *E.coli* XL1-Blue electrocompetent cells with pComb3XSS vector containing light and heavy chain genes and measurement of transformation efficiencies

The transformation of the ligated pComb3XSS-scFv plasmid and calculation of transformation efficiencies was performed via electroporation into XL1-Blue *E.coli* cells as previously described in *Section 2.6.9.1.* Following incubation, cells were plated out on SB agar plates and incubated at 37°C overnight. Transformants were also titred, using SB media as a diluent, and each dilution was plated on SB plates. Plates were scraped using SB, from which glycerol library stocks were prepared. Library stocks were flash frozen in liquid nitrogen and stored at -80°C.

2.13.13 Rescue and subsequent precipitation of scFv-displaying phage

The transformed library stock was inoculated into 400 mL of SB media with 100 µg/mL carbenicillin. The culture was grown at 37°C/250rpm until an O.D 600 of 0.4 - 0.5 was reached. Helper phage M13K07 (200 µL) was added to the culture, which was then

incubated at 37°C stationary for 30 minutes. The culture was then incubated while shaking at 30°C/250rpm for 1 hour. Kanamycin was added to the culture to a final concentration of 60 µg/mL and the culture was incubated overnight shaking at 30°C/250rpm.

An immunotube was coated with 500 µl of 100 µg/mL of diclazuril-BTG and left at 4°C stationary, overnight. An overnight culture of XL1-Blue was also prepared. This was sub-cultured in the morning by adding 500 µL of the overnight culture to 50 mL of fresh 2xTY and growing until an O.D 600 of 0.4- 0.5 was reached. Agar plates were prepared with 100 µg/mL of carbenicillin incorporated. The overnight phage culture was then removed from the 30°C incubator, transferred to sterilin tubes and centrifuged for 10 minutes at 3,220 g in an Eppendorf 5810r centrifuge. The phage supernatant was transferred to sterile sorval tubes and centrifuged at 15,300 g for 20 minutes. The supernatant was then decanted into sterile sorval tubes again and Peg and NaCl were added at concentrations of 4% and 3% (w/v) respectively. The sorval tubes were incubated on ice for 1 hour at 4°C to allow for phage precipitation. Following precipitation, the sorvals were centrifuged at 15,300 g for 20 minutes at 4°C. The phage supernatant was discarded and residual supernatant was removed by inverting the sorval tubes onto dry tissue paper. The phage pellet was then resuspended in 500 µL of PBS and 500 µL of 3% BSA. This solution was the phage input. Ten µL of this was taken for library titre estimation.

2.13.14 Enrichment of phage library via biopanning against immobilised antigens

The antigen solution was poured off and 3% BSA solution was added, filling the tube to the top. This was left incubating at RT for 2 hours. The input phage was pre-blocked by adding it to the immunotubes that were coated with 1% BSA and incubating for 1 hour at RT. The BSA solution was then poured off and the immunotubes were washed once gently with PBS. The pre-blocked phage was then added to the immunotubes and left gently shaking at RT for 2 hours. The phage was discarded and the tube was washed several times with PBST/PBS. This varied depending on the panning stage (*See Table 2.2)* for biopanning conditions. The tube was treated with trypsin to elute any bound phage. This was performed by adding 500 µL of trypsin-PBS solution (50µL of trypsin 10mg/mL in 450µL PBS) to the tube; the trypsin was made in Tris-HCl pH 7.4 with 1mM $CaCl_2$. The eluted phage was labelled the 'output'.

Table 2.2 *Biopanning conditions for diclazuril-specific murine scFv library using varying concentrations of diclazuril-BTG coated on immunotubes. The stringency of each consecutive round of panning increased by altering the number of washes with PBS and PBST and decreasing the coated antigen concentration*.

Biopanning round	Concentration of coated antigen (µg/mL)	Washing frequency
1	100	3 X PBS, 3 X PBST
2	50	5 X PBS, 5 X PBST
3	25	7 X PBS, 7 X PBST
4	10	10 X PBS ,10 X PBST

2.13.15 Re-infection of *E. coli* XL1-Blue cells with eluted phage/Library titre estimation

Subsequent rounds of panning were performed by re-infecting XL1-Blue cells with the eluted phage. This was performed as described in *Section 2.7.2* and the library titre estimation was carried out as described in *Section 2.7.3*.

2.13.16 Polyclonal phage pool ELISA and colony pick PCR

Two Nunc Maxisorb™ plates were coated with 100 µL per well of 1 µg/mL DIC-HSA conjugate and with 100 µL per well of 1 µg/mL DIC-BTG conjugate, respectively, and incubated overnight at 4ºC. The excess conjugate was discarded and the plate was then blocked with 200 µL PBS containing 3% (w/v) BSA for 1 hour at 37°C. Phage, from each round of panning diluted 3 fold in PBS containing 3% (w/v) BSA, was added to the plate in triplicate at 100 µL per well. Negative (M13 helper phage) controls were also added to the plate in triplicate. The plate was incubated for 2 hours at 37°C and washed 3 times with PBST and 3 times with PBS. Bound antibodies were detected following the addition of 100 µL of an anti-M13 HRP-labelled secondary antibody in PBS containing 1% (w/v) BSA. The plate was incubated for 1 hour at 37°C, washed as before, and TMB substrate (Sigma) was added (100 µL/well). Following incubation for 30 minutes at 37ºC, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450nm on a Tecan Safire2™ plate reader.

Twenty single colonies were randomly picked from the 4th round of panning and incorporated into a 'colony-pick' PCR to ensure the vector was harboring the scFv fragment. A sterile tip was used to pick a single colony into the following mixture, which was then placed in a Biometra $T_{GRADIENT}$ PCR machine. The amplified scFv fragments were then analysed via gel electrophoresis on a 1% (w/v) agarose gel.

Component	Concentration in 50 µL reaction
5X buffer	1X
$MgCl_2$	4 mM
dNTP	0.2 mM
Forward primer	60 pm
Reverse primer	60 pm
Single Colony	1 colony
Phusion high fidelity polymerase (5 units/µL)	1 unit

The SOE-PCR was performed in the Biometra $T_{GRADIENT}$ PCR machine under the following conditions:

Step 1	94°C for 5 minutes
Step 2	
20 cycles of;	94°C for 15 seconds
	56°C for 15 seconds
	72°C for 2 minutes
Step 3	72°C for 10minutes

2.13.17 Monoclonal soluble scFv ELISA

Individual colonies (192 in total) were picked and grown overnight at 200 rpm and 37°C in single wells containing 100 µL SB media with 100 µg/mL carbenicillin (stock plates). The stock plates were then sub-cultured into fresh 2 x TY media (180 µL) containing 1 x 505 (0.5% (v/v) glycerol, 0.05% (v/v) glucose final concentration), 1 mM $MgSO_4$ and 100 µg/mL carbenicillin. Glycerol was then added to the overnight stock plates to a final

concentration of 15% (v/v) and then transferred to a -80°C freezer for long-term storage. The sub-cultured plates were incubated at 37°C while shaking at 200 rpm, until cells reached an optical density at 600 nm (OD_{600}nm) of ~0.6. Expression was then induced by adding IPTG to a final concentration of 1 mM and incubating at 30°C (180 rpm) overnight. Two Nunc Maxisorb™ plates were coated with 100 µL of 1 µg/mL DIC-BTG conjugate and two were coated with 100 µL of 1 µg/mL DIC-HSA and incubated overnight at 4ºC. The excess conjugate was discarded and the plates were blocked with 200 µL per well of PBS containing 3% (w/v) BSA for 1 hour at 37°C. Meanwhile, the overnight plates of expressed clones were removed from 30°C and subjected to a freeze-thaw protocol for the production of scFv-enriched lysate. The plates are placed at -80°C until frozen and then thawed at 37°C. This step was repeated 3 times in total. The plates were then centrifuged at 3,220 g for 15 minutes to obtain the scFv-enriched lysate supernatant. The lysate supernatant (100 µL) was added to the corresponding well in each ELISA plate, mixed gently and incubated for 1 hour at 37ºC. The plates were washed three times with PBST and three times with PBS, followed by the addition of 100 µL per well of HRP-labelled anti-HA antibody, at a 1 in 2,000 dilution in PBST containing 1% (w/v) BSA. The plate was incubated for 1 hour at 37°C, washed as before and TMB substrate was added (100 µL/well). Following incubation for 30 minutes at 37ºC, the reaction was stopped by the addition of 10% HCl and the absorbance read at 450nm.

2.13.18 Competitive ELISA for scFv characterization

To determine if any clones were specific for binding to free diclazuril, a competitive ELISA was performed. Clones exhibiting binding towards both of the diclazuril conjugates were taken forward for competitive analysis. An ELISA plate was coated with 1 µg/mL of DIC-HSA. Single clones were grown and expressed as described in *Section 2.13.17*. The scFv-enriched lysate (50 µL) was added to one half of the plate with 50 µL of PBS (A0) and to the other half of the plate with 50 µL of 20 µg/mL free diclazuril (competitor). The free and immobilised diclazuril compete for binding to the scFv. The plates were washed 3 times with PBST and 3 times with PBS, followed by the addition of 100 µL per well of HRP-labelled anti-HA antibody, at a 1 in 2,000 dilution in PBS containing 1% (w/v) BSA. The plate was incubated for 1 hour at 37°C, washed as before, and TMB substrate was

added (100 µL/well). Following incubation for 30 minutes at 37ºC, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450 nm.

2.14 Production and characterization of polyclonal antibodies to toltrazuril

2.14.1 Production of trifluoraminoether hapten-protein conjugates

Diazonium groups react with active hydrogens on aromatic rings to give covalent diazo-bonds. Generation of a diazonium-reactive group usually occurs when an aromatic amine is reacted with sodium nitrite under acidic conditions (Hermanson, 2005). Trifluoraminoether (TFAE) was conjugated to BSA and BTG by exploiting this diazo-coupling method. *See* **Figure 5.0** for a schematic of the reaction mechanism for the preparation of the conjugate. TFAE (10 mg) was initially dissolved in 0.5 M H_2SO_4. 1 mL of sodium nitrite was then added drop wise to the TFAE over three minutes. This was allowed to react for a further three minutes. The diazotized derivative was then added to BSA (5mg) and BTG (5mg) each dissolved in borate buffer. This was reacted for 4 hours at room temperature. The conjugate was then concentrated by ultrafiltration (Amicon, Vivaspin,).

2.14.2 Immunisation of New Zealand white rabbits and antibody titre determination

The immunisation of ten New Zealand white rabbits was performed externally by the CER Groupe, Belgium. Five rabbits were immunised with the prepared TFAE-BSA conjugate and five rabbits were immunised with a commercially purchased toltrazuril-BSA conjugate (Randox, Cat. No PAS9456). An immunisation schedule was performed over a period of 9 months, upon which time the animals were sacrificed. Serum from each of the rabbits was obtained from the CER Groupe, and analysed by ELISA.

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

Nunc Maxisorb™ immunoplates were coated with 100 µL per well of the toltrazuril-BTG (Randox) and the TFAE-BTG conjugates at a concentration of 1 µg/mL and incubated overnight at 4ºC. Excess conjugate was discarded. The plates were then blocked with PBS containing 5 % (w/v) Milk Marvel for 1 hour at 37ºC. Serial dilutions of each of the rabbit anti-serum in PBS containing 1% (w/v) Milk Marvel were added to the wells and allowed to bind at 37ºC for 1 hour. The plate was washed once with PBS. This was followed by the

addition of a 1 in 2,000 dilution of HRP-labelled goat anti-rabbit antibody (Sigma) in PBS containing 1 % (w/v) Milk Marvel. The plate was incubated for 1 hour at 37°C, washed as before, and TMB substrate was added (100 µL/well). Following incubation for 30 minutes at 37ºC, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450nm on a Tecan Safire2^{TM} platereader.

2.14.4 Competitive ELISA for rabbit antiserum

Nunc Maxisorb^{TM} plates were coated with the appropriate concentration of conjugate and blocked as described in *Section 2.14.3*. Standards with decreasing concentrations of free analyte, were prepared in PBS from a 1 mg/mL stock solution of ponazuril. Toltrazuril is metabolized to toltrazuril-sulfone or 'ponazuril', as it is commonly referred to, which is the marker residue for toltrazuril. Each ponazuril standard was added to the microtitre plate with an equal volume (50 µL) of each of the rabbit anti-sera, prepared in PBST containing 1 % (w/v) Milk Marvel, at the optimal dilution. Bound polyclonal antibody was detected as before (*Section 2.14.3*).

2.14.5 Protein G purification of anti-toltrazuril polyclonal antibody from rabbit serum

A 2 mL suspension of immobilised protein G (immobilised on Sepharose 4B, stored in sterile-filtered PBS containing 20% (v/v) ethanol), was equilibrated in a column with 30 mL of sterile-filtered PBS. Five mL of serum from each of the 'toltrazuril-specific' serum batches, as determined by ELISA (*Section 2.14.4*), were pooled and made up to a final volume of 20 mL with sterile-filtered 1 x PBS (pH 7.4). All 20 mL of the diluted pAb-rich serum was then passed through the column, the eluant collected and passed through the column a second time. A total of 30 mL of wash buffer (sterile-filtered PBS) was passed through the column and, finally, the retained protein was eluted with 0.1 M glycine-HCl buffer (pH 2.5). Fractions of eluate were collected in micro-centrifuge tubes containing 150 µL of neutralisation buffer (2 M Tris-HCl, pH 8.5). This served to rapidly neutralise the highly acidic environment of the elution buffer, thereby, preventing denaturation of the eluted IgG fraction. Each of the fractions was quantified on the Nanodrop, using the pre-programmed 'IgG' option (mass extinction coefficient of 13.7 at 280 nm). The fractions

containing high concentrations of IgG were pooled. The purified IgG was then buffer exchanged into PBS and concentrated using an Amicon 5 kDa cut-off (Vivaspin). This was stored at -20°C until required for further use.

2.15 Toltrazuril antibody characterisation

2.15.1 Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of toltrazuril

2.15.1.1. Checkerboard ELISA for determination of optimal polyclonal antibody dilution and optimal coating concentration of protein conjugates

A Nunc Maxisorb^{TM} plate was coated with 100 µL of varying concentrations (1- 8 µg/mL) of Toltrazuril-BTG, prepared in PBS overnight at 4ºC. Plates were blocked with 200 µL of PBS containing 5 % (w/v) Milk Marvel for 1 hour at 37ºC. The plates were then washed 3 times with PBST and 3 times with PBS. Serial dilutions (1:5) of the purified polyclonal antibody were prepared in PBST containing 1% (w/v) Milk Marvel and 100 µL of each dilution was added to the plates in triplicate. The plates were then incubated at 37ºC for 1 hour and washed as previously described. This was followed by the addition of 100 µL of a 1 in 2,000 dilution (0.5 µg/mL) of HRP-labelled goat anti-rabbit antibody (Sigma) in PBST containing 1% (w/v) Milk Marvel. The plates were again incubated for 1 hour at 37°C, washed as before, and TMB substrate was added (100 µL/well). Following incubation for 30 minutes at 37ºC, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450 nm on a Tecan Safire2^{TM}.

2.15.1.2 Determination of optimal conjugate coating buffer for ELISA detection of toltrazuril

A Nunc Maxisorb^{TM} plate was coated with 100 µL of the optimal concentration of TLZ-BTG prepared in several different buffers at 4ºC overnight. One µg/mL of TLZ-BTG was coated in each of the buffers; 100mM acetate buffer, pH 4.8, 100mM TBS buffer, pH 7.4, 100mM TBS, pH 4.2, 100mM carbonate buffer, pH 10.3 and 100mM PBS, pH 7.4. Plates were blocked with 200 µL of PBS containing 5 % (w/v) Milk Marvel for 1 hour at 37ºC. The plates were then washed 3 times with PBST and 3 times with PBS. Serial dilutions (1:5) of the purified polyclonal antibody were prepared in PBST containing 1% (w/v) Milk

Marvel and 100 µL of each dilution was added to the plates. The plates were then incubated at 37ºC for 1 hour and washed as before. This was followed by the addition of 100 µL of a 1 in 2,000 dilution of HRP-labelled goat anti-rabbit antibody (Sigma) in PBST containing 1% (w/v) Milk Marvel.The plates were again incubated for 1 hour at 37°C, washed as before, and TMB substrate was added (100 µL/well). Following incubation for 30 minutes at 37ºC, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450nm on a Tecan Safire2™.

2.15.1.3 Inter/Intra-day variability studies for the detection of toltrazuril

Nunc Maxisorb™ plates were coated with 2 µg/mL of TLZ-BTG conjugate and blocked, as described in *Section 2.15.1.2.* A 1 mg/mL stock of Ponazuril (toltrazuril-derivative) was prepared in 10% (v/v) Methanol. Standards of decreasing concentrations of analyte were prepared in PBS. Each standard was added to an equal volume (50 µL) of toltrazuril-specific antibody at the optimal concentration in PBST containing 1% (w/v) Milk Marvel. The samples were incubated for 30 minutes at room temperature and added to the microtitre plate in triplicate. Bound antibody was detected following the addition of HRP-labelled anti-rabbit antibody in PBST containing 1% (w/v) Marvel. The plate was incubated for 1 hour at 37°C, washed as before and SureBlue TMB substrate (Sigma) was added (100 µL/well). Following incubation for 30 minutes at 37ºC, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450nm on a Tecan Safire2™. This was the intra-day study, performed in triplicate within the same assay. The inter-assay study was performed in the exact same manner, except it was performed on three separate days.

2.15.1.4 Precision assay and determination of the analytical limits of the ELISA for toltrazuril-detection

A Nunc Maxisorb™ plate was coated with 2 µg/mL of TLZ-BTG conjugate and blocked, as described in *Section 2.15.1.2.* An inhibition ELISA was performed using 20 blank replicas (1 % (w/v) Milk Marvel in PBST) with 20 replicas of 900 pg/mL of ponazuril diluted in 1 % (w/v) Milk Marvel PBST. Binding of anti-toltrazuril antibodies was detected using a 1/2,000 dilution of goat-anti-rabbit-HRP-labelled antibody. The plate was

incubated for 1 hour at 37°C, washed as before and TMB substrate was added (100 µL/well). Following incubation for 30 minutes at 37ºC, the reaction was stopped by the addition of 10% (v/v) HCl and the absorbance read at 450nm. The precision assay was used to determine the concentration of toltrazuril that can be detected with 95 % confidence, where at least 19 out of 20 positive samples are mixed with 20 negative samples.

2.15.2 Development of a SPR-based immunoassay for the detection of toltrazuril using a BIAcore 3000^{TM} instrument

Biacore binding assays were performed using a Biacore 3000™ (GE Healthcare) instrument on a Series 'S' CM5 sensor chip (GE Healthcare) immobilised with a TLZ-BTG conjugate using EDC/NHS coupling. The chip matrix is carboxymethylated dextran covalently attached to a gold surface. Data analysis was performed using BiaEvaluation™ software. The running buffer for all Biacore experiments was PBST buffer, pH 7.4, and 0.05% (v/v) Tween 20. The running buffer was filtered (pore size of 0.2 µm) and degassed using a Millipore Filtration Apparatus (Millipore Sintered Glass Filtration Unit) immediately before use.

2.15.2.1 Preconcentration studies for the immobilization of toltrazuril on a CM5 Biacore sensorchip

Preconcentration studies were performed as described in *Section 2.11.3*. TLZ-BTG solutions were prepared in 10 mM sodium acetate at a range of different pH values. These solutions were passed over an underivatised chip surface, with the degree of electrostatic binding monitored. TLZ-BTG conjugate was diluted in 10 mM sodium acetate that had been adjusted to pH values of 4.0, 4.2, 4.4, 4.6, and 4.8, using 10% (v/v) acetic acid. The conjugate was diluted to a working concentration of 10 µg/mL for each respective pH value and sequentially passed over an un-activated carboxymethylated CM5 sensor chip. The pH at which highest preconcentration of protein on to the underivatised surface was observed, was chosen as the pH for immobilisation.

2.15.2.2 Immobilisation studies for toltrazuril-BTG on the surface of a CM5 sensorchip

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

2.15.2.3 Biacore inhibition immunoassay on immobilised sensor chip surface

Standards of free ponazuril were prepared at varying concentration ranges. Each standard was incubated with an equal volume of toltrazuril-specific antibody and allowed to equilibrate for 30 minutes at RT. Each sample was then injected over the immobilised sensor surface in triplicate and the surface was regenerated with 20mM NaOH. A calibration curve was constructed by plotting the change in response (RU) for each standard, against the log of the concentration. A four-parameter equation was then fitted to the data using BIAevaluation software 4.0.1.

2.15.3 Development of a lateral-flow based immunoassay for toltrazuril-detection using coloured carboxylated-polystyrene microspheres

Preliminary lateral-flow studies were performed using a toltrazuril-BTG conjugate, toltrazuril-specific polyclonal antibodies, a HRP-labelled anti-rabbit secondary antibody and TMB substrate. This format was assessed for proof-of-concept of the lateral-flow immunoassay, prior to the development of the final lateral flow ‘multianalyte’ assay. The final assay was performed using coloured carboxylated microspheres.

2.15.3.1 Development of a competitive lateral-flow based assay for the detection of toltrazuril using TMB substrate

HiFlow Plus HF135 nitrocellulose membrane (Millipore) was sprayed using a Linomat 5 system (CAMAG) machine with 20 µL of approximately 10 µg/mL anti-toltrazuril polyclonal antibody across the bottom of a 3 cm wide nitrocellulose strip. This process was repeated for both the toltrazuril-BSA conjugate (20 µg/mL) at the test line, and a HRP-labelled anti-rabbit antibody (1 µg/mL) at the control line. This was performed at a flow-rate of 600 nL/sec using the Linomat 5 system (CAMAG). Several different spotting buffers were tested for optimal performance, these included; 2mM borate buffer pH 7.0 (0.1% (w/v) BSA, 0.05% (v/v) Tween, 0.01% (v/v) Triton X), 50mM sodium phosphate, pH 7.5, (1% (w/v) trehalose) and 15mM sodium citrate, pH 7.0, (0.01% (v/v) Triton X). The trehalose is used in the spotting buffer to stabilise the conjugate when it is dried onto the conjugate pad. The membranes were incubated at 37°C for 10 minutes and the sprayed membrane was cut into 3 identical strips each 1 cm thick. All 3 strips were then blocked by incubation with 5% (w/v) Milk Marvel at room temperature for 2 hours with agitation. The blocking solution was discarded and the membranes were washed once with PBS. The running buffer utilized in the assay was optimised by assessing the performance of the following running buffers; 2mM borate buffer, pH 7.0, (0.05% (v/v) Tween), 50mM sodium phosphate, pH 7.5, (0.05% (v/v) Tween) and 15mM sodium citrate, pH 7.0, (0.05% (v/v) Tween). Each of the strips was placed into 5 mL solutions containing varying concentrations of ponazuril in the optimal running buffer. Once the solution had visibly travelled to the top of the strip, the ponazuril solutions were discarded and the membranes were washed in the appropriate running buffer (x3) with 0.05% (v/v) Tween included (x3). Following the wash step, the membranes were incubated with a 1 µg/mL solution of HRP-labelled anti-rabbit antibody in running buffer. The anti-rabbit antibody was allowed to interact with the membrane for 2 hours at room temperature with agitation, and subsequently the membranes were washed as previously described. Development of each of the membrane strips was achieved via the addition of 1 mL of liquid TMB substrate to each membrane strip. This reaction was stopped by multiple washes with distilled water

2.15.3.2 Covalent coupling of antibodies to carboxylated polystyrene microparticles by carbodiimide coupling

Five hundred µL of green-coloured carboxylated microspheres were diluted in 0.1 M carbonate buffer, pH 10.8. This mixture was centrifuged for 5 minutes at 17,500 g in a microcentrifuge. The supernatant was carefully removed and discarded. The pellet was resuspended in 1.5 mL of 0.1 M carbonate buffer and the centrifugation/resuspension process was repeated another three times. The pellet was then resuspended in 750 µL of 0.1 M MES buffer. This was centrifuged, as previously described, and the supernatant discarded. This process was repeated a further two times. The pellet was resuspended in 625 µL of 0.1 M MES buffer. An equal volume of carbodiimide (2%) was added dropwise to the resuspended particles. This mixture was allowed to incubate for 3-4 hours at room temperature. The particles were pelleted by centrifugation as before, and the pellet was once again resuspended in 750 µL of 0.1 M MES buffer. The centrifugation/resuspension process was repeated a further three times to get rid of any unreacted carbodiimide. Following this, the pellet was resuspended in 1.2 mL of 0.2 M borate buffer. Approximately 400 µg of antibody was added to the solution and allowed to mix gently overnight at room temperature on an end-to-end mixer. The following day the mixture was centrifuged for 10 minutes at 15,300 g, the pellet was then resuspended in 1.2 mL of borate buffer. Fifty µL of 0.25 M ethanolamine was added to the solution and allowed to mix gently for 30 minutes to block any unreacted carbodiimide sites on the microparticle. The blocked microspheres were then centrifuged at 15,300 g for 10 minutes and the pellet was resuspended in 1 mL of a 10 mg/mL (w/v) BSA solution in 0.2 M borate buffer. The resuspended microparticles were mixed gently at room temperature for 30 minutes. This step serves to block any remaining non-specific protein binding sites. The treated microparticles were centrifuged in a microcentrifuge at 15,300 g for 5 minutes, the supernatant discarded and the pellet resuspended in the BSA solution again. This process was repeated twice. Finally the microparticles were resuspended in 0.01 M phosphate buffer (pH 7.4), 1% (w/v) BSA, 0.1% (w/v) sodium azide and 5% (v/v) glycerol.

2.15.3.3 Analysis of antibody-polystyrene microsphere covalent coupling via lateral-flow immunoassay

To determine whether coupling of antibody to the polystyrene beads had occurred efficiently a lateral flow-immunoassay was performed. HiFlow Plus HF135 nitrocellulose membrane (Millipore) was sprayed with 20 µL of approximately 1 µg/mL secondary antibody specific for each of the three covalently bound antibodies. For analysis of anti-halofuginone bound microspheres, anti-HA antibody was used, for anti-toltrazuril, anti-rabbit polyclonal antibody was used and for anti-diclazuril rabbit anti-goat antibody was used. The antibodies were sprayed across the top of a 3 cm wide nitrocellulose strip at a flow-rate of 600 nL/sec, using the Linomat 5 system (CAMAG). Thirty µL of the antibody-bead complex was sprayed at the bottom of the strip at the same flowrate previously mentioned. The strip was then dipped into running buffer to allow the antibody-microsphere complex to migrate up the nitrocellulose and bind to the secondary antibody control line.

2.15.3.4 Adsorption of recombinant/polyclonal antibodies onto coloured-polystyrene microparticles

Adsorption of antibodies onto microspheres is primarily through hydrophobic interactions such as Van Der Waals' attractions between the hydrophobic portion of the adsorbed ligands and the polymeric surface of the microspheres. The microspheres used in this assay are carboxylated, hence, adsorption is achieved via both ionic and hydrophobic interactions. Five hundred microlitres of the green carboxylyated microparticles were diluted with 1 mL of 0.1 M borate buffer, pH 8.5. The beads were pelleted by centrifugation in a microcentrifuge at 15,300 g. The supernatant was discarded and the beads were resuspended in 1 mL of 0.1 M borate buffer once again. This centrifugation/resuspension process was repeated a further two times. Following the final centrifugation the beads were resuspended in 1 mL of borate buffer, and 400 µg of the antibody to be coupled. This suspension was left to react overnight at room temperature. The following day the mixture was centrifuged at 15,300 g for 10 minutes. The pellet was resuspended in 1 mL of 10 mg/mL BSA in borate buffer and left to incubate for 30 minutes at room temperature with gentle mixing. The blocked-beads were centrifuged again, and

BSA was added as before. This process was repeated a further two times. Finally, the pellet was resuspended in 1mL of PBS, pH 7.4, containing 10 mg/mL BSA, 0.1% (w/v) sodium azide, and 5% (v/v) glycerol. The beads were then stored at 4 ºC.

2.15.3.5 Dynamic Light Scattering (DLS) protocol

To determine whether the carboxylated polystyrene beads were conjugated with antibody, size analysis through light scattering was performed. The Delsa Nano C light scatterer provides information regarding the size of particles in a suspension. It does this by measuring the cumulatant result, the intensity, volume and number distributions and the polydispersity index (PDI) of the suspension to be analysed. Six hundred µL of the antibody-conjugated carboxylated green polystyrene beads were added to a cuvette and placed into the DLS apparatus. Measurements were carried out in triplicate at room temperature at an angle of 90°. Results were analysed by the Delsa Nano UI 2.21 software, which in turn showed the polydispersity index, viscosity, average diameter of particles and refractive index for the solution.

2.15.3.6 Multi-analyte detection of toltrazuril, halofuginone and diclazuril using a competitive lateral-flow based format.

Antibody-coated-colored microspheres are diluted 1 in 4 in sodium citrate buffer supplemented with 1.4% (v/v) Triton X-100 and 0.1% (w/v) SDS to reduce microsphere aggregation. HiFlow Plus HF135 nitrocellulose membrane (Millipore) was sprayed with the antibody-coated microspheres at a flow-rate of 600 nL/sec using the Linomat 5 system (CAMAG) across the width of the bottom of the strip. A control test line was then sprayed at the top of the strip with a species-specific antibody, analogous to the antibody-coated on the microspheres. Test lines of conjugates were also sprayed on the middle of the strip pertaining to each of the drugs to be tested. For preliminary analysis, only one of each of the conjugates was sprayed on separate strips, to determine the detection range of the assay for each antibody. The three conjugates, halofuginone-HSA, toltrazuril-BSA and diclazuril-BTG were sprayed at concentrations of 20 µg/mL across three different areas of the strip. The membranes were incubated at 37°C for 10 minutes. The sprayed membrane was cut into 3 identical strips each 1 cm thick. All 3 strips were then blocked by incubation with

5% (w/v) Milk Marvel at room temperature for 2 hours with agitation. The blocking solution was discarded and the membranes were washed once with PBS. The running buffer for the next step was optimised by assessing the performance of the following running buffers; 2mM borate buffer, pH 7.0, (0.05% (v/v) Tween), 50mM sodium phosphate, pH 7.5, (0.05% (v/v) Tween) and 15mM sodium citrate, pH 7.0, (0.05% (v/v) Tween). When extracted egg samples were used the buffers included 5% (v/v) methanol also. Each of the strips were placed into 5 mL extracted egg solutions containing concentrations of halofuginone, ponazuril and diclazuril at 10 µg, 1 µg, 100 ng, 10 ng, 1 ng and 0 ng/mL in the optimal running buffer. Once the beads had visibly travelled up the strip, the membranes were washed in the appropriate running buffer (x3) and with 0.05% (v/v) Tween included (x3). This was a one-step competition assay, whereby high concentrations of free drug in solution were bound to the antibody on the coloured polystyrene beads, inhibiting its binding to any of the test lines during transport up the strip. In the presence of no free drug, three coloured test lines and a control line are clearly visible.

Chapter 3

Development of validated ELISA and Biacore detection methods for Halofuginone, using a light-chain shuffled scFv

Chapter outline

The focus of this chapter was to generate highly-specific antibody fragments, for the detection of halofuginone (HFG). A chicken was subjected to a typical immunisation routine with a HFG-HSA conjugate. After each injection, a bleed was taken, and the serum titre analysed. Following three boosts, the spleen cells were harvested from the chicken and the RNA was subsequently extracted. This RNA was converted to cDNA and a scFv library was constructed. The library was biopanned against a HFG-transferrin conjugate, to enrich and select for HFG binders. A monoclonal ELISA was performed to identify single clones exhibiting HFG binding. The best clone isolated from the library had a detection capability of 30 ng/mL as determined by ELISA. However, the minimum 'Maximum Residue Limit' (MRL) for HFG in certain foodstuffs can be as low as 1 ng/mL, far below the sensitivity of the selected antibody. The HFG phage pool was then affinity maturated by light chain shuffling to further improve the selected antibodies. The HFG-specific heavy chain pool of the biopanned library was assembled with the light chain repertoire amplified from the original pre-panned library. This resulted in a heavy chain-biased library, from which a scFv with the potential to detect HFG residues as low as 80 pg/mL was isolated, a 185-fold improvement over the original scFv. This new chain shuffled scFv was incorporated into validated (according to EU regulation 2002/657-EC) ELISA and Biacore assays for the sensitive detection of HFG in spiked processed egg samples.

3.1 Introduction

Coccidiosis is one of the most economically important diseases of poultry (Cantecessi *et al.*, 2008) as these animals are very susceptible to the disease due to the intensive rearing conditions employed, particularly with broilers. Halofuginone (HFG), an antiprotozoal agent, is a quinazolinone derivative used for the prevention of coccidiosis. HFG is licensed for use in broilers and turkeys and is marketed by Intervet, either in the hydrobromide form as Stenerol® (Intervet, Mechelen, Belgium) or the lactate salt form as Halocur® (Intervet, Mechelen, Belgium). Ingestion of HFG is recognised to have adverse effects in the cardiovascular and central nervous systems in rat and mouse models (Mortier *et al.*, 2005a). For this reason, the European Agency for the Evaluation of Medicinal Products (EMEA) has set maximum residue limits for HFG in certain foodstuffs. HFG was named as the marker residue for HFG detection. The EU has specific legislation in place under Commission Regulation

(EC) No. 2430/1999 which permits HFG for use in laying hens but no residues may be found in eggs. In February 2009, under EU legislation, a commission decision regarding "unavoidable contaminating residues'' of coccidiostats was amended (2009/8/EC). This introduced MRLs for HFG in certain foodstuffs that were not previously required. The MRLs for HFG for food of animal origin from animal species other than chickens for fattening, turkeys and bovines (except dairy cattle) were set as follows: eggs, 6 μg/kg; liver and kidney, 30 μg/kg; milk, 1 μg/kg; and other food, 3 μg/kg.

To ensure that food is safe for human consumption, the EU have legislation in place whereby any detection test must be validated according to the methodologies outlined in the implementation of the Commission Decision (2002/657/EC) if intended for regulatory use within the EU. This ensures that all results of tests implementing directive 96/23/EC are interpreted in a standardised manner. The LOD is defined as is the lowest analyte concentration likely to be reliably distinguished from the blank standard and at which detection is feasible. The LOQ is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. Similarly, the detection capability (CCβ) is defined as the smallest concentration of HFG that may be identified with an error probability of 'β'. This β-error relates to incorrectly declaring truly non-compliant samples as compliant (Van Loco *et al*., 2007). The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of 'α' (i.e. the number of truly compliant samples that may be erroneously declared as non-compliant) that a sample is non-compliant (Van Loco *et al*., 2007). It should be noted that in this chapter the CCα and CCβ were determined via the verification of the identification criteria on fortified solutes, and not by ISO 11843-2 weighted regression calculations.

Currently, there are several detection systems available for the monitoring of HFG residues in foodstuffs (*See* **Table 3.0**). These are primarily based around detection methods such as chromatography (Mortier *et al*., 2003; Mortier *et al*., 2005a), mass spectrophotometry (Dubois *et al.,* 2004) and immunoassay technology (Yakkundi *et al*., 2002). Enzyme immunoassays are advantageous in residue detection due to their reliability, ease-of-use, cost effectiveness and rapidity.

Table 3.0. *Functional limit of detection values from literature of previously reported halofuginone detection methods.*

Assay Method	Functional Limit of Detection	Reference
Time resolved flurometry	1 ng/mL	Hagren *et al.*, 2005
Competitive ELISA	<500 pg/mL	Huet *et al.*, 2005
LC-MS	600 pg/mL	Yamada *et al.*, 2006
LC-MS/MS	770 pg/mL	Dubreil-Chéneau *et al.*, 2009
HPLC	1 ng/mL	Kinabo *et al.*, 1989
LC-MS-MS	1.62 ng/mL	Mortier *et al.*, 2004
LC-MS-MS	2 ng/mL	Mortier *et al.*, 2003
Monoclonal Ab-based Immunoassay/HPLC	38 ng/mL and 50 ng/mL	Beier *et al.*, 1998
LC-MS-MS	600 pg/mL	Dubois *et al.*, 2004
LC	40 ng/mL	Yamamoto *et al.*, 2001
Mass spectrometry	35.4 ng/mL	Yakkundi *et al.*, 2002
LC-MS	10 ng/mL	Ding *et al.*, 2005

Critical to immunoassay development is the generation of a high affinity antibody that is specific for the target analyte. In this chapter the development of a recombinant-avian antibody to HFG and its subsequent incorporation into validated ELISA and Biacore assays for HFG are reported.

Avian immunoglobulin sequences consist of one variable segment at both the heavy and light chains. These loci can be re-arranged into functional immunoglobulin V-D-J or V-J sequences (Leonard *et al.*, 2007). Individual recombination events are further diversified by transplanting blocks of sequences from upstream pseudogenes in both the V_H and V_L chain regions. Single primer sets for each of the heavy and light chain sequences, complementary to the conserved regions flanking the unique functional V_H and V_L genes can, therefore, be used to amplify the complete spectrum of rearranged variable fragments. This conserved recombination enables the cloning of

highly diverse chicken immunoglobulin repertoires with relative ease (Leonard *et al.*, 2007). These advantages over other animal-models made chicken a good choice for immune library production for HFG.

Repertoires of polymerase chain reaction (PCR) amplified variable heavy and variable light chain antibody gene fragments from the RNA of a halofuginone-sensitised chicken were cloned into a phagemid vector to produce a library of scFv-displaying phage particles. Phage display technology, an important tool in molecular biology for the isolation of proteins or peptides against specific targets, was used to enrich for antibodies capable of binding HFG. The phage display vector pComb3XSS was employed to express HFG-specific scFvs on the surface of filamentous phage particles. The phage library was subjected to rounds of panning against a HFG-bovine thyroglobulin (BTG) conjugate. Clones with good binding specificity towards HFG were isolated and characterised by competitive ELISA. The clone exhibiting the highest displacement of free HFG did not meet the sensitivity required to be used for a validated HFG detection method. Hence, an antibody with greater sensitivity towards HFG and the potential to detect free HFG at the MRL was required. In order to isolate an antibody of higher affinity, the library was diversified via chain shuffling. A new library was constructed by amplifying the V_H region from the HFG-panned library pool and re-assorting this with the V_L regions from the unpanned library pool. This heavy chain-biased library was subjected to more stringent panning than the previous selection to isolate an antibody with greater specifity than the original scFv.

Validated ELISA and Biacore methods, using the chain-shuffled anti-HFG scFv, are described for the detection of HFG from egg in this chapter.

Biosensors use a combination of receptor compounds (e.g. antibody, enzyme, or nucleic acid) and physiochemical detector components, to observe a specific biological event in 'real-time' (e.g. antibody-antigen interaction). They can detect a broad spectrum of analytes in complex sample matrices, have the advantage of automated measurement capability and have demonstrated great promise in areas such as clinical diagnostics, food analysis and bioprocess and environmental monitoring (Ivnitski *et al.*, 1999; Fitzpatrick *et al*., 2000).

One such sensor is the Biacore. The detection principle of Biacore™ sensor systems is based on surface plasmon resonance (SPR). When a plane-polarized light beam propagates through a medium of higher refractive index (e.g. glass prism) and meets an interface of lower refractive index (e.g., sample solution), the light is reflected

above a certain critical angle. Under these conditions, an evanescent wave penetrates into the low refractive media to a magnitude of one wavelength. The evanescent wave propagates in the metal layer component of the sensor surface and causes the plasmons to resonate, resulting in a surface plasmon wave (Dunne *et al.*, 2005). In a typical hapten-based immunoassay, the ligand, usually a hapten-protein conjugate or directly immobilised hapten, is chemically attached to a sensor chip surface, which consists of a thin metal film bound to a glass surface. When the sensor is exposed to a sample containing the specific analyte, the binding of the analyte to the immobilised ligand causes a change in SPR response which is related to the concentration of analyte in the sample.

Current EU legislation regarding sampling for the official control of veterinary residues in feed is regulated under commission E.C No. 152/2009 (Huet *et al.*, 2005) This method uses high performance liquid chromatography (HPLC). However, when HPLC and SPR technology were compared for the detection of toxins, it was observed that for high throughput screening of large amounts of samples, SPR could be employed to substantially reduce the number of samples requiring confirmatory tests, thus reducing costs to the regulatory authorities (Thompson *et al.*, 2009). Hence, a method was developed for the extraction and subsequent rapid screening of samples for HFG using a Biacore 3000^{TM} instrument. A schematic representation of the SPR assay developed for HFG using a recombinant anti-HFG scFv is illustrated in **Figure 3.0.** SPR technology allows the interactions of biomolecules to be studied in 'real-time' in an automated setting. SPR is an attractive detection method in food monitoring for regulated substances, since it is possible to easily regenerate, re-use and store sensor chips for long periods of time. The method developed was directly compared to the validated ELISA method for HFG detection which is also described in this chapter.

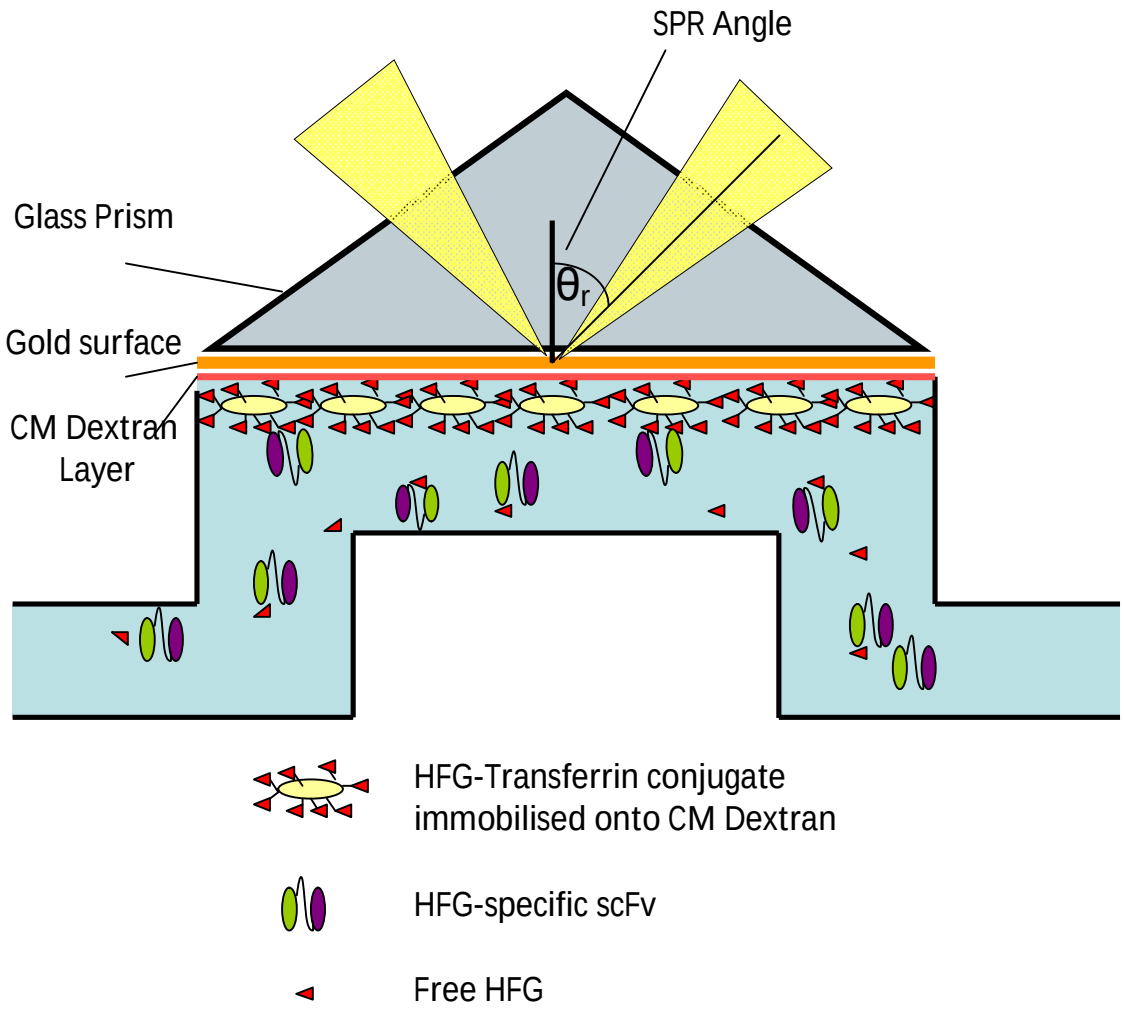

Figure 3.0. *Schematic representation of an SPR inhibition assay for the detection of HFG residues. The HFG-transferrin conjugate is immobilised on the surface of a CM5 sensor chip. HFG present in the samples to be tested competes with immobilised HFG for binding to the anti-HFG scFv. The binding of HFG to the immobilised scFv causes a change in SPR response which is inversely related to the concentration of HFG in the sample.*

3.2 Results

3.2.1 Immunisation of Leghorn variety chicken with halofuginone

A Leghorn chicken was immunised with a mixture of equal parts of HFG-HSA conjugate and Freund's complete adjuvant. The drug-conjugate was at a concentration of 200 µg/mL. A primary boost was performed by administration with 100 µg/mL of drug conjugate diluted with Freund's incomplete adjuvant. Subsequent boosts were

performed 7-10 days apart, with seven boosts in total being administered. After four boosts, a primary serum titre was determined.

3.2.1.1 Avian serum antibody titre determination

An ELISA was performed on a HFG-BTG coated immunosorbent 96-well plate using varying dilutions of serum. A very high immune response was observed for HFG, following six boosts (**Figure 3.1**). The specific antibody titre obtained was 1/1,000,000 when screened against HFG-BTG. The chicken was sacrificed to harvest the B cells from the spleen for recombinant antibody library generation.

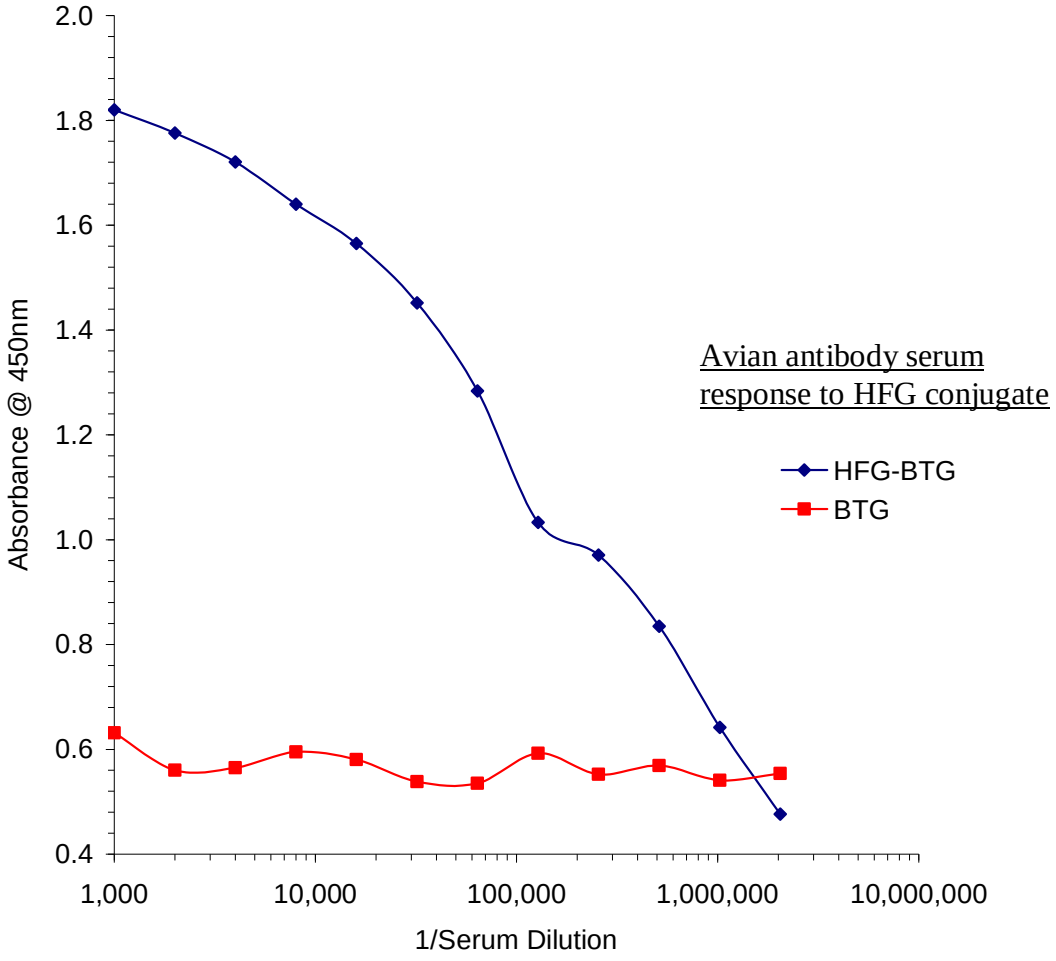

Figure 3.1 *Titration of avian serum antibodies, following HFG immunisations. Serum was harvested intravenously from the chicken immunised with the HFG-HSA conjugate and the antibody serum titre determined by ELISA.*

3.2.2 Isolation of RNA from avian B cells (spleen) and first-strand DNA synthesis

The spleens were removed from the immunised mice and carefully homogenised in TriZol reagent. The total RNA was extracted from the tissue sample, quantified using a Nanodrop ND-1000 and first-strand cDNA synthesised by reverse transcription. The cDNA template was used in the amplification of the mouse variable heavy and light chain genes.

3.2.3 PCR optimisation for murine variable heavy and variable light chain generation

A standard PCR protocol, outlined in Barbas *et al.* (2001), was employed to amplify the V_H and V_L chain genes (**Figure 3.2**) from the reverse transcribed cDNA. The V_H region amplified successfully with no subsequent optimisation required. However, the V_L region did not amplify.

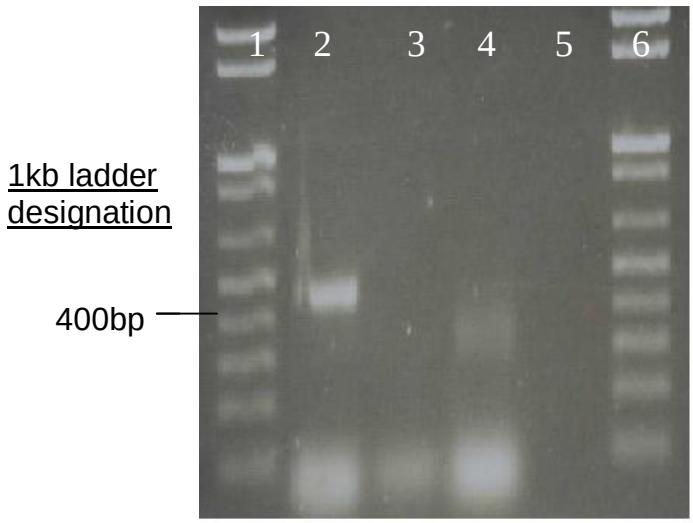

Figure 3.2 Amplification *of variable regions from synthesised cDNA derived from a HFG-immunised chicken. Amplification was performed using Go Taq polymerase and standard PCR conditions optimised by Barbas et al. (2004). Lane 1= 1kb ladder; Lane 2= V_H amplification; Lane 3= V_L amplification; Lane 4= Control PCR without polymerase; Lane 5= Control PCR without template/primers and Lane 6= 1kb ladder.*

To optimise the amplification of the V_L gene, the PCR was repeated for both the V_H and V_L genes. However, the concentration of $MgCl_2$ was increased to 6mM for the V_L amplification. **Figure 3.3** shows successfully amplified V_H and V_L chains. This suggests that a pipetting error may have occurred in the previous PCR as no product was observed. The gel-purified V_H and V_L fragments were linked by splice by overlap extension PCR (SOE-PCR).

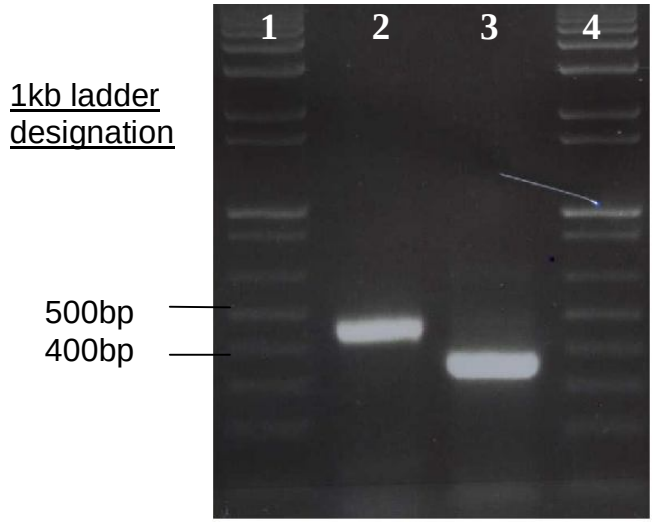

Figure 3.3 *Variable light chain amplification from the synthesised cDNA of a HFG-sensitised chicken. The amplification was performed using a magnesium chloride concentration of 6mM and standard PCR conditions. Lane 1= 1kb ladder; Lane 2= V_H amplification as before; Lane 3= V_L amplification using 6mM $MgCl_2$ and Lane 4= 1kb ladder.*

3.2.4 Avian SOE-PCR of variable heavy and light chains

The gel-purified avian variable heavy and light chain genes were used for the generation of the full-length scFv by PCR amplification. The initial PCR was performed under standard conditions, as outlined in *Section 2.6.7.* Several non-specific products were amplified in addition to the 800bp desired product (**Figure 3.4**).

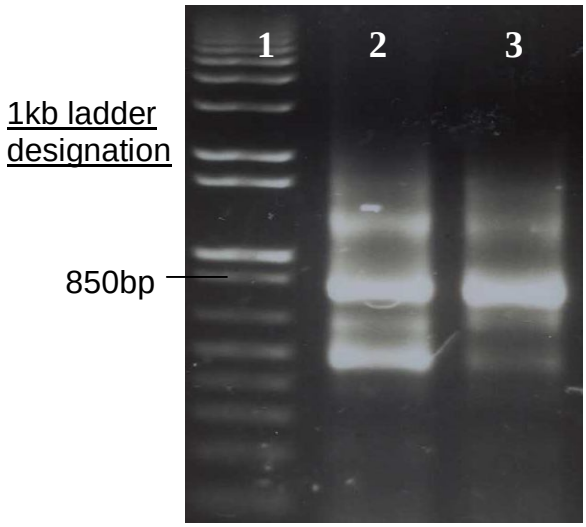

Figure 3.4 *Splice by Overlap Extension-PCR of the amplified V_H and V_L chain genes from the synthesised cDNA of HFG-sensitised mice. This primary amplification check used standard conditions for avian-derived variable domain overlap amplification, consisting of a 56^0C annealing temperature and Phusion high fidelity polymerase. Non-specific bands are observed alongside the 800bp product. Lane 1= 1kb Ladder; Lane 2 and 3= SOE amplification.*

The annealing temperature of a PCR influences the specificity of primer binding. Non-specific binding was occurring in the SOE-reaction. To circumvent this, a touchdown PCR was performed to eliminate these non-specific products. In the touchdown PCR, the annealing temperature of the reaction was incrementally decreased from 60^oC down to 56^oC over 25 cycles. This touchdown PCR increased the specificity of the reaction, resulting in only the desired 800bp product being amplified (**Figure 3.5).**

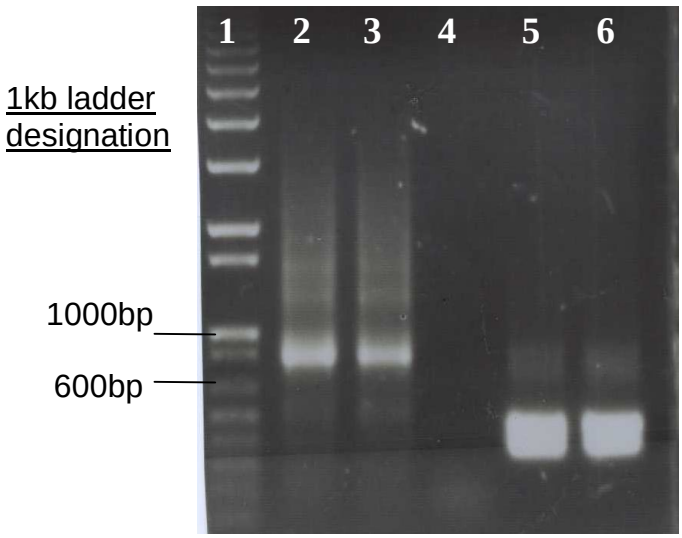

Figure 3.5: *SOE-PCR using a touchdown PCR to optimise the product yield. The touchdown PCR was performed with the annealing temperature decreasing from 60 ^{o}C to 56 ^{o}C incrementally with each subsequent cycle. Lane 1= 1kb Ladder; Lane 2 and 3= SOE amplification; Lane 4= Blank well and Lane 5 and 6= gel-purified V_H and V_L chain genes (it should be noted that the large bands observed in lanes 5 and 6 are due to overloading of the DNA).*

3.3 Halofuginone-library construction and subsequent enrichment via biopanning.

After large-scale amplification of the 800bp desired PCR fragment, it was gel purified, precipitated in ethanol, quantified and digested using *Sfi*I restriction enzyme. The scFv fragment was ligated into a digested pComb3XSS vector and then transformed into high efficiency electrocompetent XL-1 blue cells, rescued and the library size calculated. The avian HFG-scFv library size was 2.5 x 10^7 cfu/mL. HFG-specific antibody fragments were selected on HFG-BTG immobilised on the surface of an immunotube, as described in *Section 2.7.1*.

3.3.1 Selection of halofuginone-specific phage-scFv particles by biopanning

Five rounds of panning were performed and input and output titres for each round of selection determined (**Table 3.1**). During the selection process, the concentration of HFG was decreased and the number of washes was increased. Biopanning essentially mimics the *in vivo* process of selective pressure (Hoogenboom, 2005). Hence, this increase in biopanning stringency after each successive round of panning is important

to ensure positive clones are selected and it significantly reduces the levels of clones which have no specificity for the target antigen. Following the fourth round of panning, monoclonal analysis was performed as described in *Section 2.9*. From this monoclonal analysis, it was determined that 90% of the clones were HFG-specific; hence, a further round of panning was performed increasing the stringency of the process, to select for only high-affinity clones.

Table 3.1 *Panning conditions employed for each round of selection of the avian anti-halofuginone scFv library. HFG-BTG was coated on the surface of immunotubes. Phage input and output titres over the 5 rounds of biopanning are reported with each round of biopanning varying in conditions and stringency.*

Panning round	HFG coating concentration	Washing steps	Input titres Cfu/mL	Output titres Cfu/mL
1	100 μg/mL	3 X PBS, 3 X PBST	6.15×10^{11}	3.4×10^{6}
2	50 μg/mL	5 X PBS, 5 X PBST	3.5×10^{10}	1.75×10^{4}
3	25 μg/mL	7 X PBS, 7 X PBST	1.3×10^{8}	2.0×10^{3}
4	10 μg/mL	10 X PBS ,10 X PBST	1.5×10^{10}	9.0×10^{5}
5	5 μg/mL	15 X PBS ,15 X PBST	2.2×10^{10}	3.4×10^{6}

The precipitated input phage from each round of panning was incorporated into a polyclonal phage ELISA to test for enrichment against the HFG-BTG conjugate. Successful enrichment was signified by an increase in absorbance signal in the fourth round after incubating phage with an immobilised HFG conjugate and detecting bound phage, following the addition of a HRP-labelled anti-M13 secondary antibody. This can be observed in **Figure 3.6**.

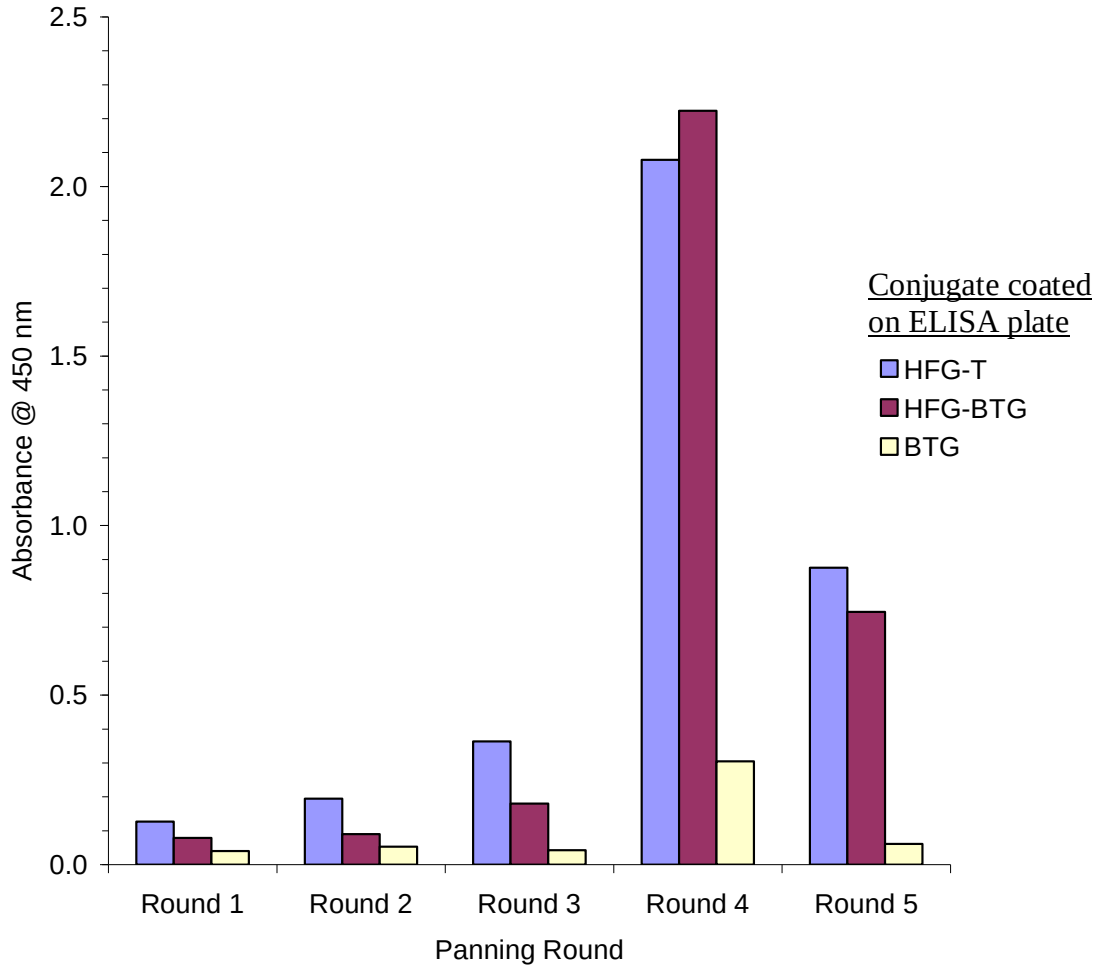

Figure 3.6 *Polyclonal phage ELISA screening for anti-HFG scFv displayed on phage, following five rounds of panning. Phage pools obtained after each round of panning were tested for binding to the HFG-BTG/HFG-Transferrin (T) conjugate by ELISA. The phage pools were also screened against BTG during each round, to ensure no non-specific binding was occurring to the conjugate carrier proteins. The scFv-displaying phage were detected using a HRP-conjugated anti-M13 antibody and the absorbance was read at 450 nm using a Tecan Safire™ plate reader following a 20 minute incubation with TMB substrate. The sharp increase in absorbance from round 4 onwards suggested the presence of HFG-specific scFv-harbouring phage within the panned library.*

3.3.1.1 Monoclonal analysis of soluble clones from halofuginone biopanning

The outputs from the fifth round of panning were infected into Top10F, a non-suppressor strain of *E.coli*. In this non-suppressor strain, the amber stop codon between the scFv and the gIII gene is not suppressed (as it is in the XL1-Blue cells), allowing production of soluble scFv antibodies fused to a C-terminal histidine (His6) and influenza hemagglutinin (HA) (YPYDVPDYAS) tag. One hundred and ninety-two single transformants were grown, induced with IPTG for expression of scFv antibody fragments and the cell lysates tested by ELISA for binding to HFG-transferrin. Any bound antibody was detected following the addition of a HRP-conjugated anti-M13 antibody (**Figures 3.7** and **3.8**). Approximately 5% of the selected clones showed binding to HFG coated on the surface of an ELISA plate from the Pan 5 output. This is quite a low percentage of specific clones, probably due to the increased specificity of the selection process, hence, these 5% of clones were subjected to competitive studies for further analysis.

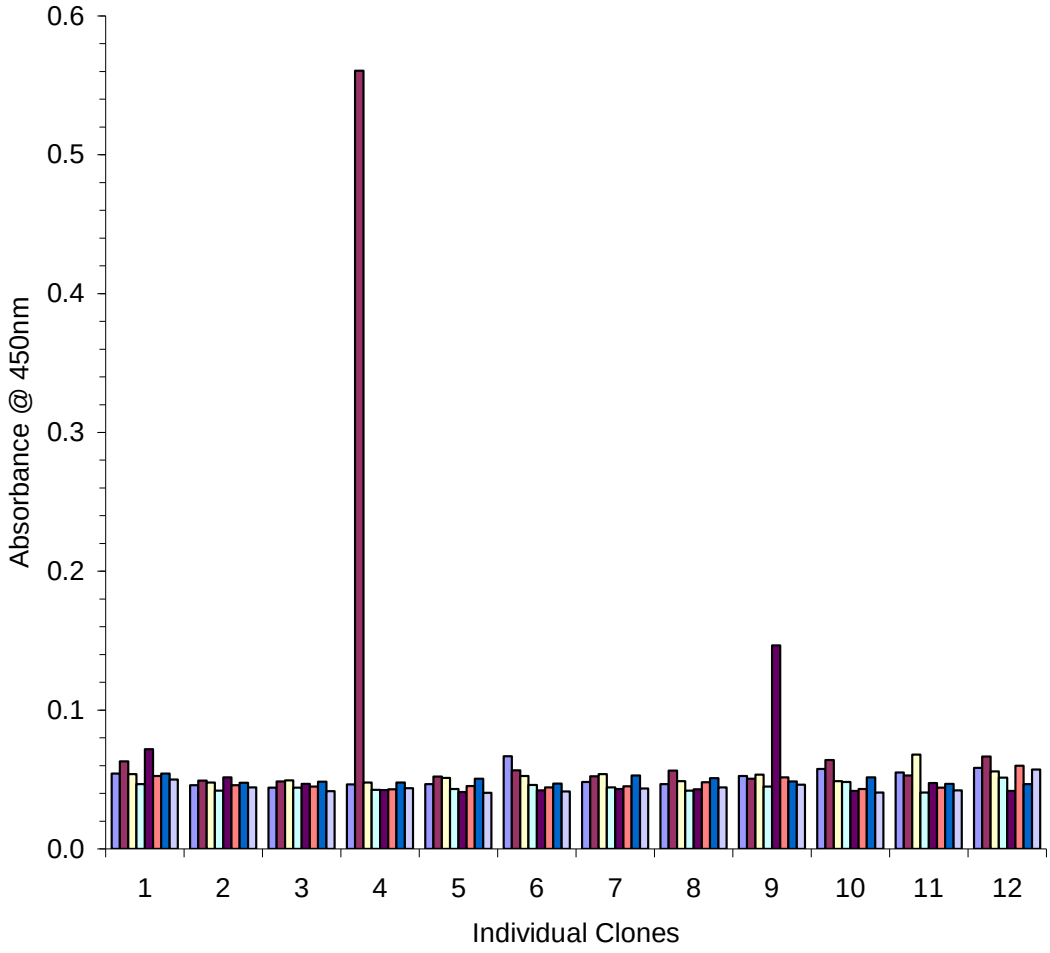

Figure 3.7 *Monoclonal ELISA to select for specific anti-HFG scFvs from randomly selected clones in the fiftth round of biopanning. The clones were solubly expressed in Top10F cells. Nunc MaxiSorp ELISA plates were coated with 1μg/mL of HFG-transferrin and specific scFvs were detected using a HRP-labelled anti-HA antibody.*

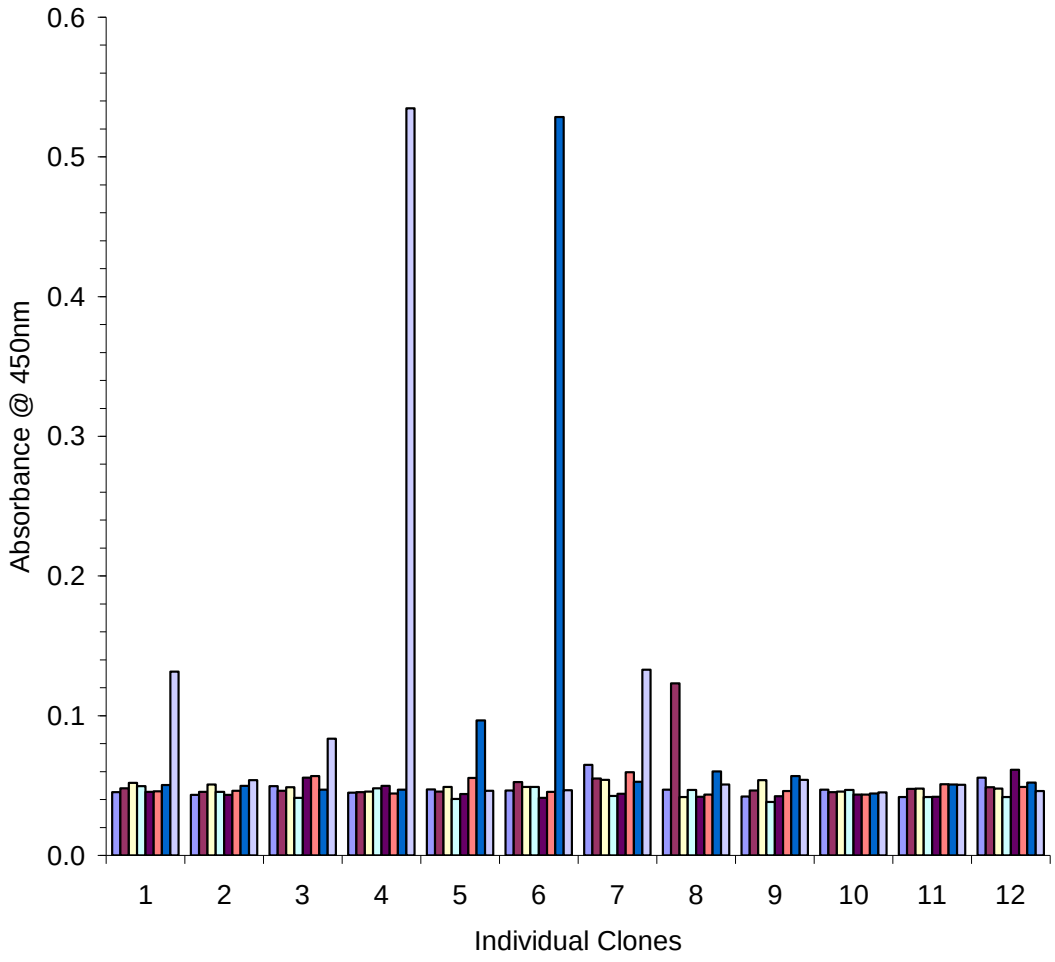

Figure 3.8 *Monoclonal ELISA to select for anti-HFG scFvs from randomly selected clones in the fifth round of biopanning). The clones were solubly expressed in Top10F cells. Nunc MaxiSorp ELISA plates were coated with 1μg/mL of HFG-Transferrin. ScFv were detected using a HRP-labelled anti-HA antibody.*

Six individual clones displaying HFG specificity from the monoclonal ELISA plates were taken forward for further characterisation. A titre of the clones against 1 μg/mL HFG-transferrin was initially performed with lysate dilutions ranging from 1 in 4 to 1 in 4,000. **Figure 3.9** shows that only three clones, namely, B4, G6, and H4 titre out on the HFG-coated plate. To verify this, a single dilution of each of the clones was screened against all three HFG conjugates; HFG-HSA, HFG-BTG, HFG-transferrin, to ensure that there was no problem with the HFG-transferrin conjugate. A BTG control was included, to ensure non-specific binding to the conjugate proteins was not occurring. **Figure 3.10** shows the specific binding of clones B4, H6 and H4 to HFG, with no cross-reactivity apparent with BTG. Very little binding is observed for clones

H1, H7 and B8 which confirms the results shown in **Figure 3.9.** This low signal may be due to problems with expression levels. The three positive clones were taken forward for further analysis.

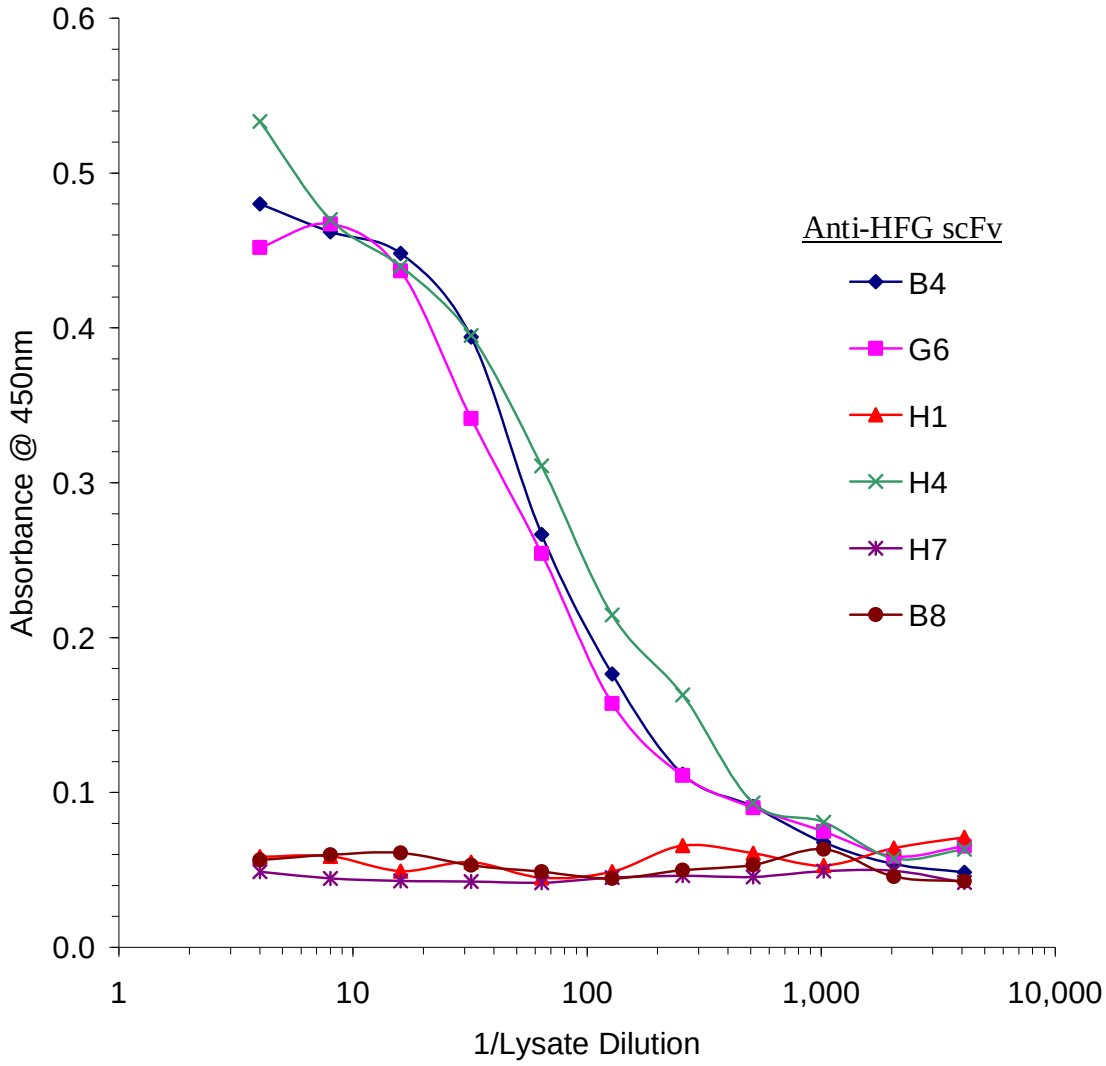

Figure 3.9 *Antibody Titre of HFG-specific clones, screened against HFG-transferrin. Dilutions of lysate from 1/4 to 1/4,000 were tested, and bound antibody was detected following the addition of a HRP-labelled anti-HA antibody.*

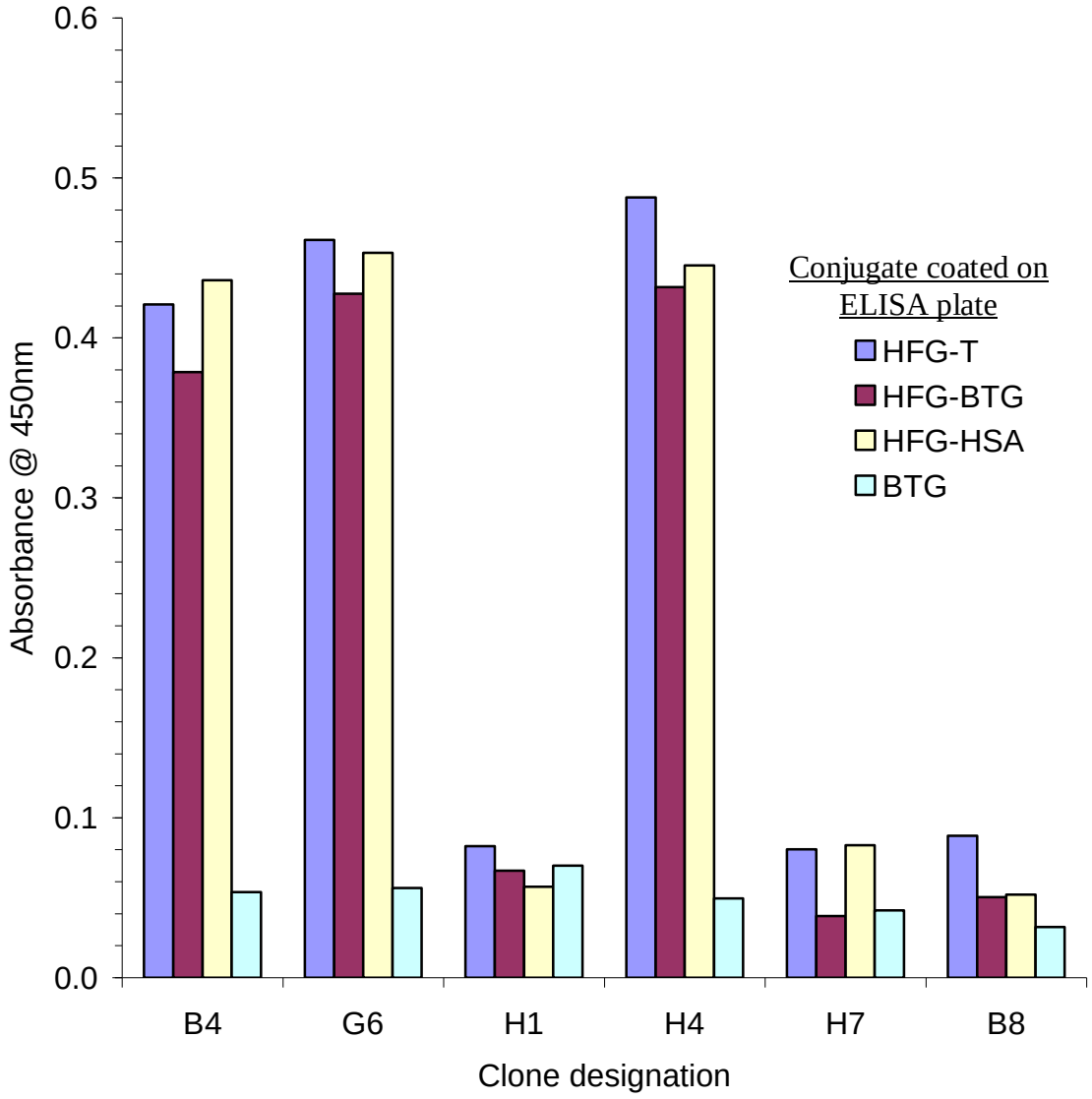

Figure 3.10 *Direct ELISA, using three different HFG conjugates, for screening of positive clones taken following monoclonal analysis. HFG-Transferrin (T), HFG-BTG, HFG-HSA and BTG, all at concentrations of 1μg/mL, were coated on the wells of an ELISA plate. Six of the positive clones identified were screened against all three of the HFG-conjugates and the BTG control.*

3.3.1.2 Expression studies of positive clones in both Top10F and XL1Blue

To optimise the expression of the three positive clones, plasmid preparations of each were transformed into the XL1-Blue *E. coli* strain by electroporation, as outlined in *Section 2.6.9.1*. The lysates from each of the clones in both Top10F and XL1-Blue,

were incubated on an ELISA plate coated with all three HFG conjugates and a BTG control. A single competitive study was performed by incubating the scFv lysates with 5 μg/mL of free HFG and observing if displacement of antibody occurred (i.e. a decrease in absorbance). **Figure 3.11** illustrates the direct ELISA performed with lysates from the three clones; B4, G6 and H4, in both Top10F and Xl1Blue cell lines. The graph also shows that binding of all three clones to immobilised HFG is inhibited in the presence of excess free HFG. This suggests that all of the clones are HFG specific. It is also apparent that for all of the clones in either cell type XL1-Blue or Top10F, the expression levels of the antibodies stayed the same, as similar results are observed in both cells. All the clones in Top10F were taken forward for competitive analysis.

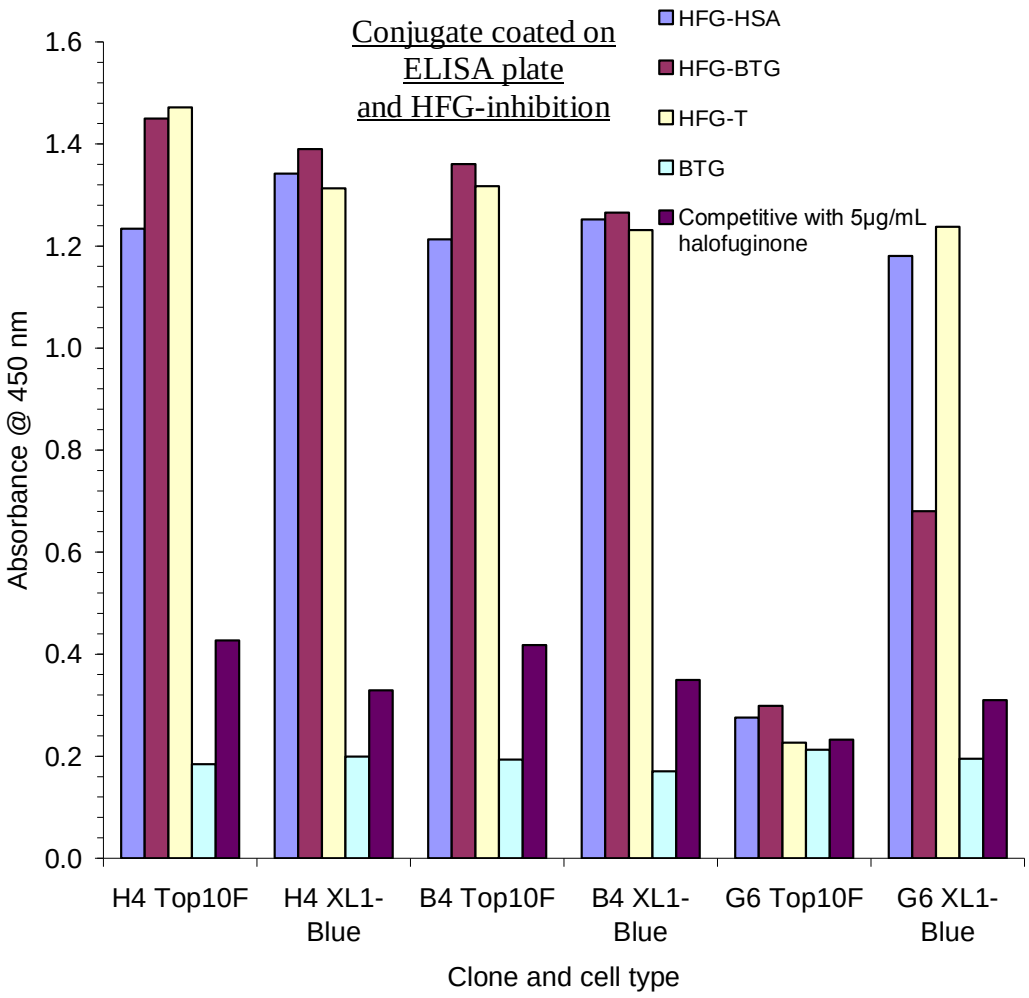

Figure 3.11 *Expression analysis of HFG-specific clones B4, G6 and H4 in both Top10F and XL1-Blue cells. A direct ELISA was performed using three different HFG conjugates, all at concentrations of 1 µg/mL. A competitive ELISA was performed using 5 µg/mL of free HFG incubated with each of the cell lysates, on HFG-transferrin (T)-coated wells. Bound antibody was detected following the addition of a HRP-labelled anti-HA antibody.*

The expression of the three HFG-specific clones in both Top10F and XL1-Blue was very similar, hence, for further competitive analysis studies, the clones were expressed in their soluble form in Top10F. It should be noted that it appears from **Figure 3.11** that the G6 clone did not actually express, however, this may have been a problem with the specific overnight culture. This was postulated since when sequenced, the G6 and B4 clones were identified as the exact same clone. A competitive ELISA was performed, as per *Section 2.9.1,* whereby cell lysates were

incubated with decreasing concentrations of HFG from 5,000 down to 39 ng/mL. It can be observed in **Figure 3.12** that all of the clones exhibit HFG specificity, and so they were sent for sequencing with MWG as outlined in *Section 2.10.1.*

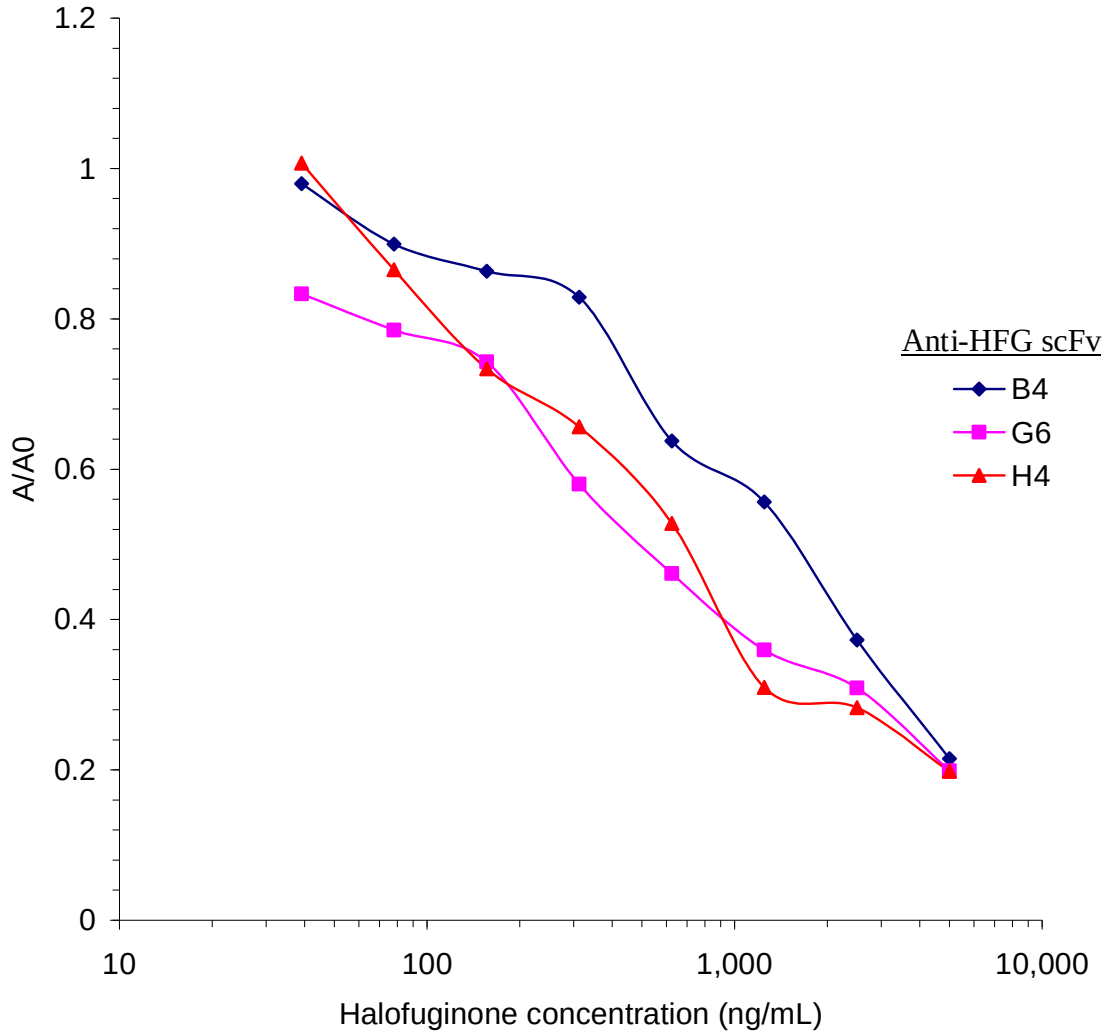

Figure 3.12 *Competitive analysis of HFG-specific clones B4, G6 and H4 in Top10F cells. A 1/1000 dilution of HFG-specific antibodies from each of the three clones, was incubated with decreasing concentrations of HFG from 5,000 to 39 ng/mL. Any bound antibody was detected following the addition of a HRP-labelled anti-HA antibody. The results are shown as A/A0, where the absorbance values of the evaluated samples (A), are normalised by expressing them as a function of the blank standard (A0).*

3.3.1.3 Sequence analysis of the halofuginone-specific recombinant antibodies

Plasmid preparations of *E. coli* Top10F (containing the pComb3X bearing the anti-HFG B4, G6 and H4 genes) were sent to MWG for sequencing. The DNA sequences were received and subsequently translated into their amino acid sequence using ExPASy translation tool. The amino acid sequences were aligned and the complementary determining binding (CDR) regions of the scFv evaluated using MultiAlin software. The CDR regions of the antibodies were highlighted according to the Kabat scheme (**Figure 3.13**). The light and heavy chain CDRs are highlighted in green and purple, respectively. The heavy and light chain sequences of the scFv B4 and G6 were found to be identical. The diversity between the B4/G6 scFv and the H4 scFv is evident, as there is only a 48 % sequence homology of the CDRs, with the CDRH3 having no sequence homology whatsoever.

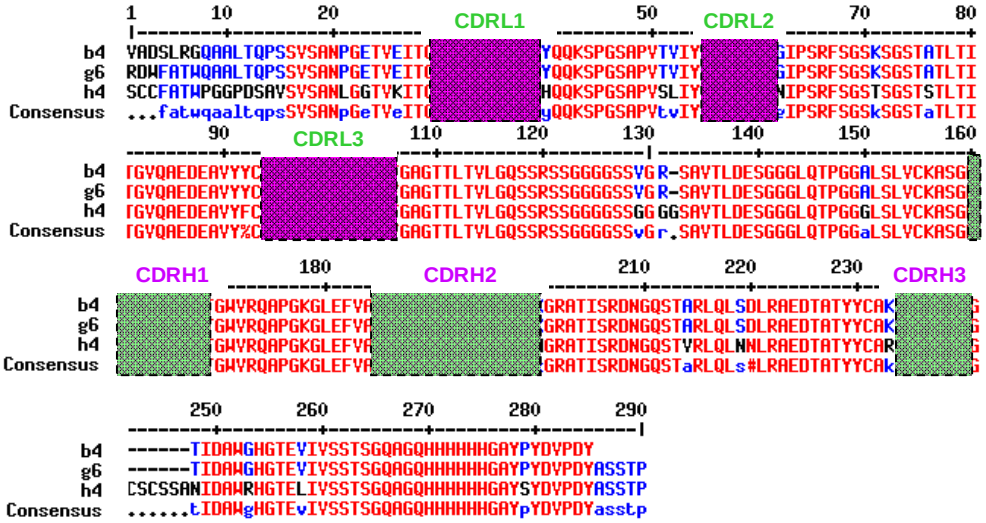

Figure 3.13 *Avian anti-HFG scFv gene sequences were translated into amino acid sequences using Expasy Translate tool and aligned using MultiAlin sequence alignment program. The highlighted sequences in green and purple show the CDRs (heavy and light chain) of the recombinant antibody fragments. The framework regions are highlighted in red and variation from the consensus of the sequences is shown in black.*

3.3.1.4 Expression and immobilised metal chromatographic purification of clones 'B4' and 'H4'

Large-scale expression of the avian anti-HFG clones B4 and H4 was performed in 500 mL cultures, as outlined in *Section 2.9.2*. The scFv B4 and G6 have the same sequence as shown in **Figure 3.13**, so only the B4 scFv was purified. The protein was extracted by sonication and the bacterial pellet removed by centrifugation. The protein lysate was passed through a 0.2 µM filter to reduce clogging of the nickel resin. It was then applied to the resin and any histidine-tagged expressed proteins captured by the nickel resin. Nickel has a high affinity for histidine residues and other proteins just pass through the column. Non-specifically bound proteins were removed by washing the resin twice. The absorbed protein was then successfully eluted using high concentrations of imidazole. Histidine has an imidazole side-chain. When high concentrations of imidazole are added to the column, in the elution buffer, they compete with histidine moieties (e.g. as in a His tag) for binding to the nickel-resin and displaces any bound His-tagged proteins. The H4 scFv was found to be very difficult to purify as a lot of non-specific binding was occurring, and to circumvent this extra wash steps were performed prior to elution (**Figure 3.14 (A)**). The IMAC-purified fractions containing the scFv were pooled, centrifuged and analysed by SDS-PAGE (**Figure 3.14**).

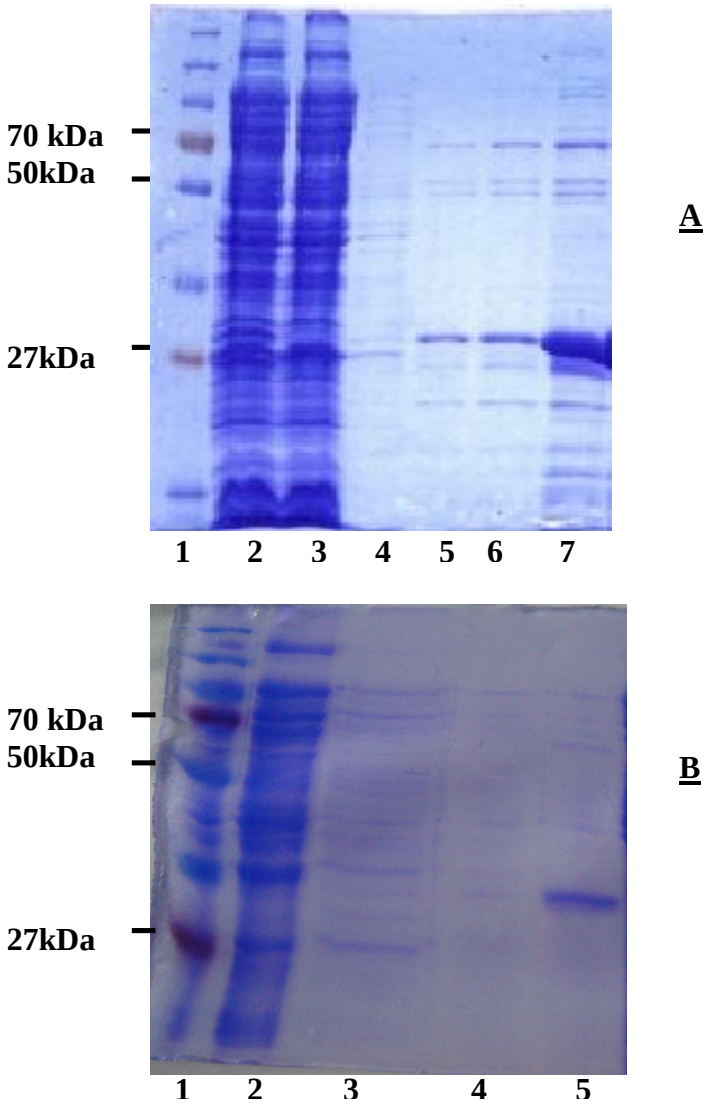

Figure 3.14 *SDS-PAGE analyses of the fractions obtained during IMAC purification of anti-HFG scFvs H4 and B4. Figure 'A' shows the purification of the 'H4' scFv, Lane 1 = Colour Burst molecular weight markers (Fermentas); Lane 2 = Lysate samples diluted 10-fold in PBS; Lane 3 = 'flow-through'; Lanes 4, 5, and 6 = washings from the column and, Lane 7 = Eluted purified antibody fractions pooled and tested undiluted.*

Figure 'B' shows the purification of the 'B4' scFv, Lane 1 = Colour Burst molecular weight marker (Fermentas); Lane 2 = Lysate samples diluted 10-fold in PBS; Lane 3 = 'flow-through'; Lane 4 =Washes 1 + 2 pooled and Lane 5 = Eluted purified antibody fractions pooled and tested undiluted. In both SDS images one distinct band is visible at the level of the 27 kDa marker, representing the purified scFv. The primary band in the eluate in Figure B appears slightly larger than in Figure A. This

may be due to some of the scFv not being fully solubly expressed and still containing part of the p3 protein.

3.3.1.5 Competitive analysis of purified 'B4' and 'H4' anti-halofuginone scFv

To determine which scFv had the greatest sensitivity to HFG and the lowest detection capability, a competitive ELISA was performed. Ten μg/mL (as determined by analysis on the Nanodrop) of each of the purified antibodies were incubated with 30,000, 10,000, 3,333, 1,111, 370, 123, 41, 13, 4.5, 1.5 and 0 ng/mL of HFG (**Figure 3.15**). The assays were performed in triplicate, and standard deviations determined, and illustrated by the error bars. The LOD for HFG for each of the scFvs was extrapolated from the competitive curves. The LOD for the H4 scFv was approximately 300 ng/mL, whereas, the LOD of the B4 scFv was approximately 100 ng/mL. However, the scFv requires even greater sensitivity than this, to be able to detect HFG below the MRL, which is approximately 1 ng/mL.

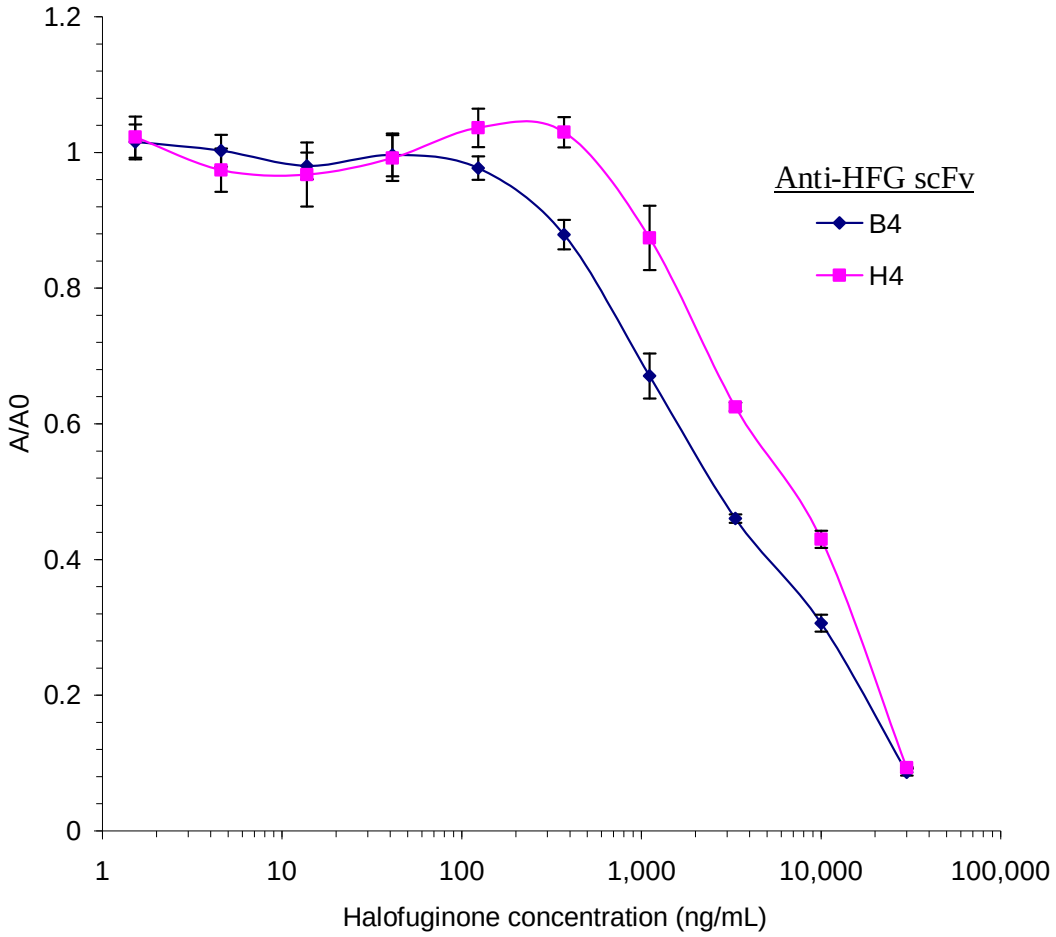

Figure 3.15 *Competitive analysis of purified anti-HFG scFvs, B4 and H4, in Top10F cells. Ten μg/mL of the antibodies were incubated with decreasing concentrations of halofuginone from 30,000 to 1.5 ng/mL. Bound antibody was detected following the addition of a HRP-labelled anti-HA antibody.*

3.4 Light chain shuffling from the halofuginone-biased phage pool

To circumvent the limited detection potential of the B4 scFv, it was postulated that mutagenesis in the form of light chain shuffling would be the most efficient method for improving the sensitivity of the antibody. This resulted in a 'heavy chain-biased' light chain-shuffled phage display library (**Figure 3.16**). This process ensures the construction of a library which contains antigen-specific heavy chains enriched during the panning process and introduces further diversity into the new library by recombining this with the un-panned arrangement of light chain encoding genes.

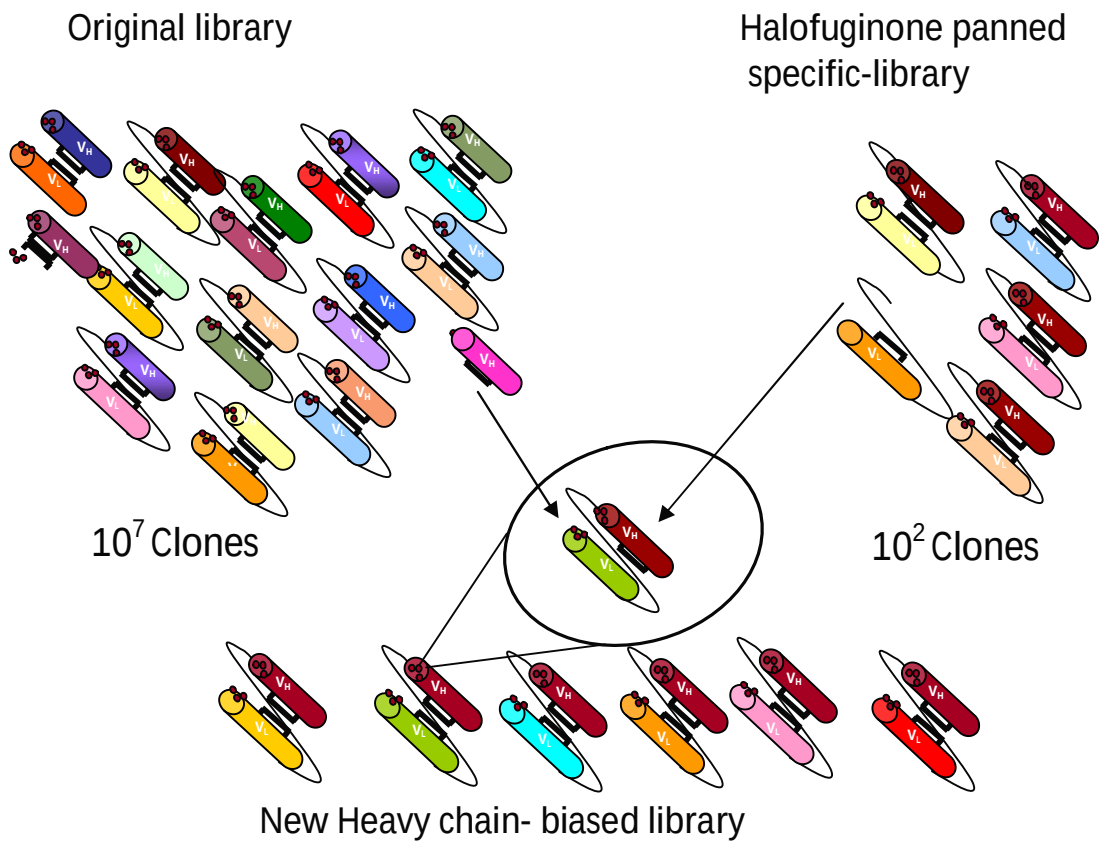

Figure 3.16 *Illustration of variable chain amplification for construction of heavy chain-biased light chain-shuffled library. The 'light chain-shuffled' library was constructed from the enriched HFG-specific heavy chain pool of the biopanned library, along with the light chain repertoire amplified originally from the cDNA. This resulted in a heavy chain-biased library.*

3.4.1 Construction of the light chain-shuffled halofuginone scFv library

In order to find the best combination of V_H-V_L pairings and to improve antibody affinity maturation, light chain shuffling was employed. The heavy chain region from the panned library, after pan 5, was amplified by PCR using the same primers that were used to construct the initial V_H fragments from the cDNA. This PCR reaction amplified the whole heavy chain pool from the enriched HFG-panned library (**Figure 3.17).** The light-chain was amplified from the original un-panned library, using the same conditions as previously described (*Section 2.6.5*). Both of these amplifications were successful at the first attempt.

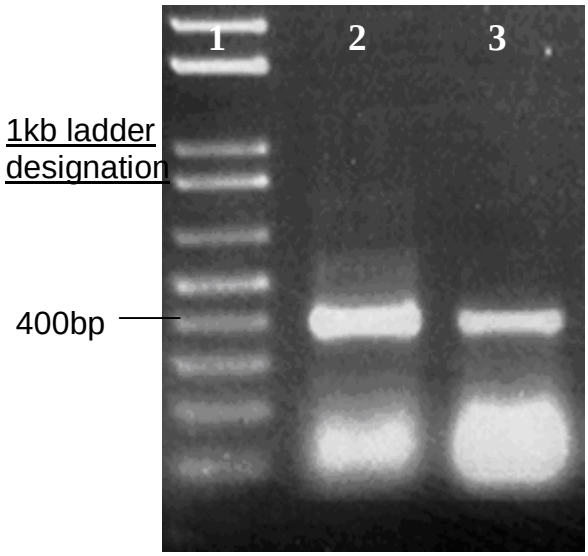

Figure 3.17 *PCR amplification of the halofuginone-sensitised V_H gene from the fifth round of panning and the V_L gene from the original un-panned library. Using the same primers employed from the initial amplification, both the variable heavy-chain region from the round 5 phage pool, and the variable light-chain region from the un-panned library, were successfully amplified. The PCR conditions are outlined in Section 2.6.5. Lane 1= 1kb Ladder; Lane 2 =V_H amplification and Lane 3= V_L amplification.*

3.4.1.1 Avian SOE-PCR of variable heavy chain from the halofuginone-panned library with the variable light chain from the un-panned library

The gel-purified avian variable heavy and light chain genes were used for the generation of the full-length chain-shuffled scFv by PCR amplification as outlined previously in *Section 3.2.4*. The SOE-product (**Figure 3.18**) was amplified with no modifications to the original PCR (*Section 2.6.7*).

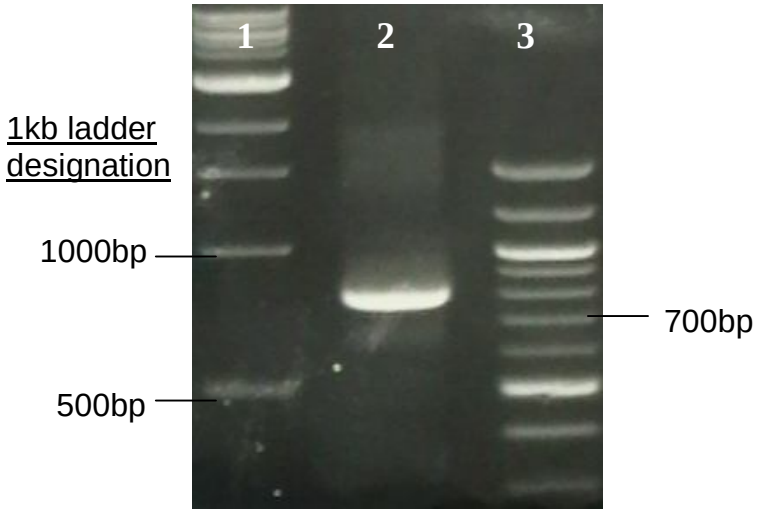

Figure 3.18 *Splice by overlap extension PCR of the halofuginone-sensitised heavy chain, and the light chain from the original constructed library. The SOE was performed under conditions as outlined in Section 2.6. This overlap yielded an 800bp heavy-chain biased scFv construct. Lane 1= 1kb Ladder; Lane 2= SOE amplification; Lane 3 = 100bp ladder*

After large-scale amplification of the 800bp required PCR fragment, it was gel-purified, precipitated in ethanol, quantified and digested using the *Sfi*I restriction enzyme. The scFv fragment was ligated into a digested pComb3XSS vector and then transformed into high efficiency electrocompetent XL-1 blue cells, rescued and the library size calculated. The avian light-chain shuffled HFG-scFv library size was 1.2 $x10^7$ cfu/mL. HFG-specific antibody fragments were selected by incubation with HFG-BTG immobilised on the surface of an immunotube, as described in *Section 2.7.1*.

3.4.2 Biopanning of the halofuginone light chain-shuffled scFv library

Biopanning was performed as previously described in *Section 3.3.2.* However, as the library was already biased for HFG, the stringency for selection was increased. **Table 3.1** shows the conditions used for biopanning the chain-shuffled scFv library.

Table 3.1 *Biopanning conditions for the light chain-shuffled halofuginone-specific scFv library using varying concentrations of halofuginone-BTG coated on immunotubes. The stringency of each consecutive round of panning was increased by increasing the number of washes and decreasing the coated antigen concentration.*

Biopanning round	**Immobilised antigen concentration**	**Washing frequency**	**Input titres (Cfu/mL)**	**Output titres (Cfu/mL)**
1	25 μg/mL	3 X PBS, 3 X PBST	5.1×10^{12}	4.2×10^{6}
2	12.5 μg/mL	5 X PBS, 5 X PBST	1.2×10^{11}	3.7×10^{6}
3	5 μg/mL	7 X PBS, 7 X PBST	3.6×10^{11}	4.8×10^{6}
4	2.5 μg/mL	10 X PBS, 10 X PBST	5.5×10^{11}	6.0×10^{7}

The precipitated input phage from each round of panning was incorporated into a polyclonal phage ELISA to test for enrichment against the HFG-BTG conjugate. Successful enrichment was signified by an increase in absorbance signal in the second round after incubating the phage with an immobilised HFG conjugate and detecting bound phage with a HRP-labelled anti-M13 secondary antibody. This can be observed in **Figure 3.19**. High-level enrichment was observed following each round of panning, alongside high input and output phage titres for each round.

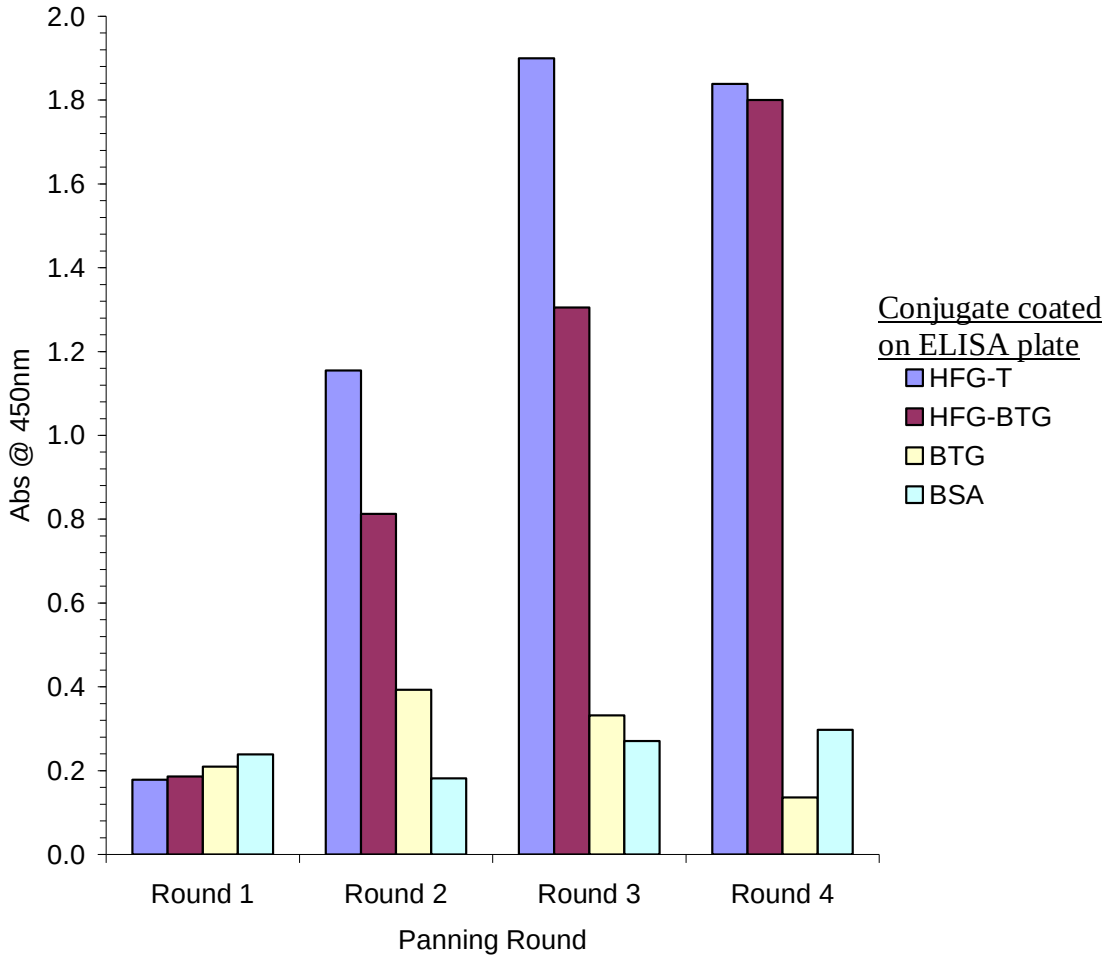

Figure 3.19 *Polyclonal phage ELISA screening for anti-HFG scFv displayed on phage, from the light chain shuffled library. Phage pools obtained after each round of panning were tested for binding to the HFG-BTG/HFG-transferrin (T) conjugate by ELISA. The phage pools were also screened against BTG during each round, to ensure no non-specific binding was occurring with the conjugate proteins. The scFv-displaying phage were detected using 500 ng/mL of a HRP-conjugated anti-M13 antibody and the absorbance was read at 450 nm using a Tecan Safire™ plate reader following a 20 minute incubation with TMB substrate. The increase in absorbance from round 2 onwards suggested the presence of HFG-specific scFvs.*

3.4.3 Monoclonal analysis of soluble clones from halofuginone biopanning

Single clone analysis of the chain shuffled library was performed as described in *Section 3.3.2.1.* From the 192 clones screened, 60 % were positive for HFG-binding

(**Figure 3.20 A & B**). These 115 clones were screened in a competitive ELISA to determine if their scFvs could bind to free HFG in solution.

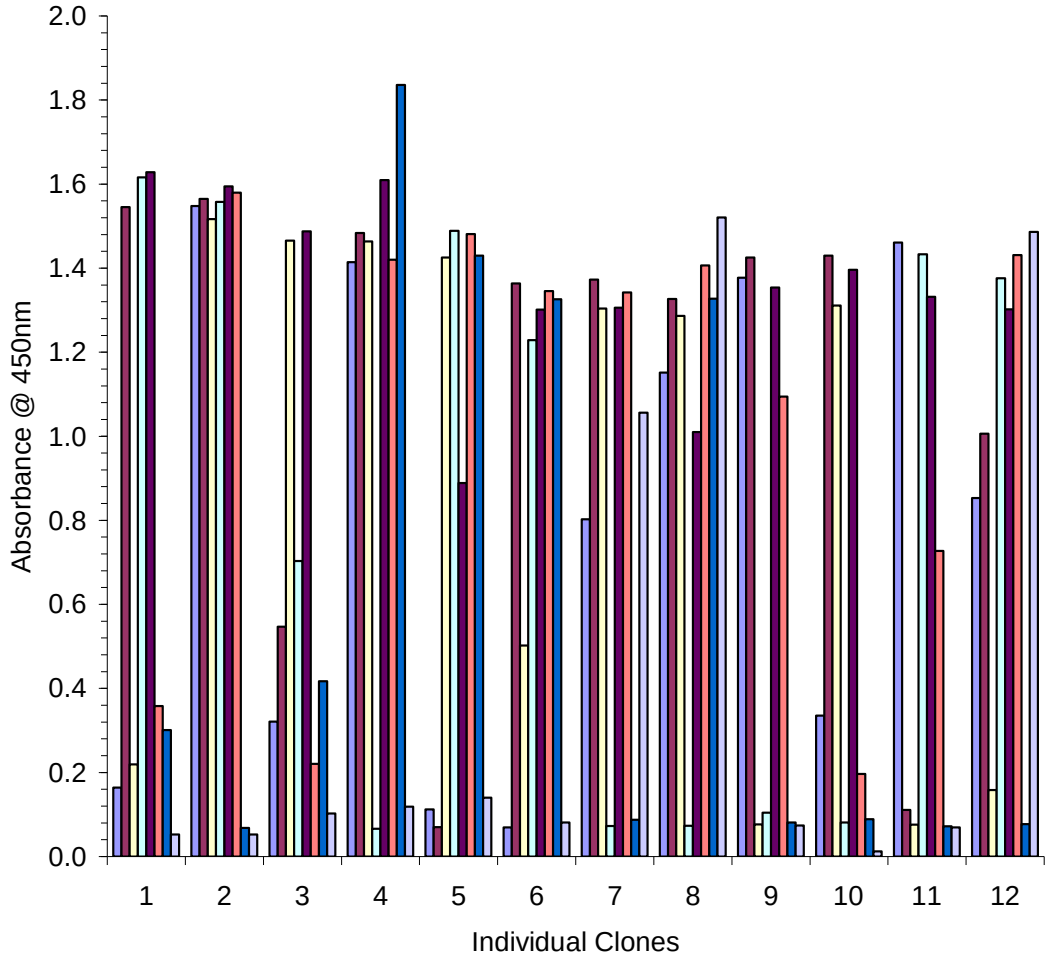

Figure 3.20(A) *Monoclonal ELISA to select for anti-HFG scFv from the chain-shuffled library in the fourth round of biopanning (Plate 1). The clones were solubly expressed in Top10F cells. Nunc MaxiSorp ELISA plates were coated with 1µg/mL of HFG-transferrin. ScFv were detected using a HRP-labelled rat-anti-HA antibody.*

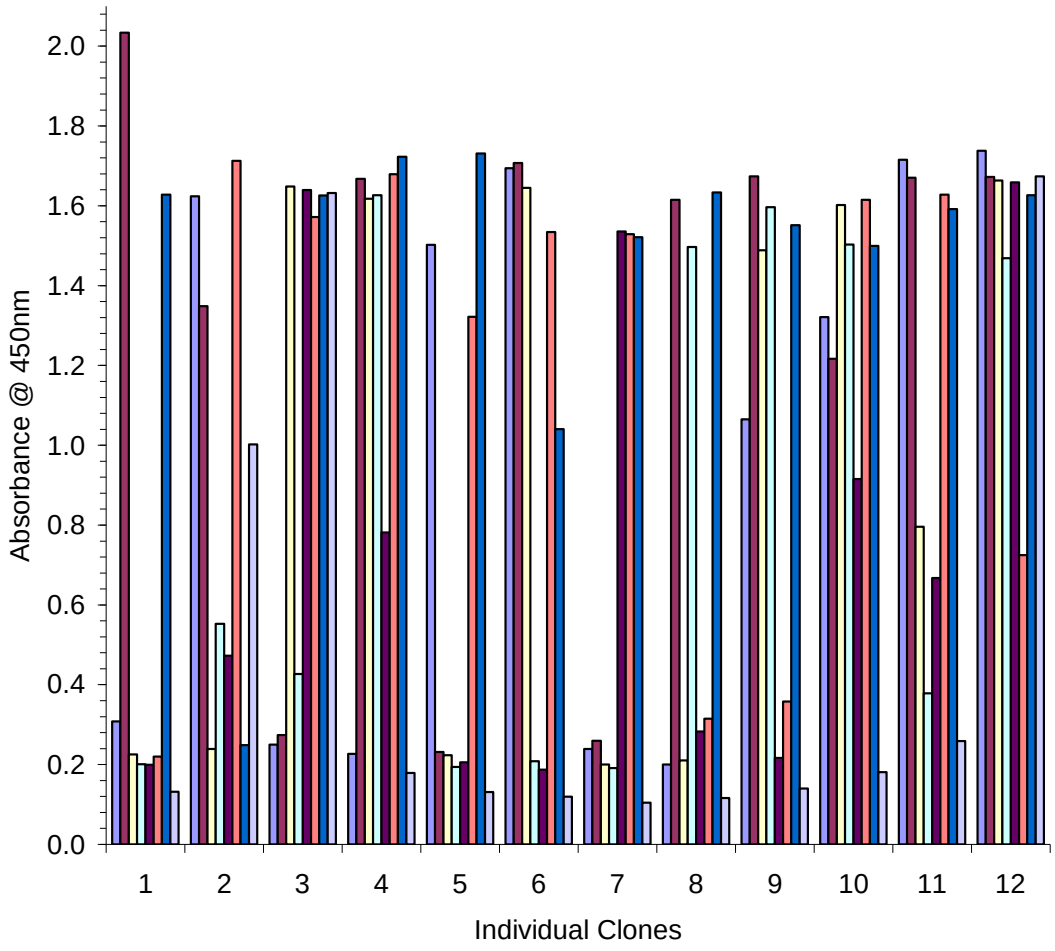

Figure 3.20(B) *Monoclonal ELISA to select for anti-HFG scFv from the chain-shuffled library in the fourth round of biopanning (Plate 2). The clones were solubly expressed in Top10F cells. Nunc MaxiSorp ELISA plates were coated with 1μg/mL of HFG-transferrin. ScFv were detected using a HRP-labelled rat-anti-HA antibody.*

3.4.4 Competitive ELISA analysis of anti-halofuginone light chain-shuffled clones

The 115 positive clones were taken forward for competitive studies. This competition ELISA incorporated lysates from each of the clones, with a high concentration (10 μg/mL) of HFG. The lysates were also analysed in the absence of HFG and the two results were compared to determine if HFG binding was inhibited by the large concentration of free HFG present. This competition ELISA identified numerous clones that appeared HFG-specific. From this, 10 clones were taken forward for broad-range competitive studies with decreasing concentrations of HFG ranging from 25 μg/mL to 127 pg/mL. Firstly, the clones were titred against HFG-transferrin to

determine the expression level of each (**Figure 3.21**). All of the clones appear to express functional scFv which titred out against HFG. The LOD of each of these clones was extrapolated from the competitive ELISA calibration curve, the results of which can be observed in **Table 3.2.** E9 displayed the lowest limit of detection with the capability to detect less than 8 ng/mL of HFG. At least five of the anti-HFG clones identified were more sensitive than the B4 wild-type clone.

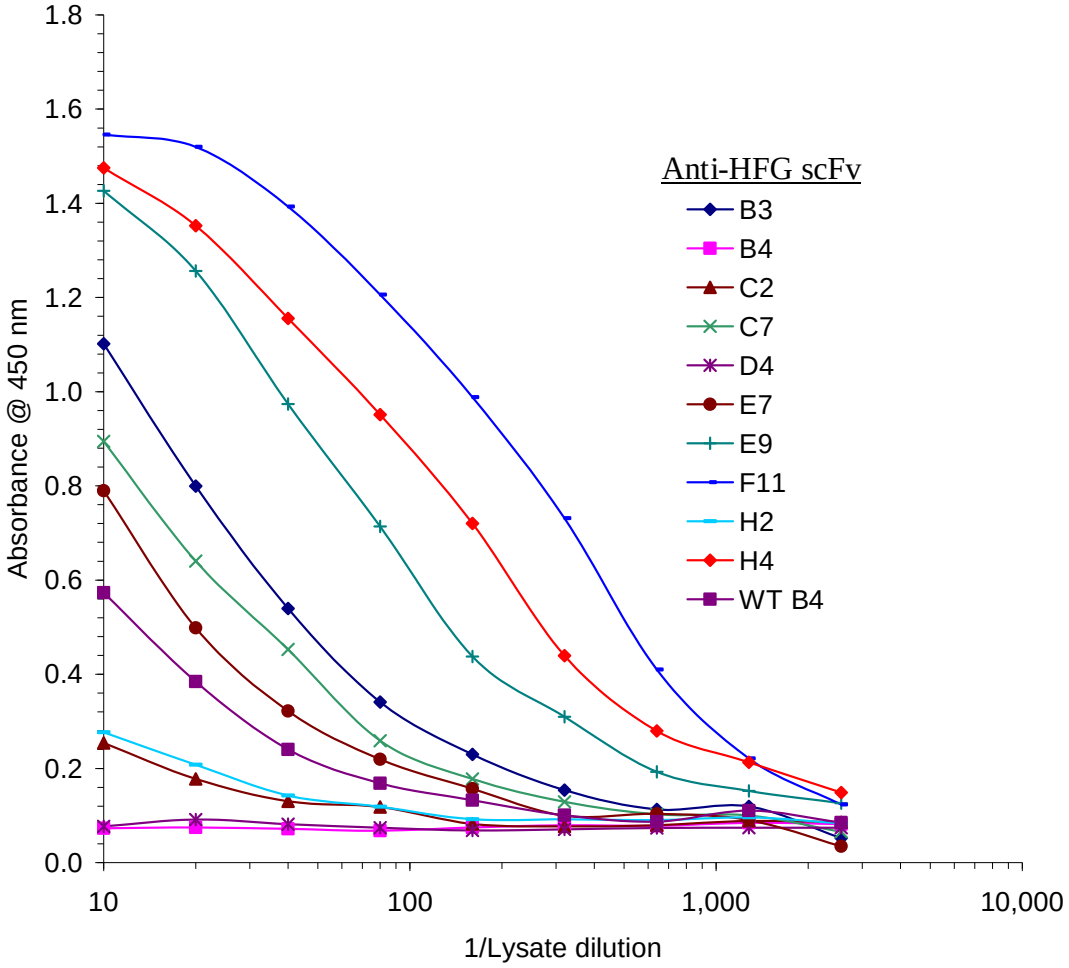

Figure 3.21 *Expression analysis of each of the competitive HFG-specific clones from the HFG chain-shuffled library. The scFv were tested at dilutions of 1/10 to 1/2,560. Any bound scFv was detected by the addition of a HRP-labelled anti-HA antibody.*

Table 3.2 *Limit of detection of each of the HFG-specific clones identified following monoclonal analysis from the light-chain shuffled HFG scFv library. The limits of detection were extrapolated from the competitive calibration curve for each clone.*

Clone	IC50 (ng/mL)
E9	>8
E7	15
H4(A)	20
C2	40
C7	100
F11	120
B3	120
D4	140
H2	150
B4(A)	200
Wild Type-B4	100

3.4.5 Sequencing of anti-halofuginone clones from light chain-shuffled library

Plasmid preparations of *E. coli* Top10F (containing the pComb3X bearing the anti-HFG chain-shuffled scFv E9, E7, H4 and C2) were sent to MWG for sequencing. These four clones exhibited the greatest sensitivity and were able to detect HFG well below the detection capability of the 'B4' clone at 100 ng/mL. The amino acid sequences were aligned and the CDRs of the antibody evaluated using MultiAlin software. The CDR regions of the antibodies were highlighted according to the Kabat scheme (**Figure 3.22**). The light and heavy chain CDRs are highlighted in green and purple, respectively. The heavy chain sequences of all three chain-shuffled scFv and the wild-type B4 scFv are all identical, with the exception of the C2 scFv which has 8 amino acid differences in the heavy chain. This sequence homology of the heavy chains was expected, as the heavy chain of the library was predisposed to bind to HFG and enrichment of individual clones with high affinity towards HFG occurred. Comparing E9, the best clone from the chain shuffled library, and the wild-type clone, B4, it is apparent from the consensus sequencing (**Figure 3.22**) of both, that the major sequence differences occur in the CDRL-3 region. These differences primarily include favoured substitutions such as asparagine at position 81 to serine (N81S), both of which are small polar amino acids or glycine at position 88 to alanine (G88A). Both amino acids are small and non-polar and hence, neither of these substitutions are likely to have a major effect on antigen binding. Also, at position 89 in the chain-shuffled E9 scFv, isoleucine

was substituted for the smaller alanine. The carbonyl group of the isoleucine could potentially interact and form hydrogen bonds with any of the upstream hydroxyl groups from the numerous tyrosines that are in close proximity to the CDRs of the light chains. It has previously been highlighted how framework region residues, that have atomic interaction with CDR residues, were found to indirectly influence the antibody binding affinity by altering the conformation of the CDR loops (Xiang *et al.,* 1999). This isoleucine to alanine substitution is the only non-conservative substitution in the CDRL3. In order to determine specific residues that contribute to the functionality of a protein, alanine mutagenesis is commonly employed to identify the contribution of individual amino acid side-chains to protein function, stability and shape (Morrison and Weiss, 2001). The pre-dominant substitution in the CDRL3 from the chain-shuffled clone was an alanine substitution. This indicates that the side-chain from the isoleucine may have partially blocked the antigen binding site or promoted steric hindrance effects during antigen binding.

The E9 scFv had the lowest IC50, and therefore was taken forward for intra/inter day competitive studies in comparison with the B4 (original) clone, to produce a validated ELISA assay.

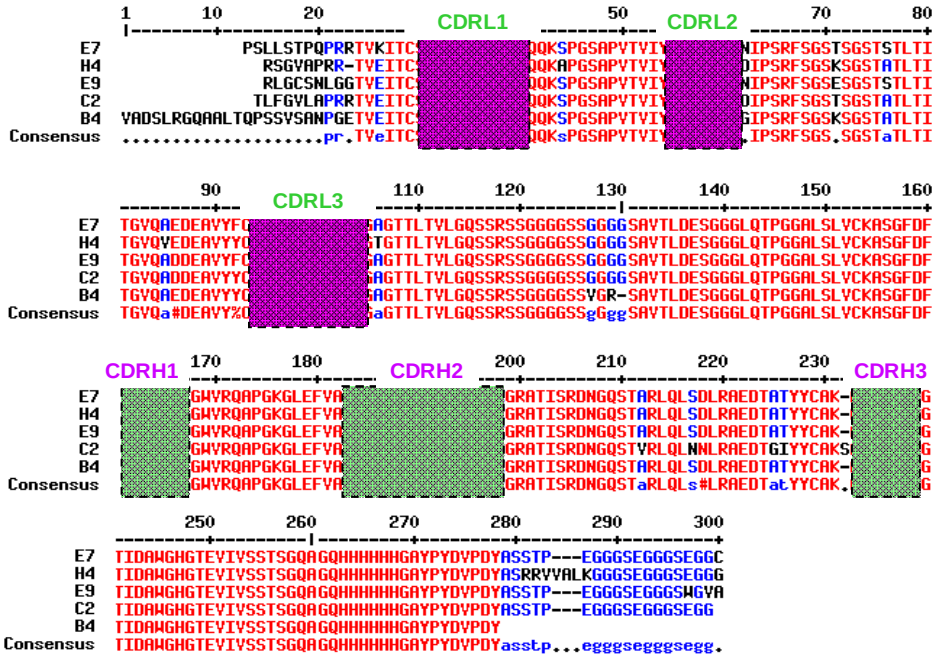

Figure 3.22 *Avian anti-HFG scFv gene sequences of selected clones following chain-shuffling. The highlighted sequences in green and purple show the CDRs (heavy and light chain) of the recombinant antibody fragments. The framework regions are highlighted in red and variations from the consensus of the sequences are shown in black.*

3.4.6 Expression and purification of the scFv E9 and subsequent characterisation by SDS-Page and Western Blotting

Large-scale expression of the avian anti-HFG E9 scFv was performed in 500 mL cultures, as outlined in *Section 2.9.2*. The scFvs were purified as described in *Section 3.3.2.4*. The IMAC-purified scFv-containing fractions were pooled, centrifuged and analysed by SDS-PAGE (**Figure 3.23**) and western-blotting (**Figure 3.24**)

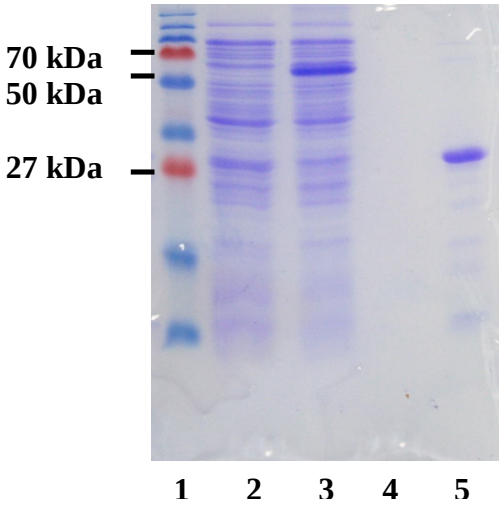

Figure 3.23 *SDS-PAGE analysis on the fractions obtained during IMAC purification of the E9 anti-HFG chain-shuffled scFv. Lanes 1 and 5 = Colour Burst molecular weight markers (Fermentas); Lane 2 = Lysate diluted 10-fold in PBS; Lane 3 = Wash 'A'; Lane 4 = Wash 'B'; Lane 5 = Eluted purified antibody fractions pooled and used undiluted. One distinct band is visible at approximately 28 kDa in the purified fractions, representing the purified scFv.*

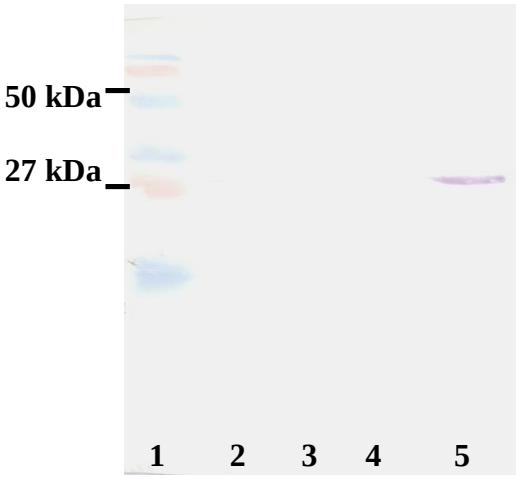

Figure 3.24 *Western blot analysis of the purified E9 anti-HFG scFv. Lanes 1 = Colour Burst molecular weight marker (Fermentas); Lane 2 = Lysate diluted 10-fold in PBS; Lane 3 = Wash 'A'; Lane 4 = Wash 'B'; Lane 5 = Eluted purified antibody fractions pooled and used undiluted. One distinct band is visible at approximately 28 kDa in the purified fraction representing the purified scFv.*

3.5 Optimisation of an ELISA for the detection of halofuginone spiked into eggs, using the purified E9 chain-shuffled scFv

Following purification of the E9 scFv, its use in a competitive immunoassay for the detection of HFG was investigated. In order to maximise the performance of the assay, several parameters were optimised. These included the optimal coating concentration of the conjugate and the optimal buffer for coating. Once these parameters were determined, intra/inter-day assays were performed to establish the reproducibility of the assay alongside comparison of assay results with the B4 wild-type scFv.

3.5.1 Selection of conjugate coating buffer for the competitive ELISA for halofuginone

To investigate the optimal pH for coating the HFG-transferrin conjugate onto the Nunc MaxiSorp plates, five different coating buffers were employed at five different pH levels. The buffers investigated were: 0.1 M acetate buffer, pH 4.8; 0.1 M phosphate buffer, pH 5.0; 0.1 M carbonate buffer, pH 10.3; 0.1 M citrate buffer, pH 3.0 and 0.01 M PBS, pH 7.4. The results are illustrated in **Figure 3.25**. The results show that the amount of antigen bound to the plate was greatest when using PBS at a pH level of 7.4, or carbonate buffer at a pH level of 10.3. PBS at a pH level of 7.4 was chosen as the conjugate coating buffer for use in subsequent assays.

3.5.2 Checkerboard ELISA for the determination of the optimum scFv dilution and optimum coating concentration for HFG-transferrin in competitive assays

It is essential to optimise both the coating concentration of the conjugate and the optimal dilution of the antibody employed in a competitive ELISA. Nunc MaxiSorp ELISA plates were coated with varying concentrations of the HFG-transferrin conjugate in the optimal conjugate coating buffer, as previously established. To determine the concentrations which would give the best assay sensitivity, a checkerboard ELISA was performed with varying dilutions of the affinity-purified antibodies. The results shown in **Figure 3.26** indicate that an optimum coating concentration of 1 µg/mL HFG-transferrin gave the highest absorbance using the most economical conjugate concentration. The optimal antibody dilution chosen, was a 1/10,000 dilution.

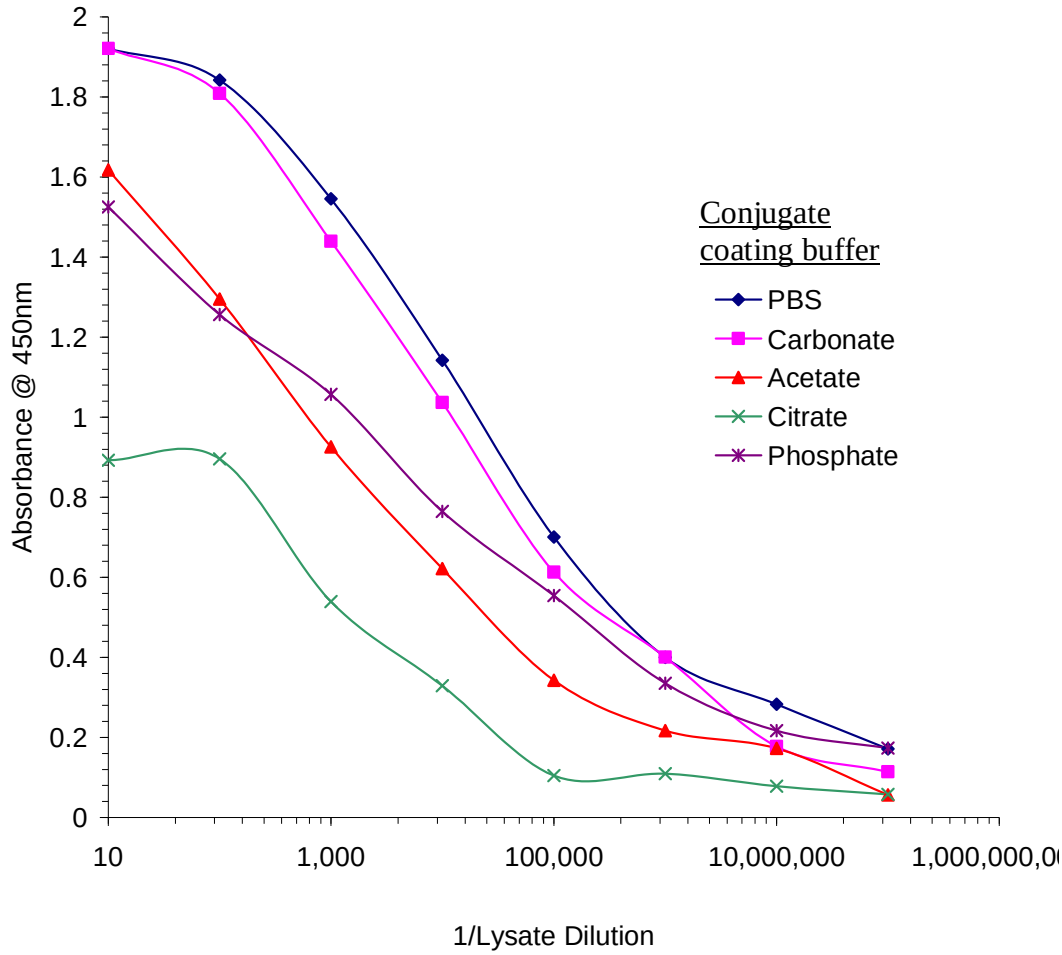

Figure 3.25 *Selection of optimal coating buffer for use in ELISA for the detection of HFG. One µg/mL of HFG-Transferrin conjugate was used. Coating buffers tested were; 0.1 M acetate buffer, pH 4.8; 0.1 M phosphate buffer, pH 5.0; 0.1 M carbonate buffer, pH 10.3; 0.1 M citrate buffer, pH 3.0 and 0.01 M PBS, pH 7.4. The anti-HFG scFv was serially diluted in buffer and added to the coated/blocked wells. Bound polyclonal antibody was then detected using HRP-labelled anti-HA secondary antibody.*

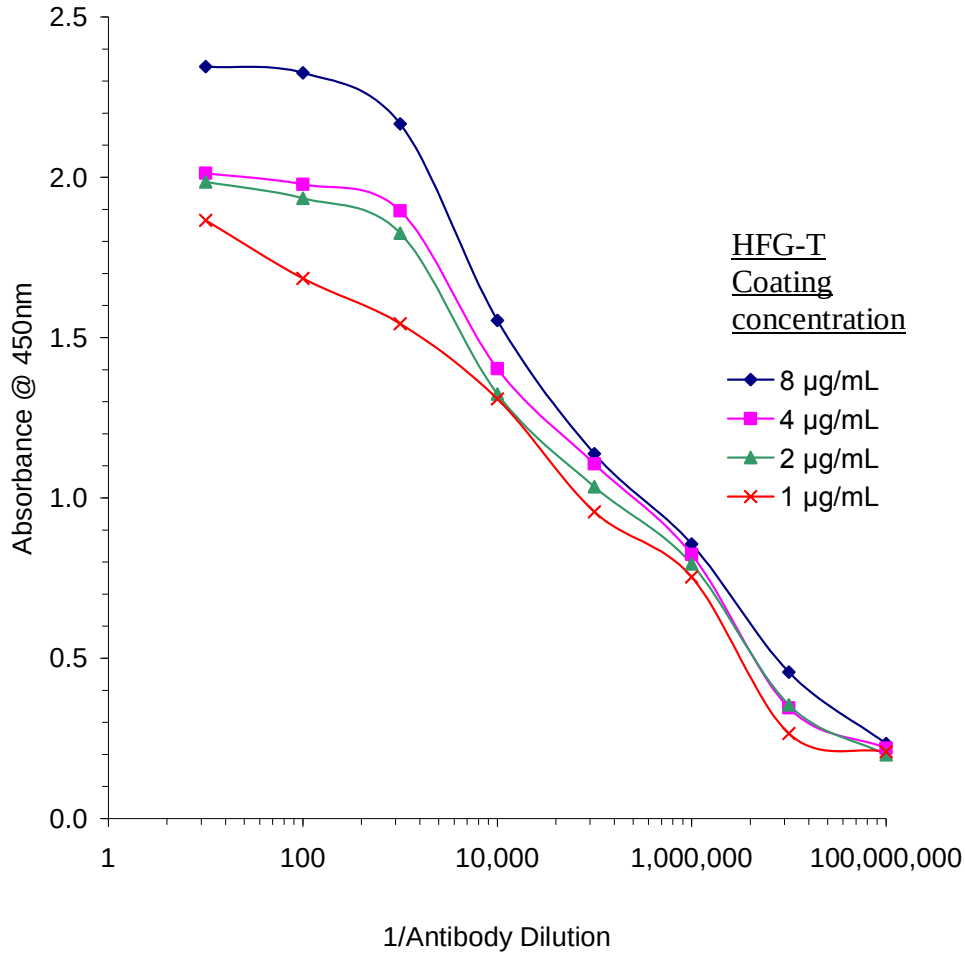

Figure 3.26 *Checkerboard ELISA for the determination of the optimal conjugate coating concentration and scFv dilution for use in a competitive ELISA for HFG. HFG-transferrin (T) concentrations of 8, 4, 2 and 1 µg/mL were used in a direct ELISA with varying dilutions of scFv ranging from 1/10 to 1/100,000,000. A 1 µg/mL HFG-transferrin (T) concentration and a 1/10,000 dilution of the scFvs were chosen for use in a competitive assay format.*

3.5.3 Cross reactivity profile of the E9 anti-halofuginone scFv

The E9 HFG-specific scFv was assessed for cross reactivity against five structurally similar veterinary drugs, which included; Toltrazuril, Ponazuril, Nicarbazin, Diaveridine, and Ethopabate, as described in *Section 2.9.3.3*. The cross reactivity profile of the E9 scFv showed 100 % cross-reactivity with the primary drug of interest, HFG, and less than 4 % cross-reactivity with any other structurally similar drug. This 4% cross-reactivity may be due to non-specific binding of the E9 scFv as it occurred with all 5 structurally similar drugs tested. The specificity of the antibody

towards HFG alone suggests that the antibody is binding to the bromine and chlorine atoms on HFG, as these atoms are not present in any of the other drugs.

3.5.4 Intra and inter-assay variability studies of the inhibition ELISA for halofuginone extracted from egg samples

Using the optimised parameters established, a competitive assay (free HFG in solution competing against immobilised conjugate for binding to the antibody), was performed in extracted egg samples as described in *Section 2.9.3.2*. Standards of HFG were prepared in 0.01 M PBS, pH 7.4, and were diluted with equal volumes of the scFv at a 1/10,000 (final) dilution. The final concentrations of HFG ranged from 5 μg/mL down to 254 pg/mL. This scFv-HFG preparation was added to an immunoplate coated with HFG-Transferrin in 0.01M PBS, pH 7.4, and blocked with 5% (w/v) Milk Marvel. HRP-labelled anti-HA antibody was then used to detect the HFG-bound scFv. Calibration curves were prepared using BiaEvaluation, and the detection capability could be extrapolated from the curve. Co-efficients of variation (CV) were determined to assess the reproducibility of the HFG assay. These are listed for 11 concentrations of free HFG within intra/inter-day assay results in **Table 3.3** with the highest CV recorded at 15.9 %. According to the 2002-657-EC Commission decision, the method reproducibility should not be greater than the corresponding reproducibility CV at a concentration of 0.5 × the permitted limit. The CVs were calculated for samples at just below half the MRL (335 pg/mL) as 9.4 % for intra-day and 10.9 % for inter-day assays, and overall CVs were calculated as 8.4 % and 9.2 % for intra and inter-day values, respectively. Hence, this assay complies with the EU guidelines for validation.

Table 3.3 *Assay performance characteristics for Halofuginone detection. Inter-day assays were plotted in BiaEvaluation™. The average percentage recovery was calculated at 91.1%.*

Halofuginone Conc. (pg/mL)	Intra-day (% C.V)	Inter-day (% C.V)	Percentage Accuracy (%)
32768	15.9	12.5	95.0
13107.2	14.1	11.4	97.0
5242.8	9.8	10.8	95.1
2097.1	5.1	7.3	95.2
838.8	3.1	6.9	89.1
335.5	9.4	10.9	99.4
134.2	11.6	11.7	93.5
53.6	6.7	9.3	53.2
21.4	5.5	6.2	97.6
8.5	7.2	6.4	-
3.4	3.8	7.2	-

A calibration curve was constructed from inter-assay results using a 4-parameter equation in BiaEvaluation™ software; from this the decision limit was calculated (**Figure 3.27**). This was estimated to be approximately 162 pg/mL for E9 and approximately 30 ng/mL for B4. The detection capability (CCβ) was then established by measuring 20 samples at the decision limit and adding 1.64 times the standard deviation of the samples to the concentration of the decision limit. This was calculated to be 163 pg/mL for E9 and 31 ng/mL for B4. These results were extrapolated from the calibration curve. However, when experimentally determining the functional limit of detection using the E9 scFv and 20 samples of varying concentrations of HFG, the lowest concentration of analyte that could be detected with 95% certainty above the blank standard was determined to be 80 pg/mL. This is illustrated in **Figure 3.28** where it can be observed that at least 19 out of 20 of the fortified samples with 80 pg/ml HFG could be correctly distinguished from the blank standard. This is a 185-fold improvement in antibody sensitivity over the original scFv.

Chain shuffling has been previously shown to enhance the properties of certain recombinant antibodies several fold. This notable increase in sensitivity highlights the importance of investigating heavy-light chain pairings.

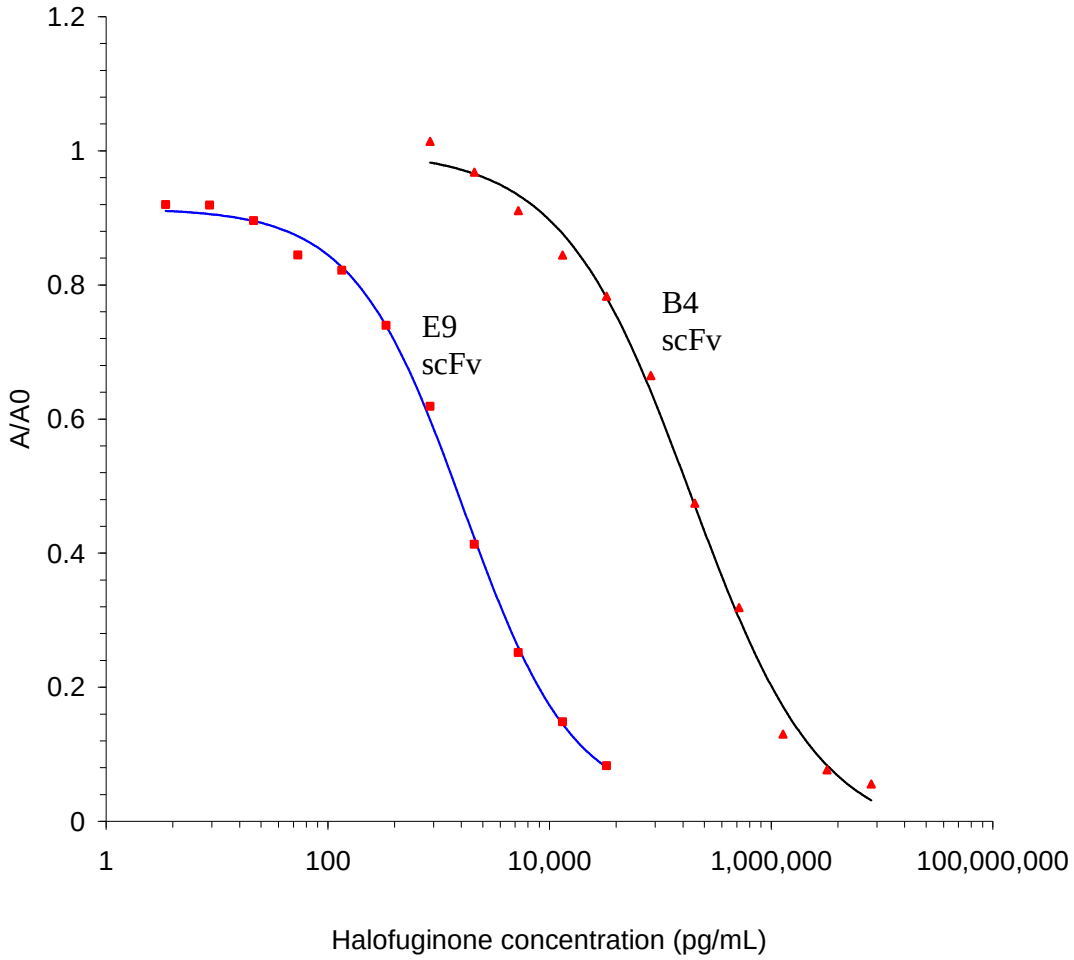

Figure 3.27 *Calibration curve of Inter-day assays for both the wild-type 'B4' and chain shuffled 'E9' scFv. The calibration curves were constructed by using a 4-parameter equation in BiaEvaluation™. The limit of detection was calculated in BiaEvaluation to be 162 pg/ml and 30 ng/mL for E9 and B4, respectively. The absorbance of the evaluated HFG samples was normalised by expressing the absorbance as a function of the blank standard (A/A0). The functional limit of detection of the E9 scFv was then experimentally determined to be below this value at 80 pg/mL.*

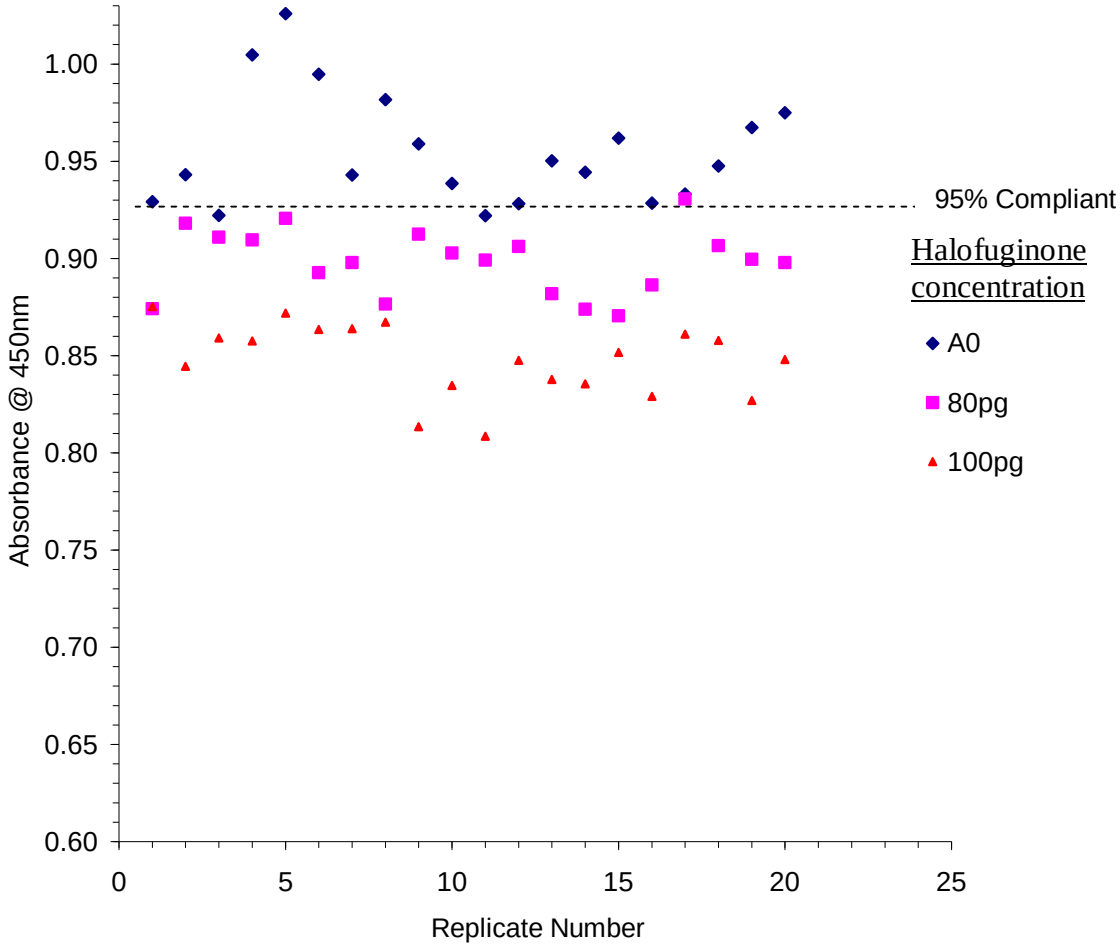

Figure 3.28 *Determination of limit of detection of the HFG-specific E9 scFv using 20 replicates of the blank standard, 20 replicates of 80 pg/mL HFG and 20 replicates of 100 pg/mL HFG in a competitive ELISA format with 1 µg/mL HFG-transferrin coated on the immunosorbent plate. HRP-labelled anti-HA Mab was used to probe for bound scFv. The dashed line indicates 95 % compliance, i.e. the ability of the antibody to detect 95 % of the samples as positive for HFG residues when compared to the blank standard.*

3.6 Development of an scFv-based Biacore assay for the detection of halofuginone in egg samples

Using the E9 chain-shuffled anti-HFG scFv previously described, a surface plasmon resonance-based assay on the Biacore 3000 system was optimised to detect HFG.

3.6.1 Preconcentration studies for the immobilisation of halofuginone onto the CM5 sensor chip surface

Preconcentration studies were performed to determine the optimum pH at which the electrostatic interactions between the HFG-transferrin and a CM5 sensor chip surface are most favourable for maximum binding to occur. A broad pH range was investigated using buffers with different buffering capacities, as described in *Section 2.11.3*. The conjugate was passed over the activated chip surface in the different buffers with pH below the isoelectric point (pI) of the protein. **Figure 3.29** demonstrates that the amount of HFG conjugate electrostatically absorbed onto the CM dextran surface is dependant on the pH of the buffer. The pH level allowing the optimum amount of conjugate to be immobilised on the surface was pH 4.0. This buffer was selected for the immobilisation of the conjugate onto the CM5 sensor chip.

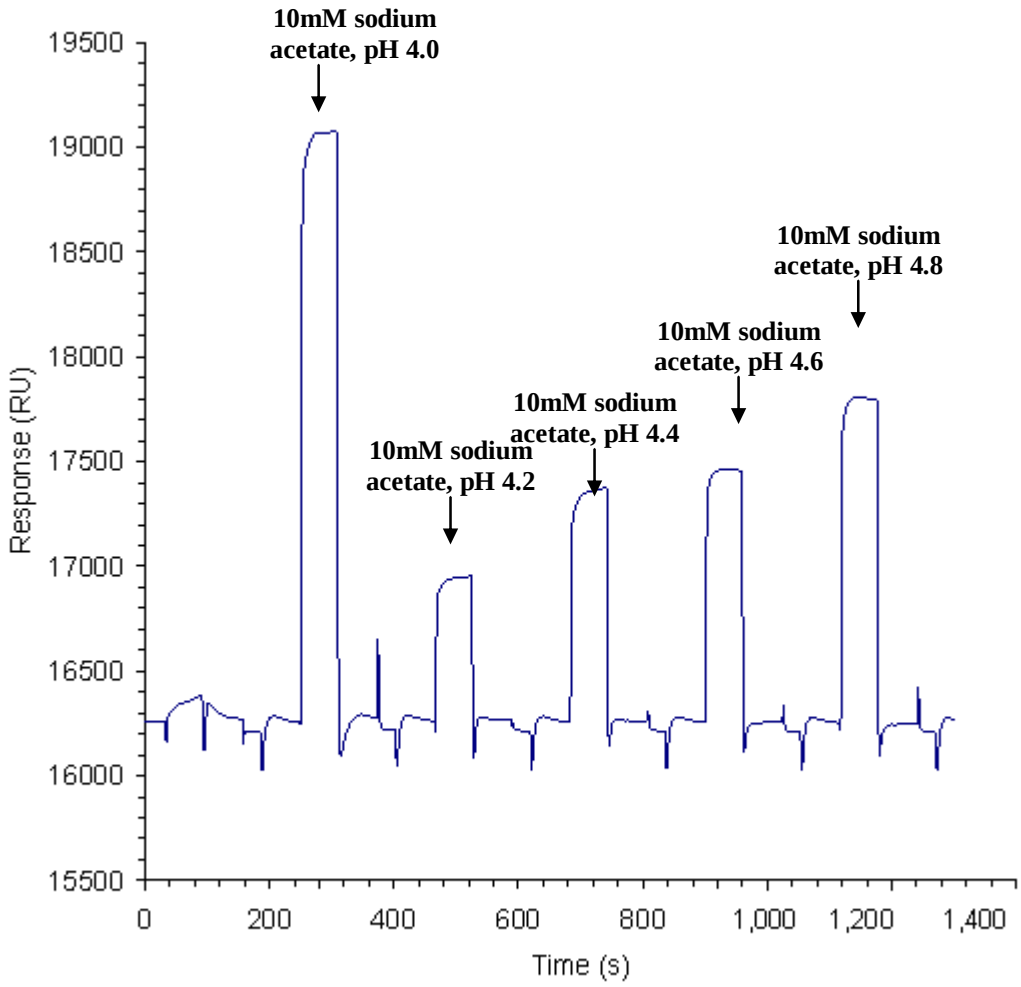

Figure 3.29 *The HFG-transferrin conjugate in 10 mM solutions of sodium acetate at pH levels of 4.0, 4.2, 4.4, 4.6, and 4.8, was passed over the surface of a CM5 dextran chip at a flowrate of 10 µl/min. The optimal pH for the immobilisation of the HFG-Transferrin conjugate to the CM5 dextran surface was 4.0 with the highest levels of apparent binding observed.*

3.6.2 Production of a directly immobilised halofuginone-transferrin CM5 sensor chip surface

HFG-Transferrin was directly immobilised onto the surface of a CM5 chip as described in *Section 2.11.4,* by coupling with free primary amine groups on the conjugate. This immobilisation process can be observed in **Figure 3.30**. The carboxymethyl groups on the chip are activated by passing a mixture of EDC/NHS over the surface. The EDC solution, in the presence of NHS, activates the CM dextran carboxyl groups into functional ester groups. The surface NHS esters react with

primary amino groups on the protein causing covalent binding of the protein to the surface. In the last step the remaining esters are transformed into amides by reaction with ethanolamine, essentially 'capping' any unreacted sites. A final level of 3880 RU of covalently attached HFG-transferrin was achieved.

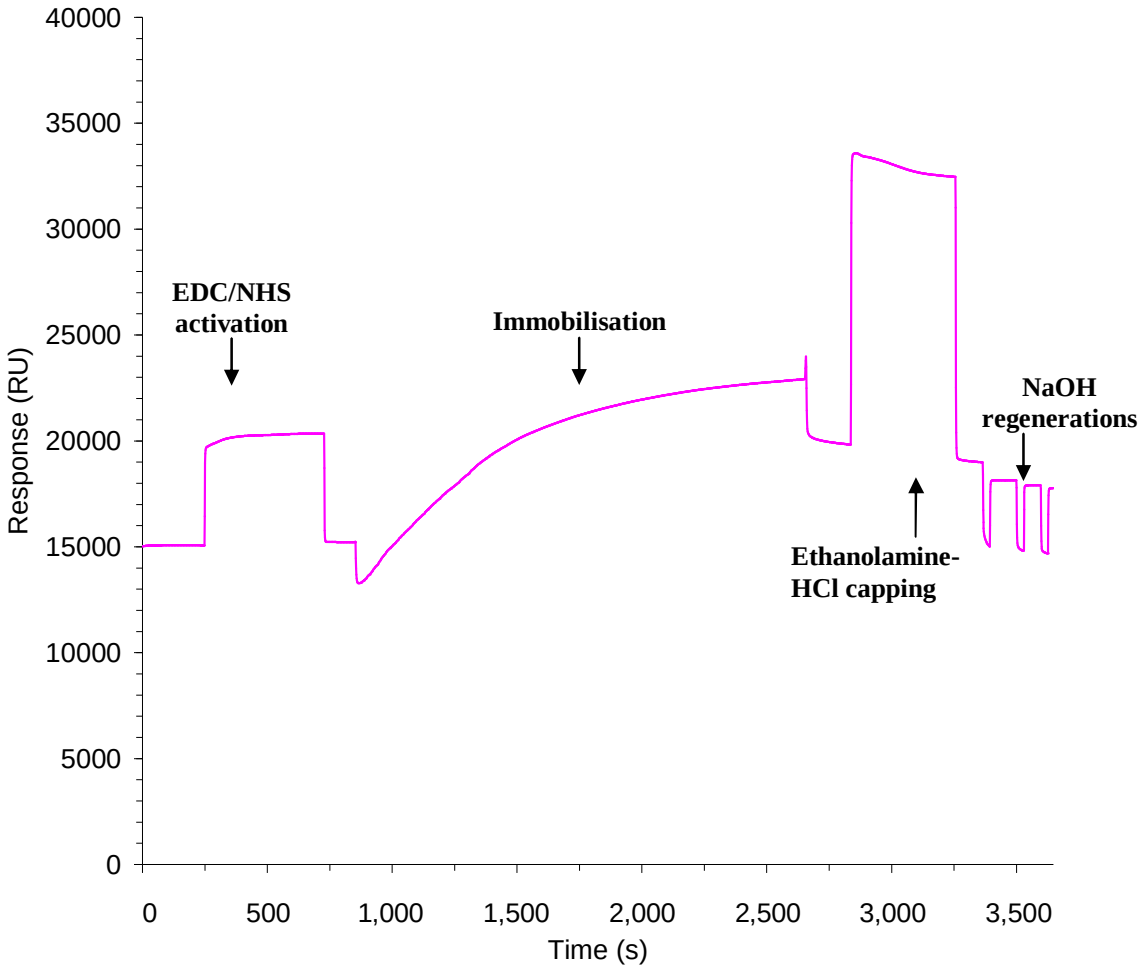

Figure 3.30 *Sensorgram illustrating the direct coupling of HFG-transferrin to a CM5 sensor chip surface. The surface was activated with EDC/NHS solution, and the HFG-transferrin was passed over the activated carboxyl surface. Deactivation of any unbound or free functional ester groups was achieved by treating the chip surface with ethanolamine-HCl, resulting in the capping of any reactive groups. The surface was then regenerated with a solution of NaOH. A final level of 3880 RU of covalently attached HFG-transferrin was achieved.*

3.6.3 Evaluation of optimal flowrate, contact time and scFv dilution for SPR detection of HFG residues in a competitive format

The optimal flowrate, contact time and dilution of the anti-HFG scFv for use on the covalently attached HFG-transferrin chip, was investigated. Contact times of 3, 6, 9 and 12 minutes, each at flowrates of 10, 20 and 30 µl/minute were evaluated. This was designed to determine which parameters gave the highest response change with respect to analyte concentration. It can be observed in **Figure 3.31** that a contact time of 6 minutes, a flowrate of 10 µl/minute and a scFv dilution of approximately 1/4,000 yields response units between 100-150 RU, which is an ideal maximum response for a competitive assay, without the assay being too time-consuming or using large quantities of antibody.

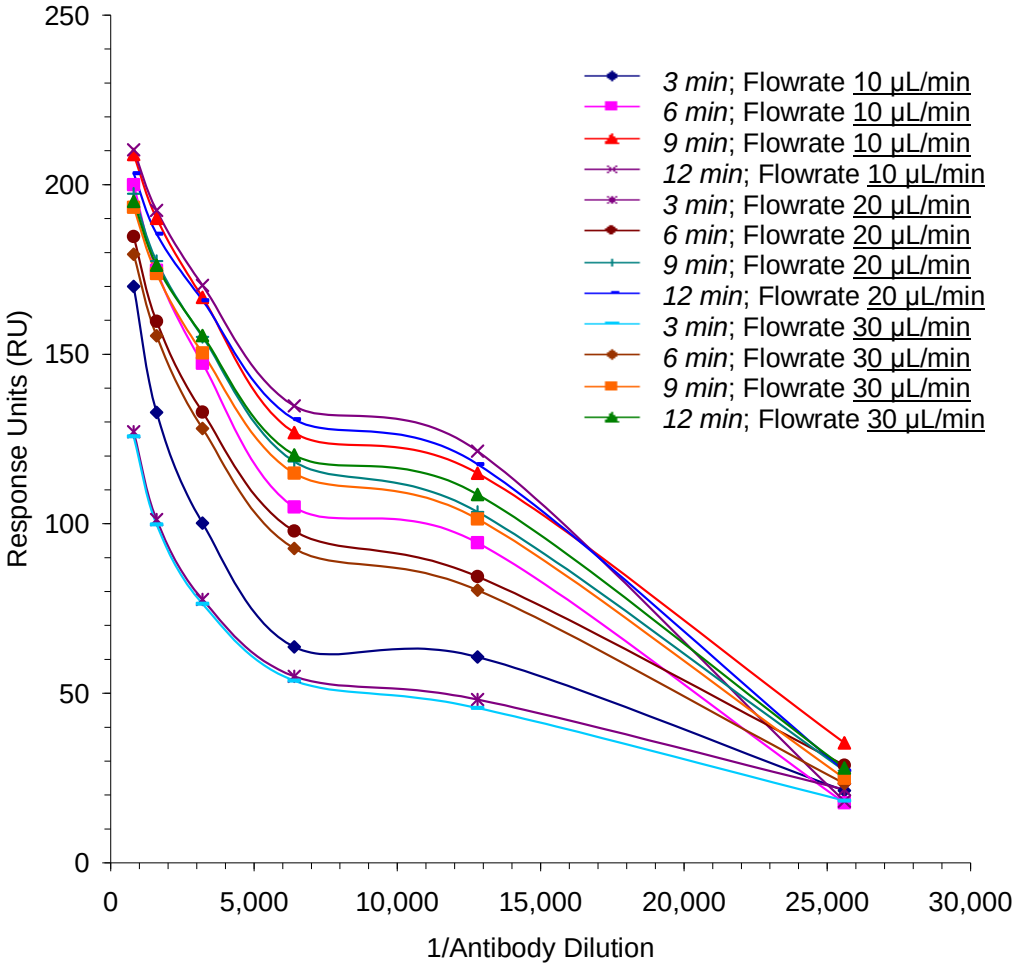

Figure 3.31 *Optimisation of halofuginone Biacore assay parameters including flow-rate, contact time and scFv dilution. Three different flowrates 10, 20 and 30 µl/minute, four different contact times 3, 6, 9, and 12 minutes, and six different scFv dilutions, 1/800, 1/1,600, 1/3,200, 1/6,400, 1/12,800, and 1/25, 600, were assessed to determine the optimal conditions for the SPR detection of HFG.*

3.6.4 Preliminary Biacore inhibition-analysis for the detection of halofuginone in PBS.

To ensure that the assay was functional in a competitive format, a preliminary competitive assay was performed in PBS using six different concentrations of free HFG ranging from 100 to 1.56 ng/mL. Each of these concentrations of HFG were incubated with a 1/4,000 (final) dilution of the E9 scFv prior to passing over the surface of the HFG-immobilised sensor-chip. **Figure 3.32** shows the sensorgram data for the preliminary competitive analysis. It is apparent from the graph that the greater the concentration of free HFG in the samples, the less scFv is available for binding to

the sensor surface. When comparing the data from the 100 ng/mL of HFG to 1.56 ng/mL of HFG, it can be observed that there is at least 200 RU less of antibody binding occurring when the higher concentration of HFG is present.

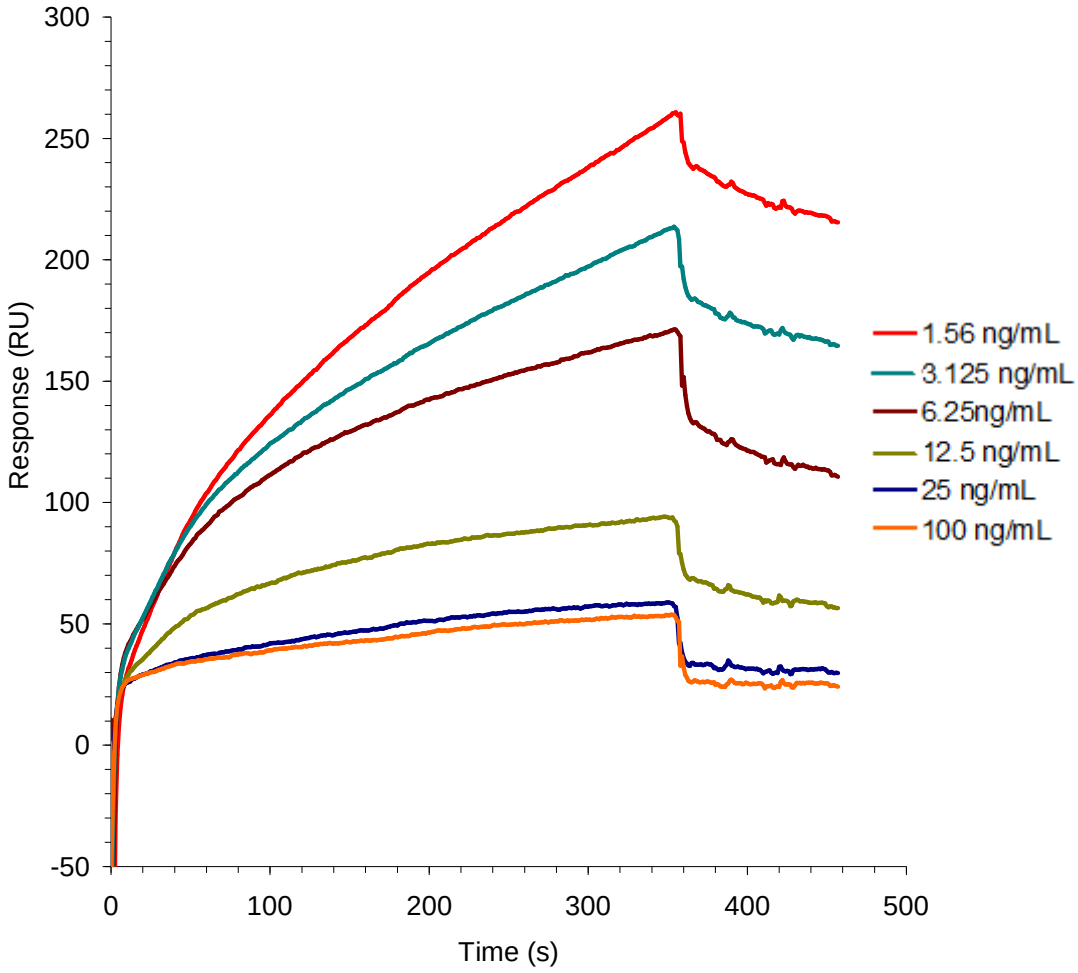

Figure 3.32 *Overlay plot demonstrating the decrease in binding of purified anti-HFG scFv to the immobilised HFG-transferrin surface when incubated with increasing concentrations of free HFG at 100, 25, 12.5, 6.25, 3.125, and 1.56 ng/mL in PBS.*

3.6.5 Analysis of Biacore-based halofuginone-detection assay performed in egg matrices.

Assay reproducibility may be affected by environmental conditions and can lead to significant variations in the analytical results. To determine the intra-day (within the same assay) and inter-day (between assays) variation of results, a calibration curve was generated for HFG. For the inter/intra-day studies, egg samples were spiked (as described in *Section 2.11.7)* with HFG at concentrations of 0, 0.78, 1.5, 3.1, 6.3, 12.5,

25, and 50 ng/mL. The spiked-HFG residues were subsequently recovered from the matrix and resuspended in PBS/ 5% (v/v) MeOH. They were mixed with an equal volume of the HFG-specific scFv at a final concentration of 10.6 µg /mL (1/4,000 dilution) prior to injection over the sensor–chip surface. Each standard was injected in triplicate at a flow-rate of 10 µL/minute for 6 minutes. Response units (RU) were recorded before and after sample injection. The normalised responses were calculated and a four-parameter equation was fitted to the data. The co-efficients of variation (CV) values between the inter and intra-day batches were calculated from the calibration curve. These are outlined in **Table 3.4**. From the CV values, it is apparent that the reproducibility of the Biacore assay is very good, as the CVs for the intra-day assay are an average of 4.2 % with the highest CV recorded at 10.6 %. For the inter-day assays the average CVs are at 4.5 % with the highest at 9.8 %. The percentage accuracy of the assay was extrapolated from the calibration curve and the average calculated at 99.5 % which is excellent for a competitive assay performed in extracted egg matrix. The effects of egg matrix and extraction recoveries are discussed further in Chapter 5.

Table 3.4 *Calculated co-efficients of variation for the chain-shuffled E9 scFv in a competitive assay format with concentrations of HFG ranging from 50 ng/ml to 780 pg/mL. Inter and intra day assays were performed. Bound scFv was recorded as response units. Percentage recoveries were extrapolated from the four-parameter calibration curve of the inter-day assays plotted in BiaEvaluation™. The average percentage recovery was calculated at 99.5%.*

Halofuginone Conc. (ng/mL)	**Intra-day (% C.V)**	**Inter-day (% C.V)**	**Percentage Accuracy (%)**
50	1.2	1.2	97.8
25	1.2	1.1	102.1
12.5	0.4	1.0	99.9
6.25	10.6	7.6	95.9
3.12	9.6	9.8	122.8
1.56	2.5	6.6	99.5
0.78	3.9	4.7	78.9

Figure 3.33 shows the inter-assay mean calibration curve in extracted egg matrix using the anti-HFG scFv. The red dashed line indicates the lowest level of HFG detectable in this matrix. The LOD of the assay in egg samples was found to be approximately 3.4 ng/mL, as extrapolated from the calibration curve.

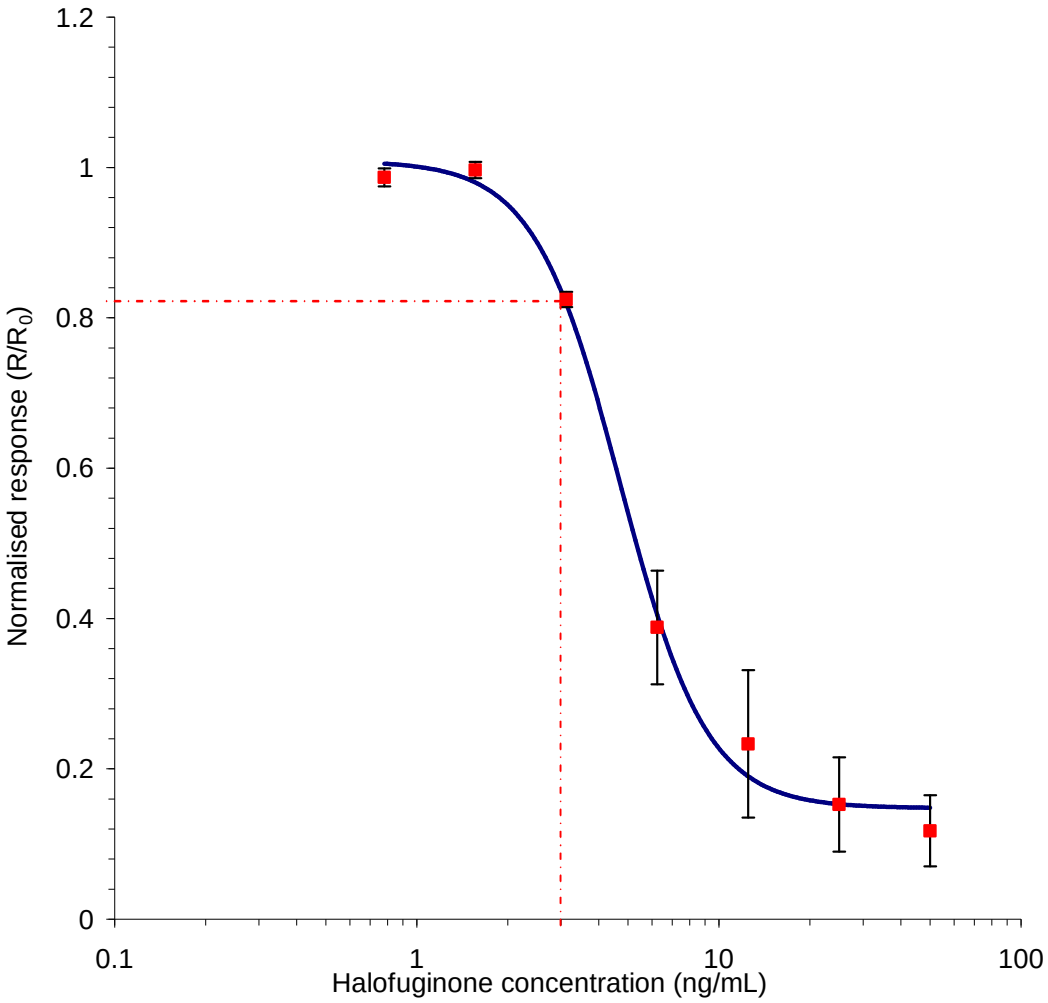

Figure 3.33 *Intra-day assay results for HFG extracted from eggs. Data was expressed as R/R0 (i.e. response at different free analyte concentrations are expressed as a proportion of response in the presence of no competing analyte.) Analysis was performed in triplicate on different sets of standards. The limit of detection of HFG as denoted by the dashed line is 3.4 ng/mL.*

The analytical limit 'CCα' (decision threshold) as discussed in *Section 3.1*, was calculated as the average analyte concentration of the blank standards plus 2.33 times the standard deviation of the blank samples. This was approximately 3.28 ng/mL. Using this value, the 'CCβ' (detection capability) could be determined by adding the result for CCα with 1.64 times the standard deviation of the samples spiked at half the MRL or 3 ng/mL (i.e. 3.28 + 1.64 (0.082)). This was calculated as 3.41 ng/mL. This is illustrated in **Figure 3.34** whereby 20 samples spiked at half the MRL and 20 blank samples were analysed. These response values were all normalised (R/R0) to estimate

the standard deviations and extrapolate corresponding concentrations from the standard curve (**Figure 3.33**). The functional LOD was experimentally verified by analysing 20 samples at concentrations of 2.8 ng/mL. It can be observed from **Figure 3.34** that 20/20 of these samples can be reliably distinguished from the blank standard. The false negative rate of (≤5%) as required by the 2002/657/EC legislation is adhered to and, therefore, after experimental verification, 2.8 ng/mL was calculated as the actual detection capability for the halofuginone Biacore-based detection assay.

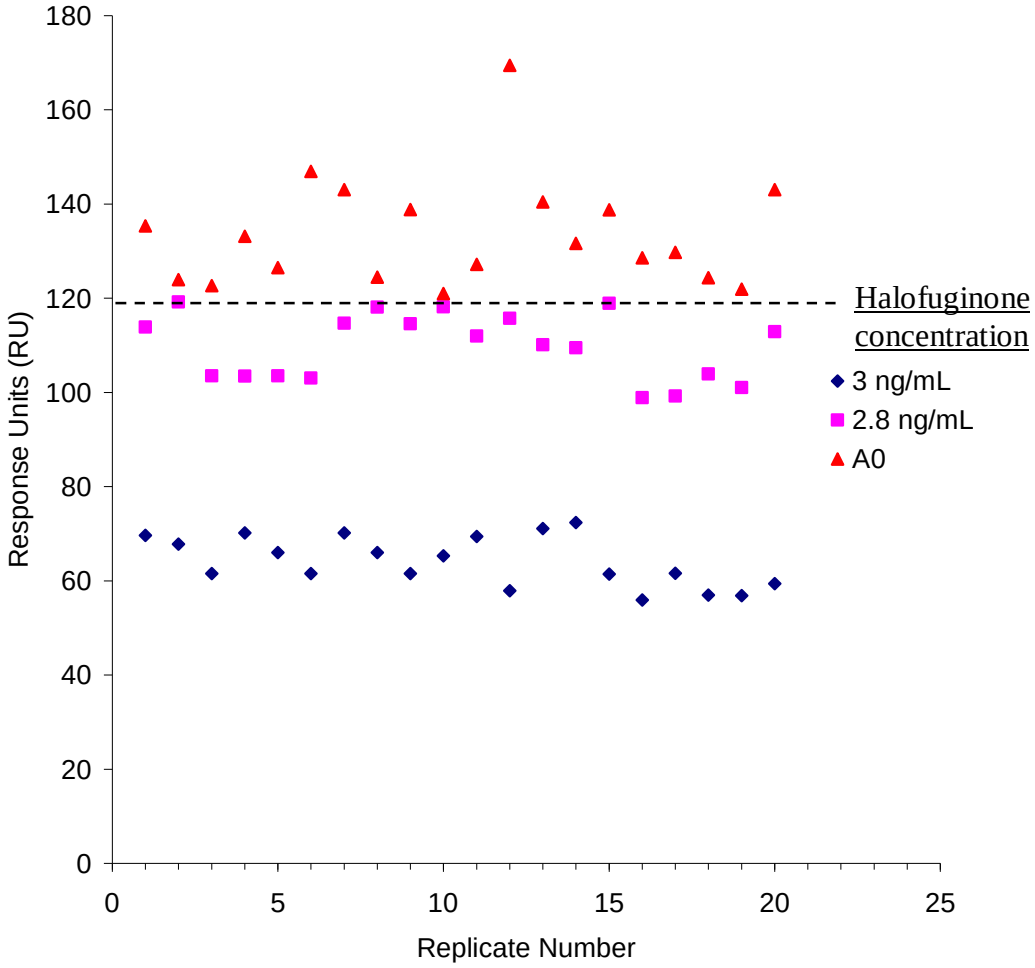

Figure 3.34 *Determination of limit of detection of HFG-specific E9 scFv using 20 replicates of the blank standard, 20 replicates of 3 ng/mL HFG (half the MRL in eggs) and 20 replicates of 2.8 ng/mL HFG in a competitive assay format. The samples were passed across the HFG-immobilised chip and the response units recorded. The dashed line indicates 100 % compliance, i.e. the ability of the antibody to differentiate 20/20 samples, spiked with 2.8 ng/mL of HFG, from the blank standard.*

3.7 Discussion and Conclusion

In this chapter light chain shuffling was employed to improve the properties of an avian-derived HFG-specific scFv antibody pool. A 185-fold improvement in sensitivity of a HFG-specific scFv was achieved. This affinity improvement stresses the importance of optimising light and heavy chain pairings for recombinant antibodies. The E9 scFv has the potential to detect down to 80 pg/mL of HFG in assay buffer and extracted samples in ELISA. Hence, this assay would be highly applicable in the diagnostic monitoring of HFG residues in the food industry.

The decision limit was calculated according to the guidelines outlined in the 2002-657-EC Regulation. A calibration curve was constructed from inter-assay results using a four-parameter equation in BiaEvaluation™ software; from this the decision limit was calculated. This was estimated to be approximately 162 pg/mL for E9 and approximately 30 ng/mL for B4. The detection capability (CCβ) was then established by measuring 20 samples at the decision limit and adding 1.64 times the standard deviation of the samples to the concentration of the decision limit. This was calculated to be 163 pg/mL for E9 and 31 ng/mL for B4. These results were extrapolated from the calibration curve. However, when experimentally determining the functional limit of detection using the E9 scFv and 20 samples of varying concentrations of HFG, the lowest concentration of analyte that could be detected with 95% certainty above the blank standard was determined to be 80 pg/mL.

From reviewing the literature, it is apparent that the lowest previously reported functional limit of detection for HFG detection was approximately 500 pg/mL. The functional limit of detection of the HFG-specific E9 scFv in the ELISA was calculated to be 80 pg/mL as shown in **Figure 3.28**. The lowest MRL listed by the EU for HFG is 1 ng/mL and, even when taking into account the limit of detection calculated from the inter-day calibration curve, which was approximately 162 pg/mL, the E9-scFv still has the capability of detecting significantly below the current MRL.

Comparing the results of the ELISA to the Biacore assay for HFG detection, the ELISA proves to be superior. In the Biacore assay, the detection capability was calculated as 3.4 ng/mL. The functional limit of detection was experimentally verified by analysing 20 samples at concentrations above and below this detection capability. As 2.8 ng/mL was detectable with 95 % confidence, this was classed as the actual detection capability of the Biacore assay. The development of this recombinant anti-HFG scFv and its subsequent incorporation into an automated detection system has

the potential to replace the current routine applications in the surveillance of HFG residues in food. The current MRL for HFG in eggs is 6 ng/mL and the analytical limit of detection of this Biacore assay is 2.8 ng/mL, verifying the ability of this assay to distinguish between compliant and non-compliant samples. This SPR assay also proved very reproducible with the highest percentage of variation between separate assays shown at 10.6 %. Such a recombinant antibody-immunosensor could potentially be the starting point for the development of a multi-residue detection sensor chip, which would allow rapid screening for a range of different anti-protozoan drug residues.

The measurement of affinity interactions between two molecules can prove challenging, particularly when dealing with drug compounds with molecular weights lower than 500 Da. A significant problem is the lack of signal strength due to the target analyte being too small to produce a substantial signal itself. If greater Biacore assay sensitivity was required for the detection of HFG, directly immobilising the target analyte onto the sensor chip surface may be an alternative solution (Townsend *et al*., 2006), as this would enable a greater density of HFG to be packed on the surface of the chip. Mitchell *et al.* (2005) showed several ways of enhancing SPR biosensing of small molecules by nanoparticle labelling with gold, observing a 13-fold improvement in the limit of detection when a sequential binding format with labelled secondary antibody was employed. SPR biosensors are very useful for food safety analysis as they are sensitive, 'real-time' interactive devices that can allow a timely response in the detection of food contaminants, as well as permitting the rapid quantification of trace drug residues in food. Also, in SPR technology, false positives are minimised due to the short contact times of analyte and antibody which favour high affinity interactions instead of low affinity matrix effects, thereby making SPR detection highly favourable in food monitoring when compared to conventional methods (Dubois *et al*., 2004).

A previous study reported how light chain shuffling was employed to overcome poor stability when no actively expressing clones were observed from the initial un-shuffled library, whereas functional antibody fragments were readily selected from the chain-shuffled library (Rojas *et al.*, 2004). The effect of light chain shuffling on the affinity of antibodies, without detrimental effects to antigen specificity, have also been noted previously (Yoshinaga et al., 2008). In that case a light chain-shuffled variant of an MCP-1 specific antibody displayed a 15-fold increase in affinity when

compared to the wild-type. Another observable benefit of antibody engineering, in conjunction with recombinant display technologies, is the selection of clones with improved expression levels.

Overall this chapter describes how chain shuffling enabled a 185-fold improvement in antibody affinity which is a very significant result with important implications for sensitive and accurate analysis of halofuginone in foodstuffs. Subsequent steps in environmental monitoring of large samples due to the ease and cost-efficiency of the assays are outlined. This work also demonstrates the simplicity of mimicking an immune process, such as somatic-hypermutation for the isolation of superior antibodies.

Chapter 4

Development of a diclazuril-specific scFv library

Chapter outline

This chapter gives an insight into the generation of recombinant antibody fragments, phage library construction, and selection of anti-diclazuril scFv fragments. Chickens were immunised with a 'diclazuril'-HSA conjugate. However, no diclazuril-specific immune response was observed. Mice were then immunised with the same conjugate, which induced a diclazuril-specific immune response in the murine models. Several expression problems were encountered during the amplification of the recombinant gene fragments. To overcome these problems the cDNA was re-synthesised and the library was constructed a second time. The scFv gene fragment was cloned into a phage-display vector, to allow for the selection of diclazuril-specific scFv on the surface of phage particles, via biopanning. However, when the scFv library was screened, no diclazuril-binding scFv fragments were present.

4.1 Introduction

Antibody engineering and production was revolutionised by the development of modern molecular biological methods for the expression of recombinant DNA. Among the various strategies developed for tailoring the affinity and specifity of recombinant antibodies, phage display has proven to be a robust and versatile technology. The two predominant formats for recombinant antibodies in phage display are generally either the single chain Fv (scFv) or Fab antibody fragments. Both antibody formats have their advantages and disadvantages. The format selected should be ideal for the purpose of the assay. In the scFv format, the Fc region, is eliminated as it contributes to immune effector functions, which may cause interference with secondary antibodies used in the assay format. In contrast, Fab fragments are generally more stable than scFv fragments and are less prone to aggregation. Quintero-Hernanádez and colleagues (2007) re-formatted a scFv to a Fab fragment and tested both in the presence of guanidium chloride and found that the stability of the antibody fragment substantially increased when it was re-formatted to a Fab fragment. The major disadvantage of Fabs relative to scFvs is the lower expression levels generally found in *E. coli* (Barbas *et al.,* 2004). ScFvs were the format of choice for the generation of an anti-diclazuril recombinant antibody library due to the presence of only one polypeptide chain in the scFv, easier production in

bacteria, and small size (25-30 kDa), which may allow increased assay sensitivity, due to more dense packing on the assay platform surface.

Diclazuril has a low molecular mass of 407.64 Daltons. It is necessary, therefore, to conjugate it to an immunogenic carrier protein in order to elicit an immune response in host animals. This is primarily performed by the linkage of suitable functional groups on the drug to a reactive grouping on the carrier protein. The presence of an accessible functional group (not a target epitope) on the hapten governs the selection of the conjugation method to be employed. Common procedures use amine, carboxylic acid, hydroxyl, or sulfhydryl groups on the hapten and the protein (Singh *et al.*, 2004). When this technique is not successful, or is not possible due to the lack of suitable functional groups on the hapten, alternative strategies must be sought. The only available functional group in the diclazuril entity is thought to be the primary epitope for antibody recognition of diclazuril and is, therefore, unsuitable for linking to a carrier protein. Fodey and colleagues (2007) report the use of antigen mimics for the generation of diclazuril-specific antibodies, whereby, an intermediate compound produced during the manufacture of diclazuril was deemed an ideal candidate for use as a diclazuril mimic. This compound, carboxydiclazuril, was conjugated to both BTG and HSA proteins using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *n*-hydroxysuccinimide (NHS) coupling chemistries (Fodey *et al.*, 2007). These conjugates were obtained from Queen's University Belfast and used for immunisation.

4.2 Results

4.2.1 Immunisation of Leghorn chicken with diclazuril

A chicken was immunised with a mixture of equal concentrations of a 'diclazuril-mimic'-HSA and halofuginone-HSA conjugates over a period of three months. After four boosts, a primary serum titre was determined by performing a direct ELISA.

4.2.1.1 Avian serum antibody titre determination

A direct ELISA was carried out using varying dilutions of serum incubated on a 'diclazuril'-BTG coated Nunc MaxiSorp 96-well ELISA plate. It can be observed in **Figure 4.0** that no antibody response was generated to diclazuril after four boosts. However, a very high antibody response was observed for halofuginone, when the same serum was analysed. This suggested, firstly, that the 'diclazuril'-HSA conjugate

used was not satisfactory for generating an antibody response and, secondly, that the avian host's immune system was actively responding to the immunisation.

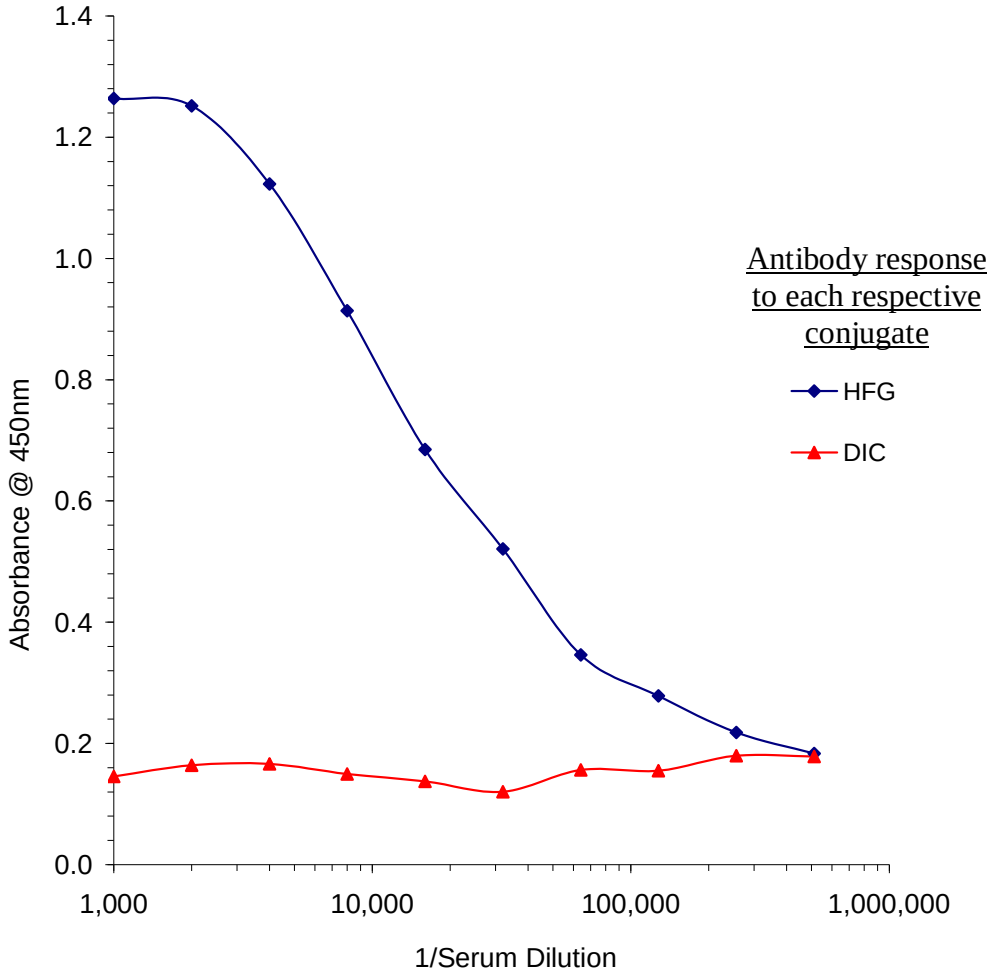

Figure 4.0 *Antibody serum titre from a chicken immunised with 'diclazuril'-HSA and halofuginone-HSA after four boosts. The antibody response for each target was determined by performing a direct ELISA on Nunc MaxiSorp 96-well plates coated with each of the screening conjugates (i.e. with a different carrier protein to that which was immunised). 'Diclazuril'-BTG and halofuginone-transferrin were coated on separate ELISA plates, which were subsequently blocked with an appropriate blocking agent. Bound avian antibodies were detected using a HRP-labelled rabbit anti-chicken IgG antibody. No significant response was observed when analysing the serum for anti-diclazuril antibodies.*

4.2.1.2 Further diclazuril boosts

Three more boosts were administered to the chicken. Seven to ten days after each boost, a bleed was taken and an antibody serum titre was performed to determine the

presence of anti-diclazuril antibodies in the serum. This antibody serum titre is shown in **Figure 4.1**. No diclazuril-binding antibodies were present in the serum after all seven boosts. This implied that the 'diclazuril' hapten was not recognised as a foreign antigen, and thus was not producing a diclazuril-specific immune response. In turn, no anti-diclazuril antibodies were being generated.

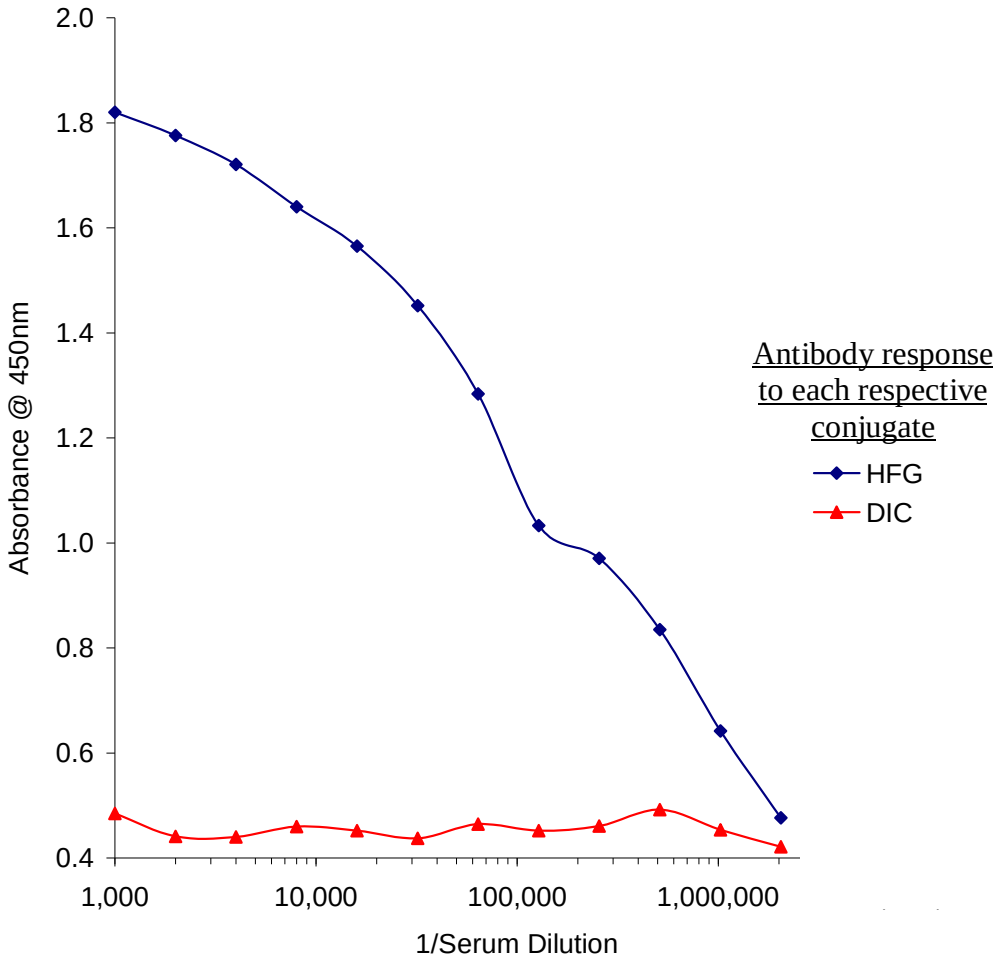

Figure 4.1 *Antibody serum titre from a chicken immunised with 'diclazuril'-HSA, and halofuginone-HSA after seven boosts. The antibody response for each target was determined by performing a direct ELISA on Nunc MaxiSorp 96-well plates coated with each of the screening conjugates. 'Diclazuril'-BTG and halofuginone-transferrin were coated on separate ELISA plates which were subsequently blocked with an appropriate blocking agent. Bound avian antibodies were detected using a HRP-labelled anti-chicken antibody. No significant response was observed when analysing the serum for anti-diclazuril antibodies, after seven boosts.*

4.2.1.3 Evaluation of 'diclazuril'-conjugates using a commercial anti-diclazuril polyclonal antibody

To determine if the diclazuril derivative 'carboxydiclazuril' was still present and intact on the conjugates used for immunisation and screening, an ELISA was performed using a commercial anti-diclazuril polyclonal antibody (Randox Ltd.). A checkerboard ELISA was carried out to analyse two 'diclazuril'-BTG and two 'diclazuril'-HSA conjugates; one set was obtained from Queen's University Belfast (QUB) and one set was prepared 'in-house' as described in *Section 2.13.1*. A range of concentrations of each of the 'diclazuril'-conjugates were coated on the surface of an ELISA plate, which was then blocked with an appropriate blocking agent. Anti-diclazuril antibody (produced in rabbit) was serially diluted and added to the ELISA plate coated with each of the respective conjugates. Bound anti-diclazuril antibody was detected using a HRP-labelled goat-anti-rabbit secondary antibody. As shown in **Figure 4.2** the anti-diclazuril commercial antibody titred out when incubated on a plate coated with decreasing concentrations of each of the 'diclazuril'-BTG/'diclazuril'-HSA conjugates. This suggested that the drug-carrier protein complex is still intact, that the 'diclazuril' derivative is still present on the conjugate, and that the commercial antibody is specific for the bound 'diclazuril' drug on all four conjugate complexes. The presence of the 'diclazuril' derivative on the conjugates was verified. Therefore, another immunisation was carried out. No chickens were available for immunisation at this time, hence, three mice were immunised with the 'diclazuril'-HSA conjugate from QUB.

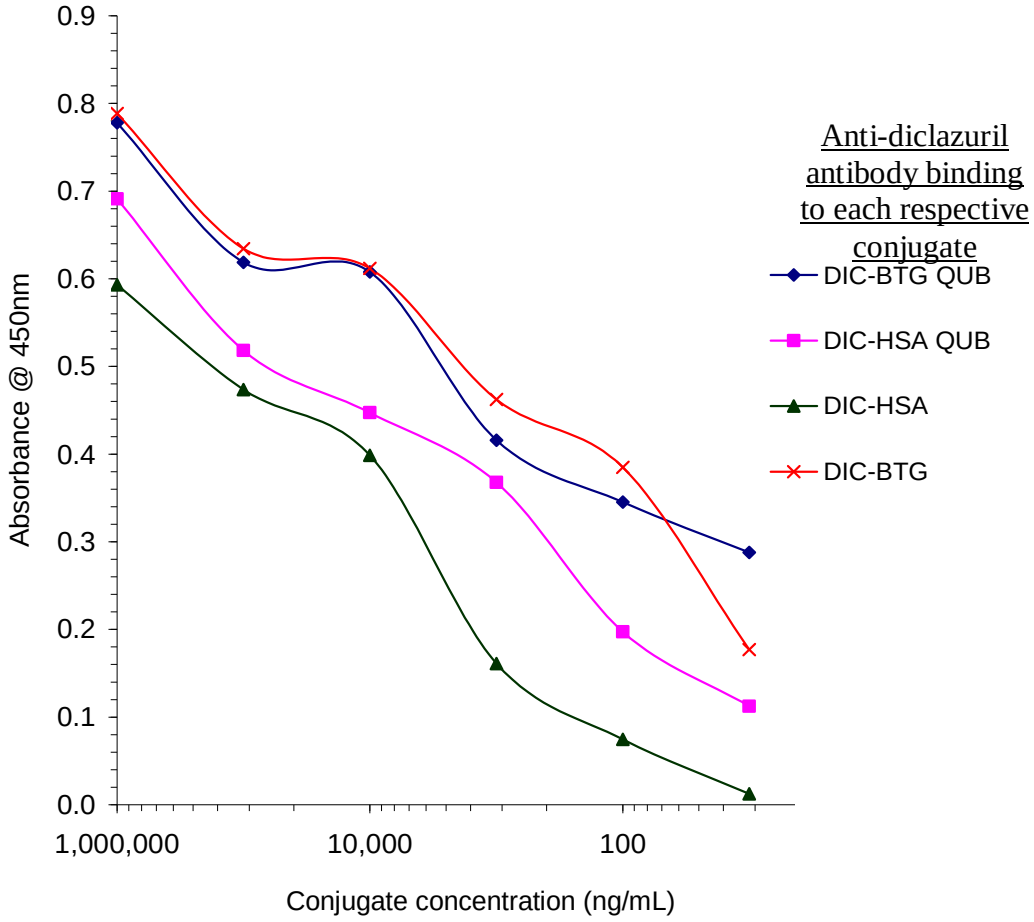

Figure 4.2 *Checkerboard ELISA to determine the presence/absence of the 'diclazuril' derivatives on each of the 'diclazuril'-conjugate complexes. Varying concentrations of each conjugate was coated on a Nunc MaxiSorp 96-well ELISA plate, and the plate was subsequently blocked by the addition of an appropriate blocking reagent. Dilutions of the anti-diclazuril polyclonal antibody were added to the plate. Antibody that bound to the immobilised 'diclazuril' on the plate surface was then detected following the addition of a HRP-labelled goat-anti-rabbit secondary antibody.*

4.2.2 Immunisation of balb/c mice with 'diclazuril'

Balb/C mice aged 5-8 weeks were initially immunised with a mixture of equal parts of 'diclazuril'-HSA conjugate and Freund's complete adjuvant. Subsequent boosts were performed with Freund's incomplete adjuvant 7-10 days apart, with five boosts given in total.

4.2.2.1 Mouse anti-serum titre determination

An antibody serum titre was performed from a tail bleed taken from mice treated with an extensive 3 month immunisation schedule for diclazuril (**Figure 4.3**). ELISAs were performed using varying dilutions of serum antibody on a 'diclazuril'-BTG-coated 96-well plate. The finding of an anti-diclazuril antibody titre of greater than 1:100,000 signified that diclazuril-antibody generation had occurred. The mice were sacrificed to harvest the B cells from the spleen for recombinant antibody library generation and selection of positive antibodies to diclazuril.

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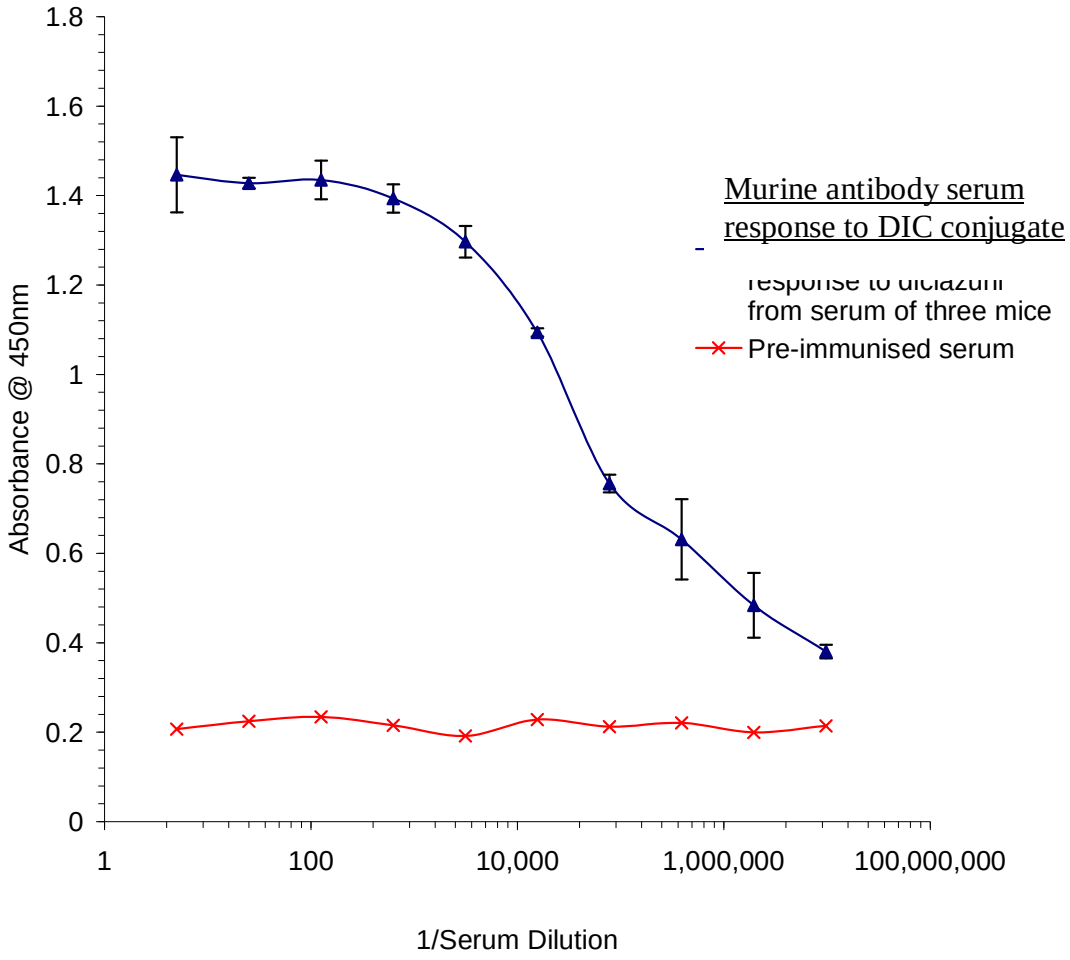

Figure 4.3 *Average antibody serum titre from three mice immunised with the 'diclazuril'-HSA, conjugate (received from QUB) after six boosts. The antibody response for diclazuril from the serum was determined by performing a direct ELISA on plates coated with 'diclazuril'-BTG. The plates were blocked with an appropriate blocking agent and varying dilutions of the murine serum were incubated on the plate. Bound murine antibodies were detected using a HRP-labelled rabbit-anti-mouse IgG. The mean diclazuril-antibody titre of serum from the three mice is illustrated, with the standard deviations from the mean depicted by the error bars. A negative control of serum taken prior to the immunisation protocol is also shown.*

4.2.3 Isolation of RNA from murine B cells (spleen) and first-strand DNA synthesis

The spleens were removed from the immunised mice and carefully homogenised in Trizol reagent. The total RNA was extracted from the tissue sample, quantified using a Nanodrop ND-1000 and first-strand cDNA synthesised by reverse transcription. The cDNA template was used in the amplification of the mouse variable heavy and light chain genes.

4.2.4 PCR optimisation for murine variable heavy and variable light chain generation

A standard PCR protocol, as outlined in Barbas *et al.* (2001), was employed to amplify the V_H and V_L chain genes (**Figure 4.4**) from the reverse transcribed cDNA. The V_H region amplified successfully with no subsequent optimisation required. However, the V_L region did not amplify.

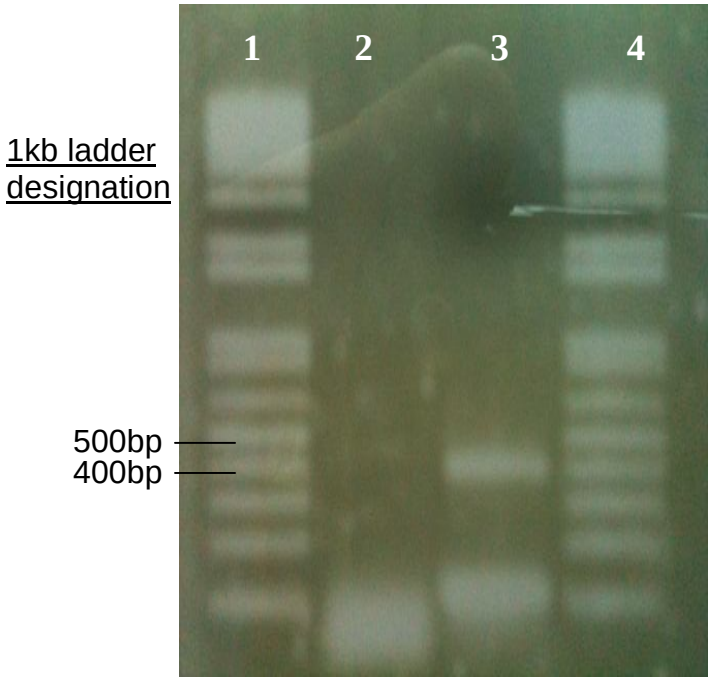

Figure 4.4 *Amplification of variable regions from synthesised cDNA of 'diclazuril' sensitised mice. Amplification was performed using Go Taq polymerase and standard PCR conditions. Lane 1= 1kb ladder; Lane 2= V_L amplification; Lane 3= V_H amplification and Lane 4= 1kb Ladder.*

To optimise the amplification of the V_L gene, several steps were taken. Initially, a high fidelity polymerase, Phusion polymerase, was used with varying concentrations of magnesium chloride. The role of $MgCl_2$ in the PCR is to increase the stringency of the DNA interactions, as many components in the PCR reaction bind magnesium ions including primers, template, PCR products and dNTPs. DNTPs quantitatively bind magnesium ions (Cobb and Clarkson, 1994) and, because it is necessary for free magnesium ion to serve as an enzyme cofactor in PCR, the total magnesium ion concentration must exceed the total dNTP concentration and, subsequently, may require optimisation for every PCR. Hence, a magnesium chloride gradient was performed (**Figure 4.5**) to determine the optimum concentration of magnesium ions required for successful amplification of the desired product. It can be observed from **Figure 4.5** that the magnesium chloride concentration which yielded the greatest amplification was 2 mM, whereas the original concentration used was 4mM. The gel-purified V_H and V_L fragments were linked by splice by overlap extension PCR (SOE-PCR), whereby the purified fragments are joined by an 18 amino acid glycine-serine linker $(G_4S)_4$. This long linker provides sufficient flexibility for the V_H and V_L domains to associate predominantly as scFv monomers, whereas the use of a smaller linker may cause multimerisation of the scFvs.

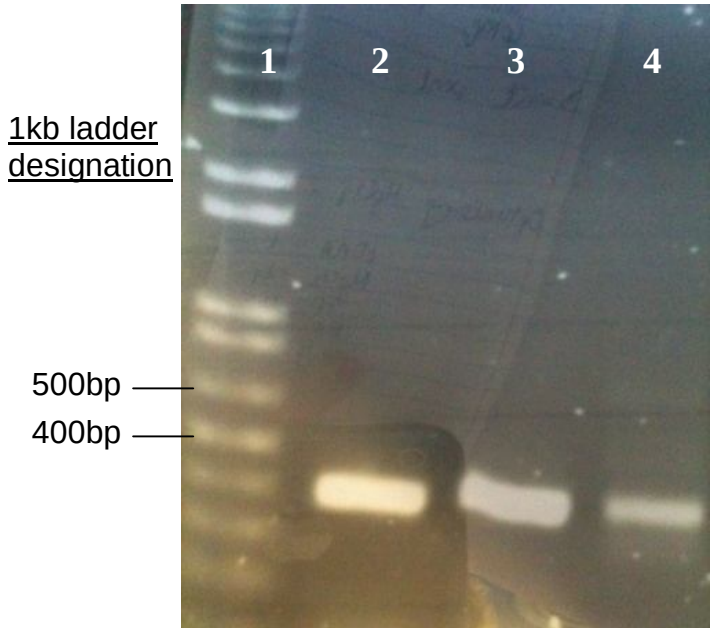

Figure 4.5 *Optimisation of variable light chain amplification from the synthesised cDNA of 'diclazuril'-sensitised mice. The amplification was performed using Phusion polymerase, a magnesium chloride gradient, and standard PCR conditions. Lane 1= 1kb ladder; Lane 2= 2 mM $MgCl_2$; Lane 3= 4 mM $MgCl_2$ and Lane 4= 8 mM $MgCl_2$.*

4.2.5 Murine SOE-PCR of variable heavy and light chains

The sense and reverse extension primers used in this SOE-PCR recognise the sequence tails that were generated in the previous amplifications of the V_H and V_L domain. A longer extension time is used here to favour the generation of a full length 800bp product. An overlap SOE product appeared to be amplified as shown in **Figure 4.6**, but the product was amplifying at 550bp instead of the desired 800bp. Optimisation of the SOE-PCR was required for a clean amplification to occur without any non-specific overlap products being generated.

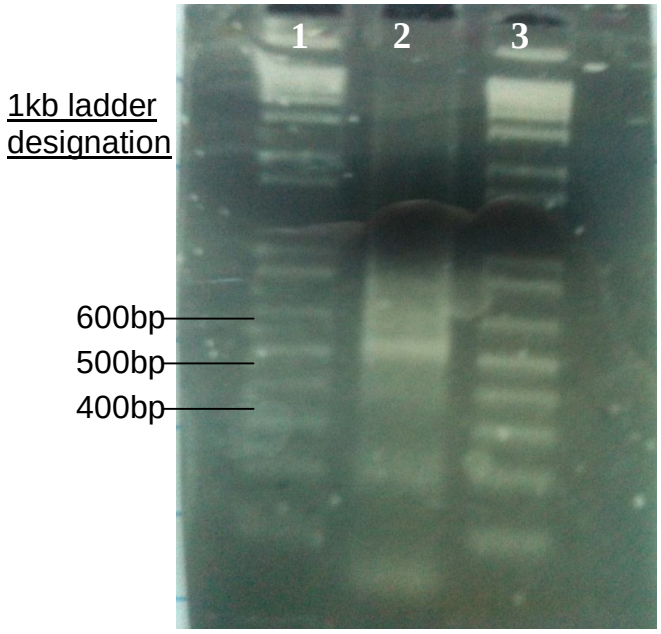

Figure 4.6 *Splice by Overlap Extension-PCR of the amplified V_H and V_L chain genes from the synthesised cDNA of 'diclazuril'-sensitised mice. This primary amplification check employed standard conditions for murine-derived variable domain overlap amplification, consisting of a 56^0C annealing temperature and Phusion high fidelity polymerase. Lane 1= 1kb Ladder; Lane 2= SOE amplification and Lane 3= 1kb Ladder.*

A magnesium chloride gradient was the first optimisation step undertaken. It can be observed from **Figure 4.7** that no specific product was amplified when the magnesium chloride concentration was varied. The suggested annealing temperature for murine derived SOE-scFv products (as outlined in Barbas *et al.*, 2004) to use for this amplification was 56^oC. The next step taken to optimise the amplification of the desired product was to vary this annealing temperature.

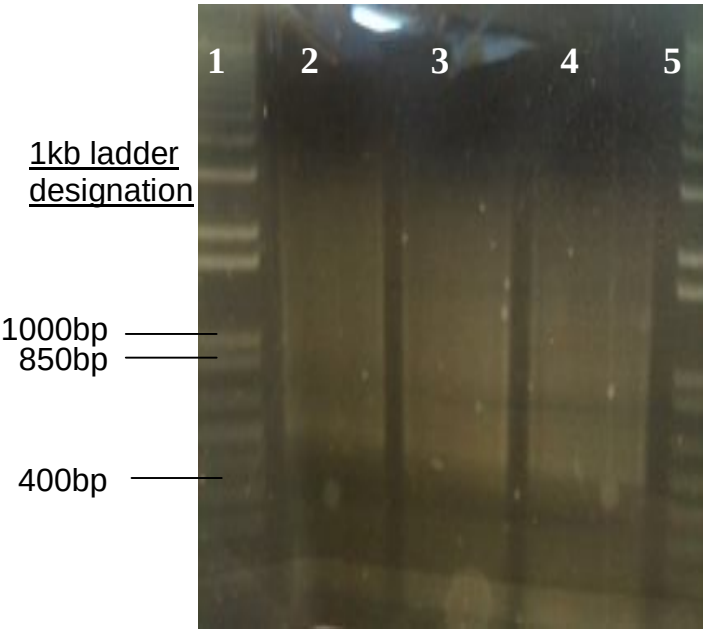

Figure 4.7 *Optimisation of the anti-diclazuril scFv SOE product amplification using a magnesium chloride gradient, Phusion polymerase and a 56^{0}C annealing temperature. Lane 1= 1kb ladder; Lane 2= 2 mM $MgCl_2$; Lane 3= 4 mM $MgCl_2$; Lane 4= 8 mM $MgCl_2$ and Lane 5= 1kb ladder.*

The melting temperature ($T_{m)}$ of the primer-template pairs is affected differentially by the individual buffer components and also the primer and template concentrations. Generally, any calculated T_m value is regarded as an approximation. Several reactions, run at temperature increments above and below the calculated $T_{m,}$ will give a good approximation of the optimum annealing temperature for a given set of reaction conditions (Roux, 1995). Annealing temperature optimisation was carried out by performing the SOE-PCR at a range of different annealing temperatures. During the annealing temperature optimisation a product was amplified, which is shown in Lane 4, in **Figure 4.8**. However, this product was not at the desired 800bp range for the SOE product. Instead it was lower, at approximately 200bp.

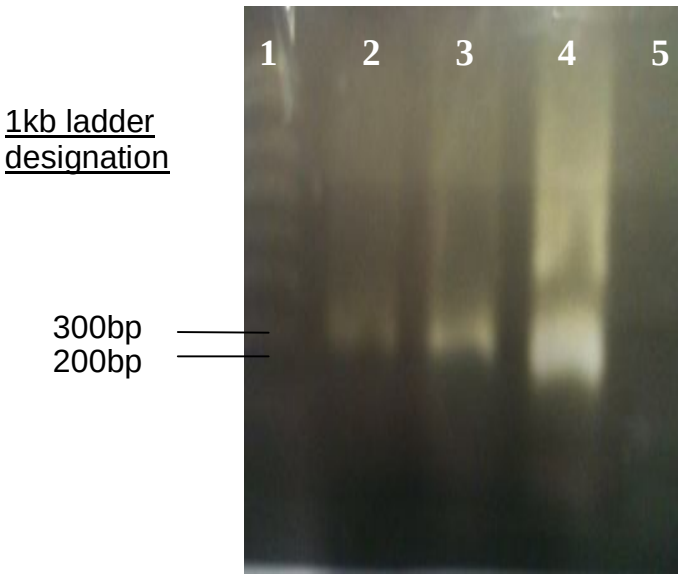

Figure 4.8 *Optimisation of the diclazuril scFv SOE product amplification using different annealing temperatures. Standard PCR conditions, Phusion polymerase and 4 mM $MgCl_2$ were used. Lane 1= 1kb ladder; Lane 2= 52^0C; Lane 3= 54^0C; Lane 4= 58^0C and Lane 5= 60^0C.*

New PCR reagents were obtained and the template ratio of variable heavy: variable light chain constructs was subsequently optimised. The optimal template ratio of variable heavy: variable light chain constructs was investigated by performing the SOE-PCR under ratios of; 1: 2 (variable heavy: variable light), 2: 1 (variable heavy: variable light), 1: 3 (variable heavy: variable light), and 3: 1 (variable heavy: variable light). The PCR products from each of these reactions were resolved on a gel, as shown in **Figure 4.9**. The DNA smears present on the resolved gel may be caused by the increased number of PCR cycles, which were used to favour the amplification of the full length 800bp product. The ratio of three times the concentration of light: heavy chain produced a weak band at the desired 800bp range.

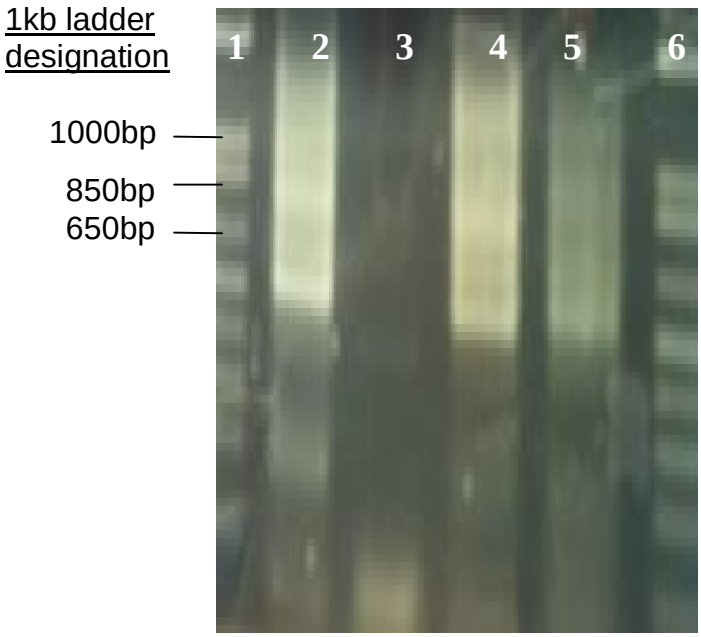

Figure 4.9 *Optimisation of the diclazuril scFv SOE product amplification using different ratios of variable heavy: variable light chains. Standard PCR conditions were used as previously outlined. Lane 1= 1kb ladder; Lane 2= 1:2 (variable heavy: variable light); Lane 3= 2:1 (variable heavy: variable light); Lane 4= 1:3 (variable heavy: variable light); Lane 5= 3:1 (variable heavy: variable light) and Lane 6= 1kb ladder.*

Using the optimal concentration ratio of variable heavy: variable light chains and new primers, a PCR was performed with a magnesium chloride gradient. The PCR products were resolved on the gel as shown in **Figure 4.10**. It can be observed that no PCR product was amplified after performing the SOE-PCR, using the optimised template ratio, the recommended annealing temperature, new PCR primers and varying the magnesium ion concentration.

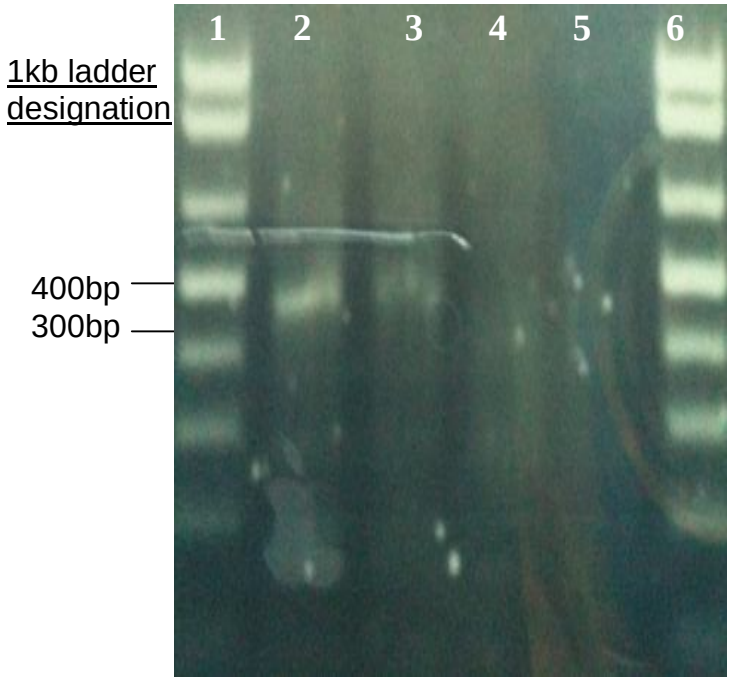

Figure 4.10 *Anti-diclazuril scFv SOE product amplification using the optimised template ratio of 3:1 VH: VK, an annealing temperature of 56^{o}C, new primers, Phusion polymerase and a magnesium chloride gradient. Lane 1= 1kb ladder; Lane 2= 2 mM $MgCl_2$; Lane 3= 4 mM $MgCl_2$; Lane 4= 6 mM $MgCl_2$; Lane 5= 8 mM $MgCl_2$ and Lane 6= 1kb ladder.*

The last variable to optimise was the selected polymerase. Five different polymerases, Go-Taq^{TM}, Phusion^{TM}, Accuzyme^{TM}, BioTaq^{TM} and Platinum Taq^{TM} were used under standard amplification conditions of a 56^{0}C annealing temperature with 4 mM $MgCl_2$. The products of these amplifications were resolved on an agarose gel as shown in **Figure 4.11**. Phusion was the only polymerase that appeared to amplify the desired 800bp SOE product (*Lane 5*). However, it can be observed that DNA smearing was still occurring when the product was resolved on a gel.

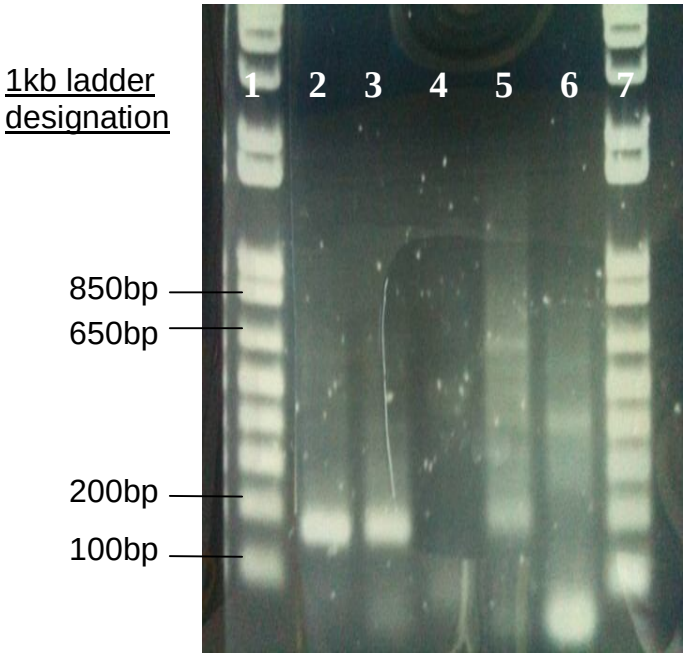

Figure 4.11 *Anti-diclazuril scFv SOE product amplification with polymerase optimisation. The product was amplified using the optimised template ratio of 3:1 VH: VK, new primers, 4 mM $MgCl_2$ and a panel of polymerases. Lane 1= 1kb ladder; Lane 2= BioTaq™ polymerase; Lane 3= Accuzyme™ polymerase; Lane 4= Platinum™ polymerase; Lane 5= Phusion™ polymerase; Lane 6= GoTaq™ polymerase and Lane 7= 1kb ladder.*

The full-length SOE product was proving too difficult to optimise with the purified gene fragments. Hence, it was decided to repeat the cDNA synthesis from the RNA preparation and amplify the variable gene regions again. It can be observed in **Figure 4.12** that, even when using the re-synthesised cDNA, the amplification of the 800bp product is still very weak. This is because the annealing of both variable regions is not occurring efficiently. The large band observed between 400-500bp corresponds to both of the variable chain genes which have not annealed.

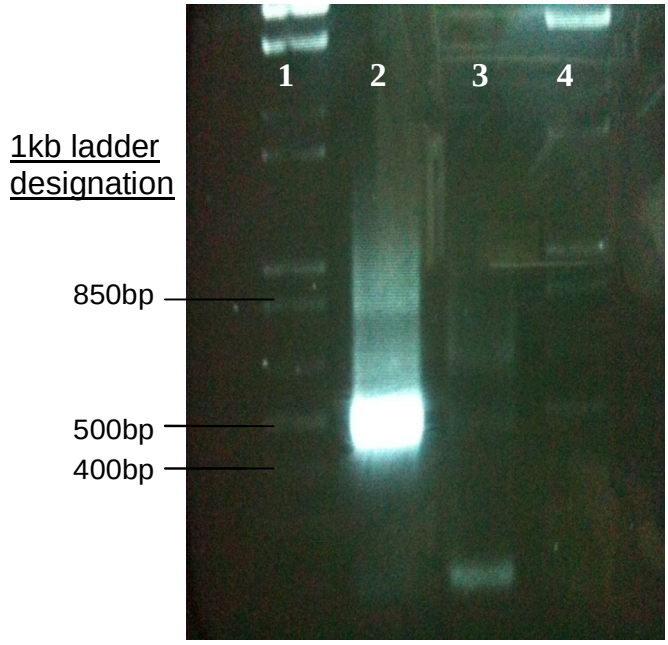

Figure 4.12 *Anti-diclazuril scFv SOE amplification using variable heavy and variable light chain domains amplified from the re-synthesised cDNA preparation. The SOE-PCR was amplified using standard amplification conditions. Lane 1= 1kb ladder; Lane 2= SOE amplification using Phusion polymerase; Lane 3= SOE amplification using GoTaq polymerase and Lane 4= 1 kb Ladder.*

Previous experience in the laboratory (data not shown) has proved that DreamTaq™ polymerase worked efficiently when no other polymerase would work. The DreamTaq™ product claims that their proprietary formulation of the DreamTaq ™ buffer is optimised for robust performance in PCR, subsequently minimising any extensive optimisation stages. Therefore, DreamTaq™ polymerase (Fermentas) was employed to determine if the amplification of the SOE product could be enhanced. When DreamTaq™ polymerase was used with a standard annealing temperature of 56^0C, and 8 mM $MgCl_2$, the desired 800bp product was efficiently produced, as shown in *Lane 4* in **Figure 4.13**.

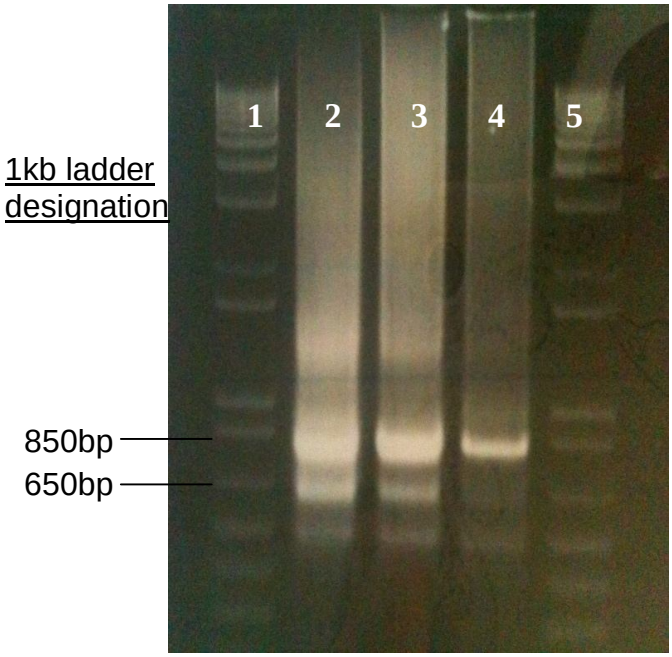

Figure 4.13 *Anti-diclazuril scFv SOE product amplification, using DreamTaq™ polymerase, and variable chain genes amplified from the re-synthesised cDNA preparation. The SOE-PCR was performed under standard amplification conditions incorporating a magnesium chloride gradient. Lane 1= 1kb ladder; Lane 2= 2 mM $MgCl_2$; Lane 3= 4 mM $MgCl_2$; Lane 4= 8 mM $MgCl_2$ and Lane 5= 1kb ladder.*

4.2.6 Library construction and subsequent enrichment via biopanning

After large-scale amplification of the 800bp product, it was gel-purified, ethanol precipitated, quantified and digested using *Sfi*1 restriction enzyme as described in *Section 2.13.8.1*. The digested overlap product and vector DNA were then purified from a 1% (w/v) and 0.6% (w/v) agarose gel, respectively. The purified SOE product and vector were concentrated via ethanol precipitation and quantified using the Nanodrop ND-1000. Following quantification, the SOE product was then cloned into the pComb3XSS phage display vector using the restriction enzyme *Sfi*I. The successfully ligated product, as shown in **Figure 4.14** was transformed into electrocompetent *E. coli* cells by electroporation using a Gene Pulser™ apparatus. Several methods exist for the introduction of DNA into a bacterial host. One commonly used method is the chemical transformation or 'heat-shock' technique, which may be employed when high efficiencies are not essential. In the case of

diverse library building when high transformation efficiencies are required, a method such as electroporation is favourable (Dower *et al.*, 1988). The transformed mouse anti-diclazuril scFv library produced had a size of 1.26 x 10^7 cfu/mL. Barbas *et al.* (2004) suggest that for antibody libraries derived from immune animals, reasonable library sizes are in the range of 10^7 to 10^8 transformants. This library would generally be classed as medium in size, as highly diverse phage libraries with up to 10^{11} transformants have previously been reported (Sblattero and Bradbury, 2000; Lloyd *et al.*, 2009). However, high affinity antibodies specific for environmental contaminants have been isolated from libraries as small as 10^6 library transformants (Shaw *et al.*, 2008). The murine-derived diclazuril-scFv library was then subjected to phage display biopanning against an immobilised 'diclazuril'-BTG conjugate.

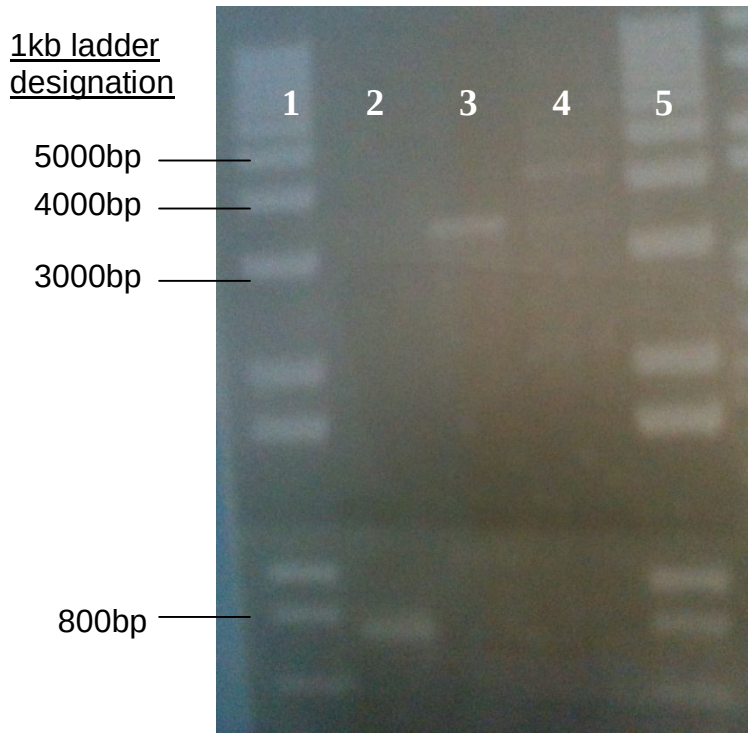

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Figure 4.14 *Analysis of the ligation reaction product of the Sfi1-digested murine-derived SOE product and the Sfi1-digested pComb3XSS phage display vector. Lane 1= 1kb ladder; Lane 2= digested and purified SOE product at 800bp; Lane 3= digested and purified double-cut pComb3XSS vector at 3300bp; Lane 4= ligated SOE and vector at approximately 4100bp and Lane 5= 1kb ladder.*

4.2.6.1 Selection of -specific phage-scFv particles by biopanning

Four rounds of affinity selection through biopanning were performed on 'diclazuril'-BTG-coated immunotubes. Enrichment of the library was facilitated by altering the stringency of each consecutive panning step. This was achieved by increasing the number of wash steps and decreasing the concentration of immobilised antigen on the immunotube. The panning conditions are outline in **Table 4.0**.

Table 4.0 *Panning conditions employed for each round of selection of the mouse anti-diclazuril scFv library. Phage input and output titres over the 4 rounds of biopanning are reported with each round of biopanning varying in conditions and stringency.*

Panning round	'Diclazuril' coating concentration	Washing steps	Input titres cfu/ mL	Output titres Cfu/ mL
1	100 μg/mL	3 X PBST 3 X PBS	7.3×10^{11}	3.4×10^{6}
2	50 μg/mL	5 X PBST 5 X PBS	1.2×10^{12}	4.8×10^{7}
3	25 μg/mL	7 X PBST 7 X PBS	6.9×10^{11}	2.6×10^{6}
4	5 μg/mL	10 X PBST 10 X PBS	1.0×10^{12}	4.3×10^{7}

The precipitated input phage from each round of panning was incorporated into a polyclonal phage ELISA to test for enrichment against the 'diclazuril'-BTG conjugate. Successful enrichment was signified by an increase in absorbance signal after incubating phage with the immobilised 'diclazuril' conjugate and detecting bound phage with a HRP-labelled anti-M13 secondary antibody. This can be observed in **Figure 4.15**. A competitive polyclonal phage ELISA was also performed to ensure diclazuril-specific scFv-displaying phage were present (**Figure 4.16**).

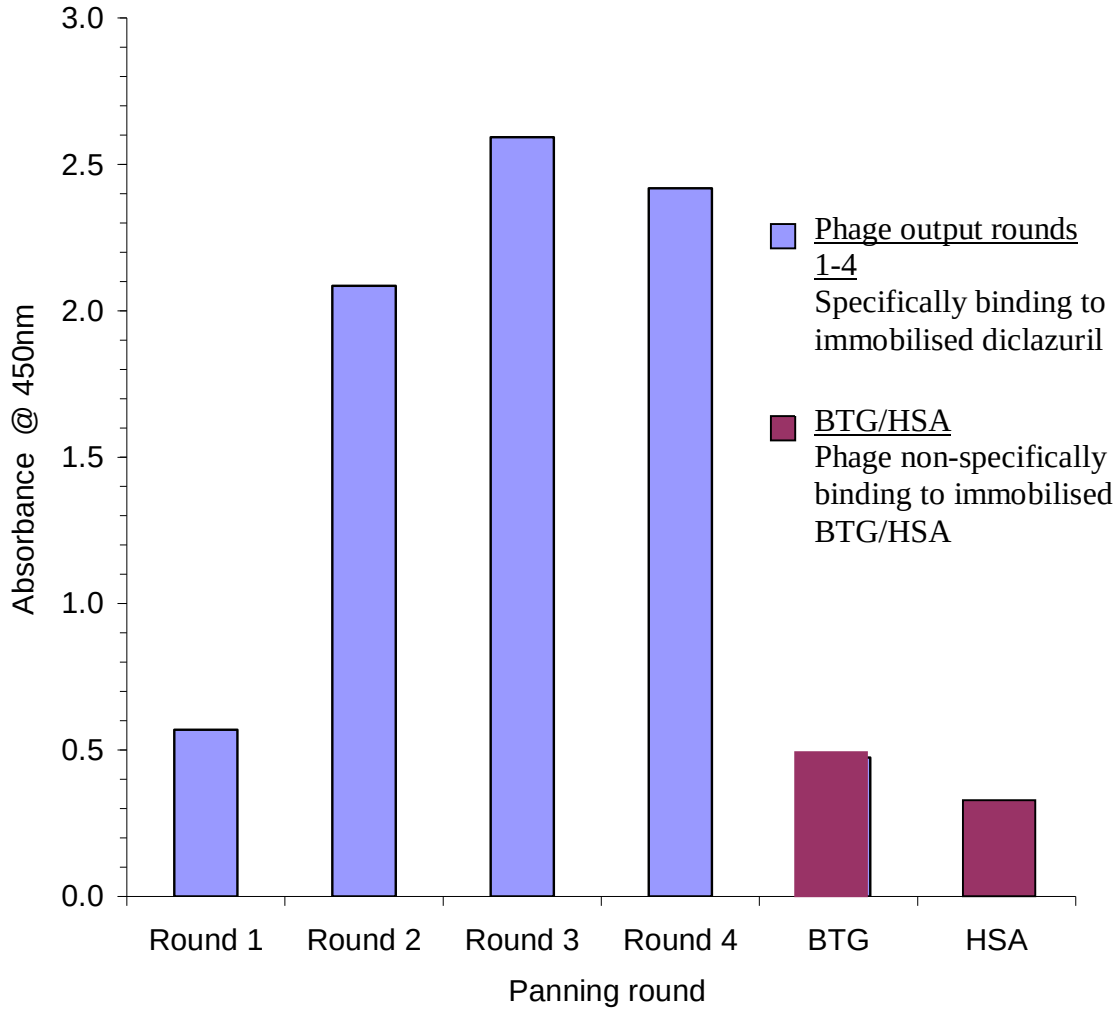


Figure 4.15 *Polyclonal phage ELISA screening for anti-diclazuril scFv displayed on phage, following four rounds of panning. Phage pools obtained after each round of panning were tested for binding to the 'diclazuril'-HSA conjugate by ELISA. BTG and HSA were also coated on an ELISA plate as negative controls. The phage pool from round 4 was added to the BTG/HSA wells to determine if the phage was non-specifically binding to the conjugate proteins. The scFv-displaying phage were detected using 500 ng/mL of a HRP-conjugated anti-M13 antibody and the absorbance was read at 450 nm using a Tecan Safire™ plate reader following a 20 minute incubation with TMB substrate. The sharp increase in absorbance from round 2 onwards suggested the presence of diclazuril-specific scFv-harbouring phage within the panned library.*

A competitive phage ELISA (**Figure 4.16**) was performed to determine the presence of diclazuril-specific scFv in each of the phage pools. Phage from each round was incubated with a high concentration (20 μg/mL) of free diclazuril. This phage-mixture was then incubated on a 'diclazuril'-HSA-coated ELISA plate. The free diclazuril would displace any diclazuril-binding phage present, and a low signal would be expected. This was directly compared to phage incubated on the 'diclazuril'-HSA coated plate, without any competing analyte present. It can be observed in **Figure 4.16** that no significant displacement of phage occurred from any round of biopanning. This suggests that apparent phage binding in the polyclonal ELISA were binding non-specifically to the carrier protein or linker region of the conjugate. This indicated that no phage-displaying scFv were diclazuril-specific.

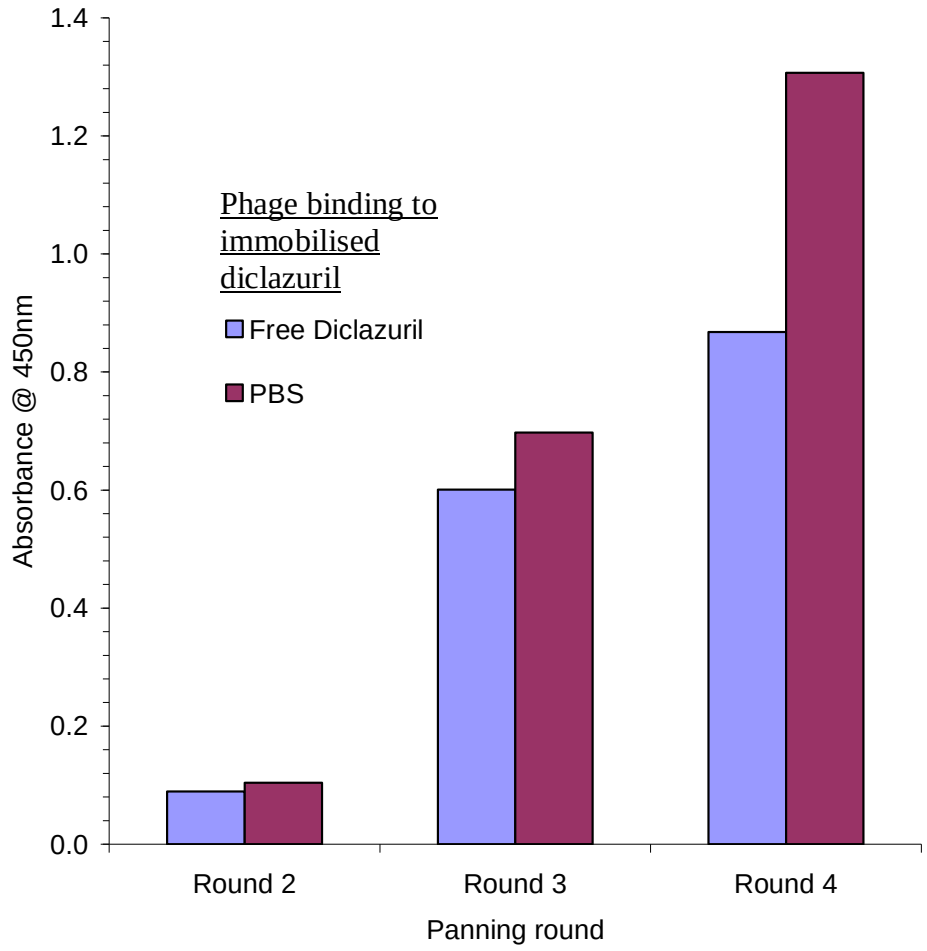


Figure 4.16 *Competitive phage ELISA, analysing the phage pools from rounds 2, 3 and 4 of panning, for binding to free diclazuril. Phage from each round were incubated with an equal volume of a high concentration (20 μg/mL) of free diclazuril. This was directly compared to phage from each round incubated with an equal volume of PBS. The phage and free diclazuril mixtures were then added to a 'diclazuril'-HSA coated Nunc MaxiSorp 96-well ELISA plate, whereby free and immobilised diclazuril compete for binding to the scFvs. Diclazuril-binding phage were displaced and bound phage were detected by the addition of a HRP-labelled anti-M13 secondary antibody.*

To determine whether any diclazuril-binding phage were present, even in low copy number, the library was screened by solubly expressing randomly selected scFv. ScFv are solubly expressed by infection of the phage pool into a non-suppressor *E. coli* strain as described in *Section 2.13.17*. The phage display vector Pcomb3XSS contains an amber stop codon (TAG) between the scFv and the gene III of the phage. When the

phage are infected into a non-suppressor *E. coli* strain which reads the amber stop codon, it facilitates the soluble expression of scFv particles. However, it has been noted that native scFv expression can be achieved without infection into a non-suppressor strain. Even in suppressor strains such as XL1-Blue, suppression is only 50 % and, after the addition of IPTG, adequate amounts of scFv can be produced (Marks and Bradbury, 2004).

Phage from rounds three and four of selection were infected into Top10F cells for soluble expression of scFv. Ninety-six clones were picked from each round and expressed via IPTG induction. The scFvs were harvested from the cells by performing a freeze-thaw procedure for cell lysis. The lysate was then screened for anti-diclazuril scFv by performing a direct ELISA (**Figures 4.17 and 4.18**). 'Diclazuril'-HSA was coated on an ELISA plate, and the plate was blocked by the addition of an appropriate blocking agent. Bound scFv was then detected using a commercial HRP-labelled anti-HA antibody (Sigma). **Figures 4.17 and 4.18** show the binding of individually selected scFv fragments to the 'diclazuril'-HSA conjugate from rounds three and four of panning.

Eleven of the clones selected were taken for further characterisation. Each clone was expressed in 5 mL small-scale cultures. The scFvs were grown overnight and expression was induced by the addition of IPTG. The eleven expressed clones were titred on a 'diclazuril'-HSA-coated and blocked ELISA plate, and bound scFv was detected by the addition of a HRP-labelled anti-HA secondary antibody. However, none of these scFv exhibited any binding towards diclazuril in a direct ELISA format (data not shown). The polyclonal ELISA results suggested the presence of anti-diclazuril scFv, yet the monoclonal ELISA results suggest no diclazuril-binding scFv were present after four rounds of selection. The phage used for the polyclonal ELISA was possibly too concentrated and a significant amount of non-specific binding may have occurred, leading to a false-positive result. In a library of 10^7 clones, the incidence of diclazuril-binding clones should be far greater than eleven, after four rounds of affinity selection. This may indicate that the library was not sufficiently diverse initially, and many of the clones analysed were multiple copies of the same clone. It may also indicate that the panning conditions at the start of the selection process were too stringent and did not allow for enrichment of all of the clones in the library, hence library diversity was lost too soon.

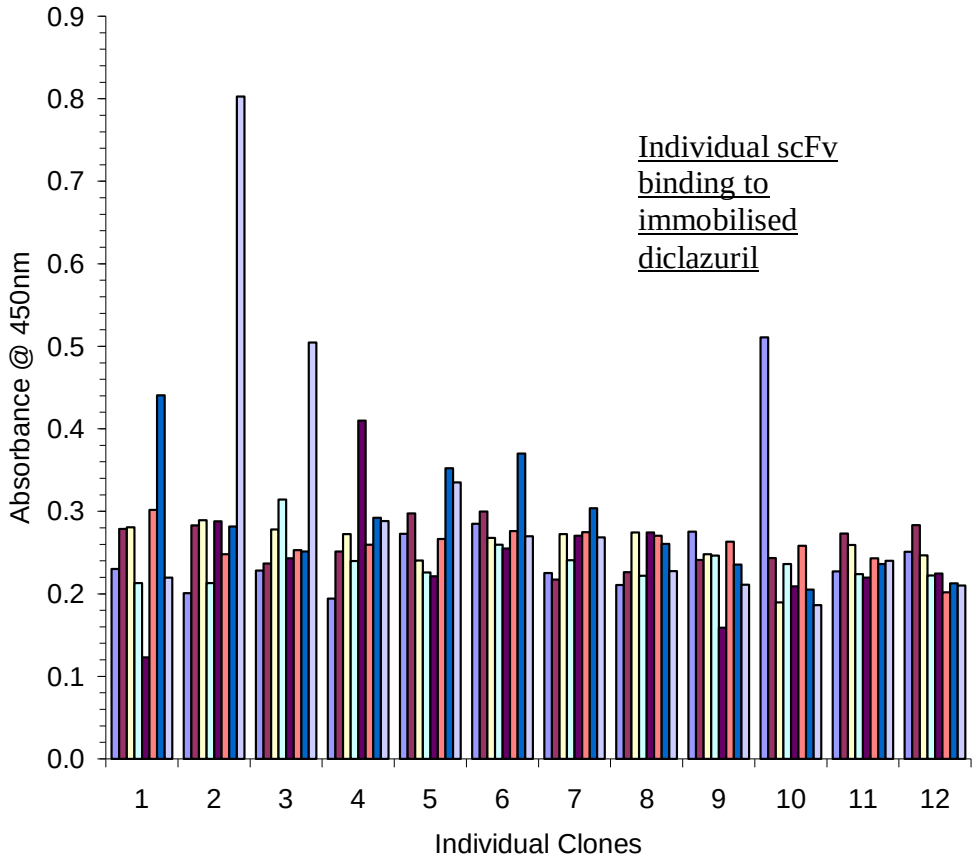


Figure 4.17 *Monoclonal ELISA with randomly selected clones from round three of panning. The clones were solubly expressed in Top10F cells. Nunc MaxiSorp ELISA plates were coated with 1 μg/mL of 'diclazuril'-HSA and blocked with an appropriate blocking agent. ScFv were detected using a HRP-labelled anti-HA antibody. Six of these clones were grown overnight in 5 mL cultures for further characterisation studies.*

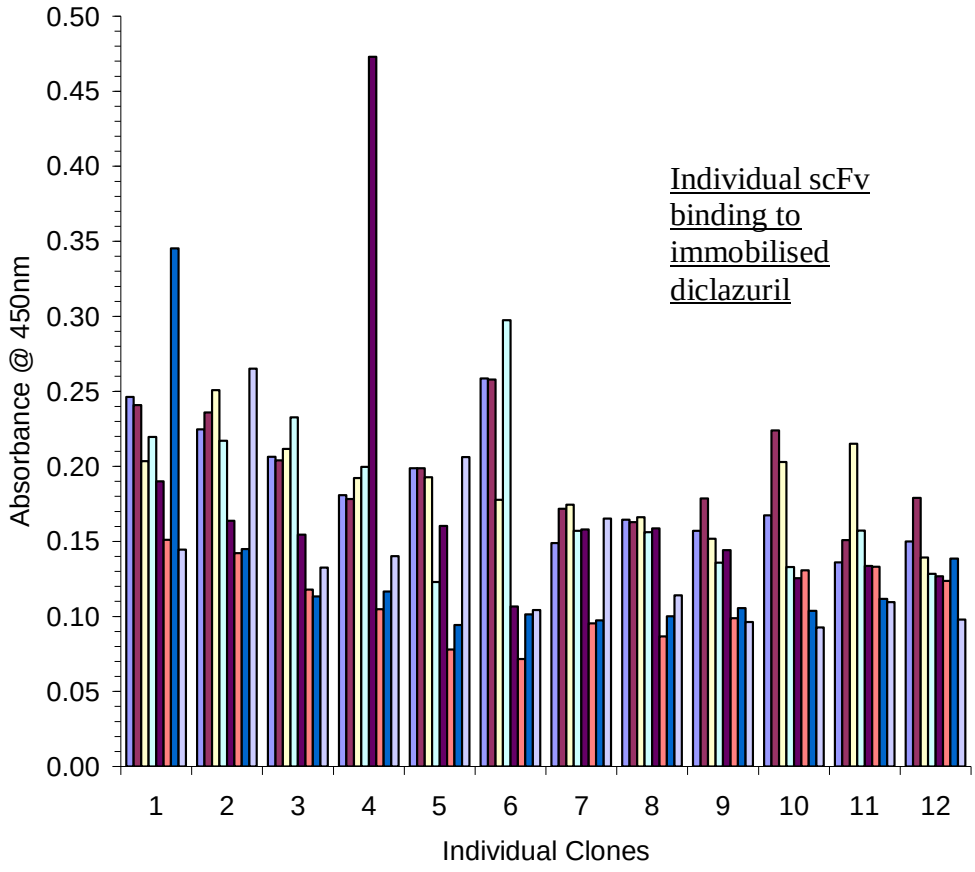


Figure 4.18 *Monoclonal ELISA to select for anti-diclazuril scFv from randomly selected clones in the fourth round of biopanning. The clones were solubly expressed in Top10F cells. Nunc MaxiSorp ELISA plates were coated with 1μg/mL of 'diclazuril'-HSA and blocked with an appropriate blocking agent. ScFv were detected using a HRP-labelled anti-HA antibody. Five of these clones were grown overnight in 5 mL cultures for further characterisation studies.*

It was decided to decrease the stringency of the panning, by increasing the initial antigen concentration, yet keeping the number of washes the same. This method does not involve selection by 'off-rate'. It relies on a balance of both 'off-' and 'on-rate' selection by antigen reduction. If high affinity binders were solely required, they could be selected afterwards via antigen-challenge. Biopanning was repeated again using the conditions shown in **Table 4.1.**

Table 4.1 *Panning conditions employed for each round of the repeated selection for the mouse anti-diclazuril scFv library. Phage input and output titres over the 4 rounds of biopanning are shown, with each round of biopanning varying in conditions and stringency.*

Panning round	Diclazuril coating concentration	Washing steps	Input titres cfu/ mL	Output titres cfu/ mL
1	200 μg/mL	3 X PBST 3 X PBS	4.4×10^{11}	5.3×10^{5}
2	10 μg/mL	3 X PBST 3 X PBS	2.1×10^{11}	1.9×10^{5}
3	50 μg/mL	3 X PBST 3 X PBS	1.6×10^{10}	2.6×10^{5}
4	10 μg/mL	3 X PBST 3 X PBS	2.4×10^{11}	6.7×10^{5}

The results of the polyclonal ELISA, after repeating the biopanning selection process, indicate a very low number of phage binding to the 'diclazuril'-conjugate (**Figure 4.19)**. These results suggest that little or no phage are displaying diclazuril-specific scFv. To confirm this, a monoclonal ELISA was performed on randomly selected individual clones from rounds three and four. This is illustrated in **Figures 4.20 and 4.21**.

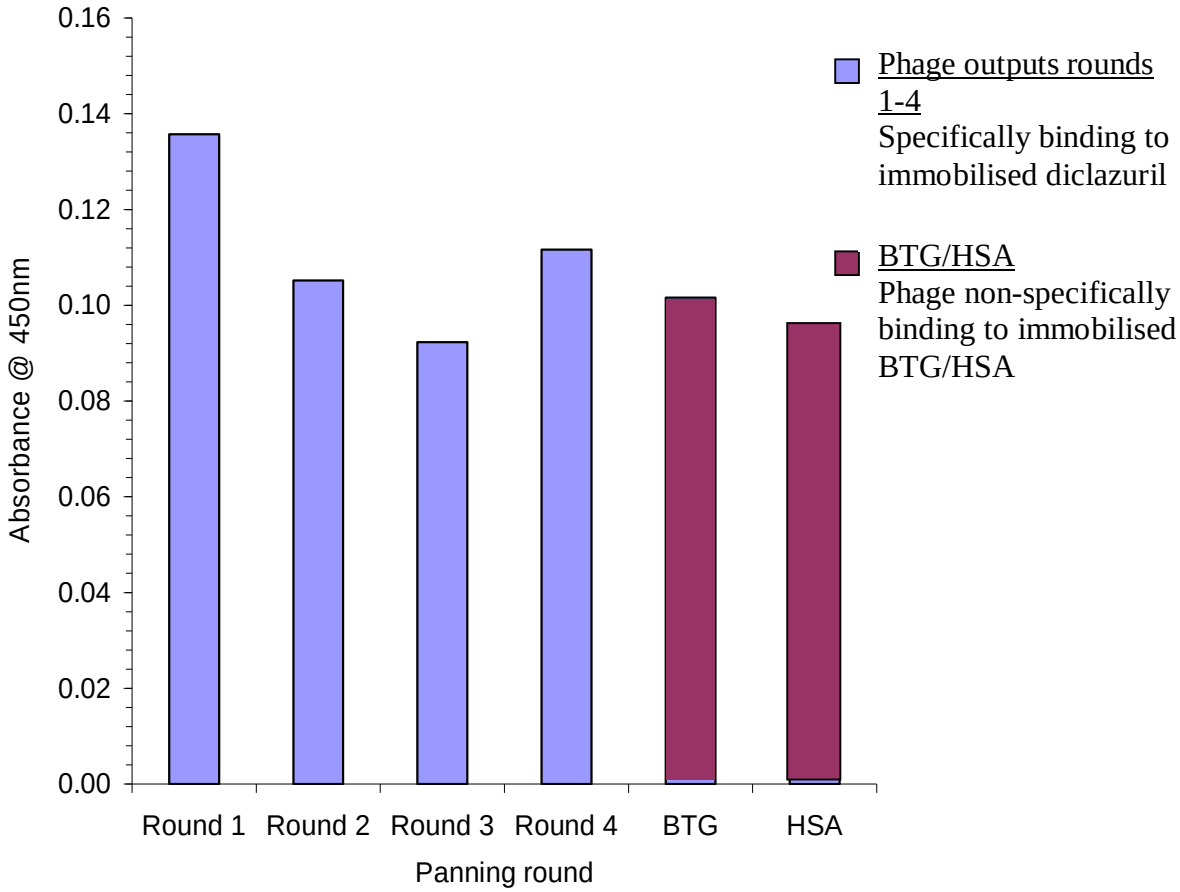


Figure 4.19 *Polyclonal phage ELISA screening for anti-diclazuril scFv displayed on phage, following four rounds of the repeated panning procedure. Phage pools obtained after each round of panning were tested for binding to 'diclazuril'-HSA conjugate by ELISA. BTG and HSA were also coated on a Nunc MaxiSorp ELISA plate as negative controls. The phage pool from round 4 was added to the BTG/HSA wells to determine if the phage were non-specifically binding to the conjugate proteins. The scFv-displaying phage were detected using 500 ng/mL of a HRP-conjugated anti-M13 antibody and the absorbance was read at 450 nm using a Tecan Safire™ plate reader following a 20 minute incubation with TMB substrate. No variation in absorbance was observed over any of the biopanning rounds.*

The monoclonal soluble-phage ELISA from the repeated biopanning selection confirmed that no expression of diclazuril-specific scFv had occurred (**Figures 4.20 and 4.21**). No clones exhibited significant binding to the 'diclazuril'-conjugate after

the repeated bio-panning selection. The absorbance of the monoclonal ELISAs did not reach more than approximately 0.23 at 450nm, which would generally be considered as background in most assays under these conditions.

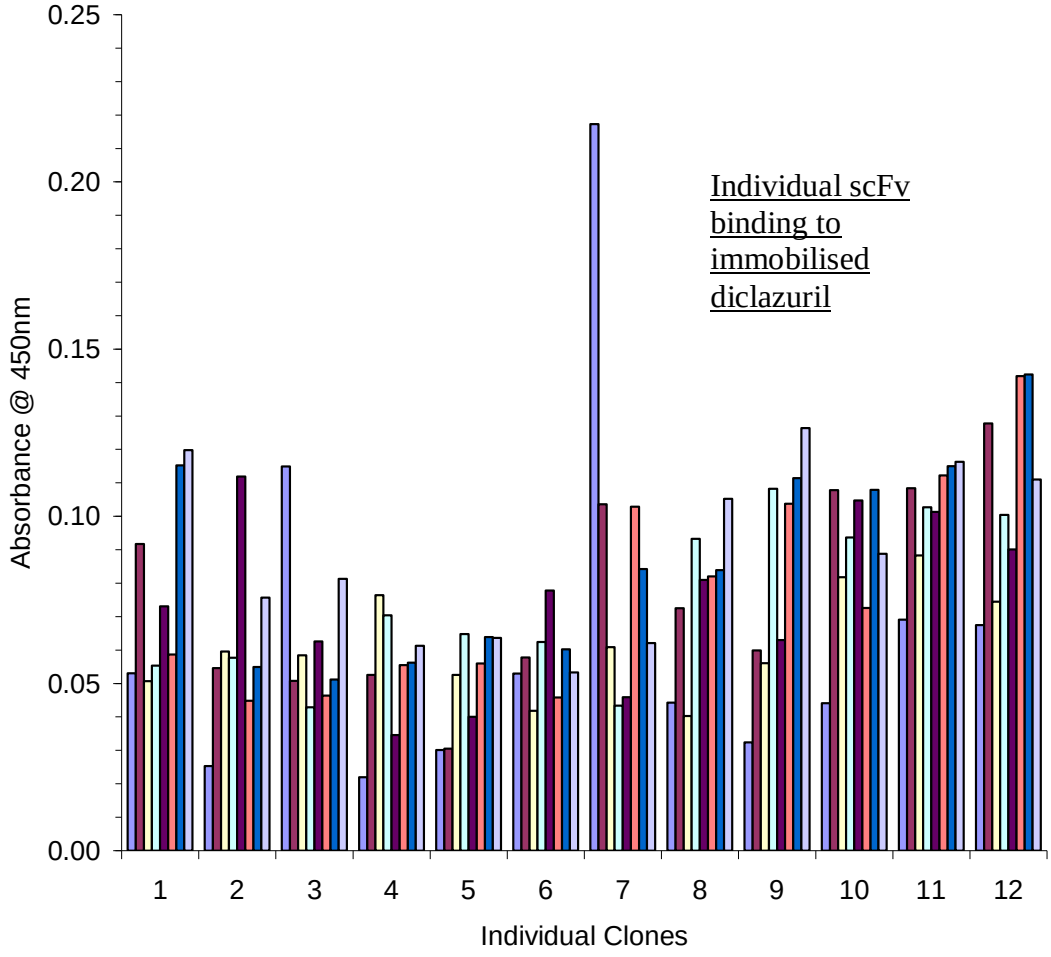


Figure 4.20 *Monoclonal ELISA to select for anti-diclazuril scFvs from randomly selected clones in round three of the repeated biopanning selection procedure. The clones were solubly expressed in Top10F cells. MaxiSorp 96-well ELISA plates were coated with 1 μg/mL of 'diclazuril'-HSA and blocked with an appropriate blocking agent. ScFv were detected using a HRP-labelled anti-HA antibody.*

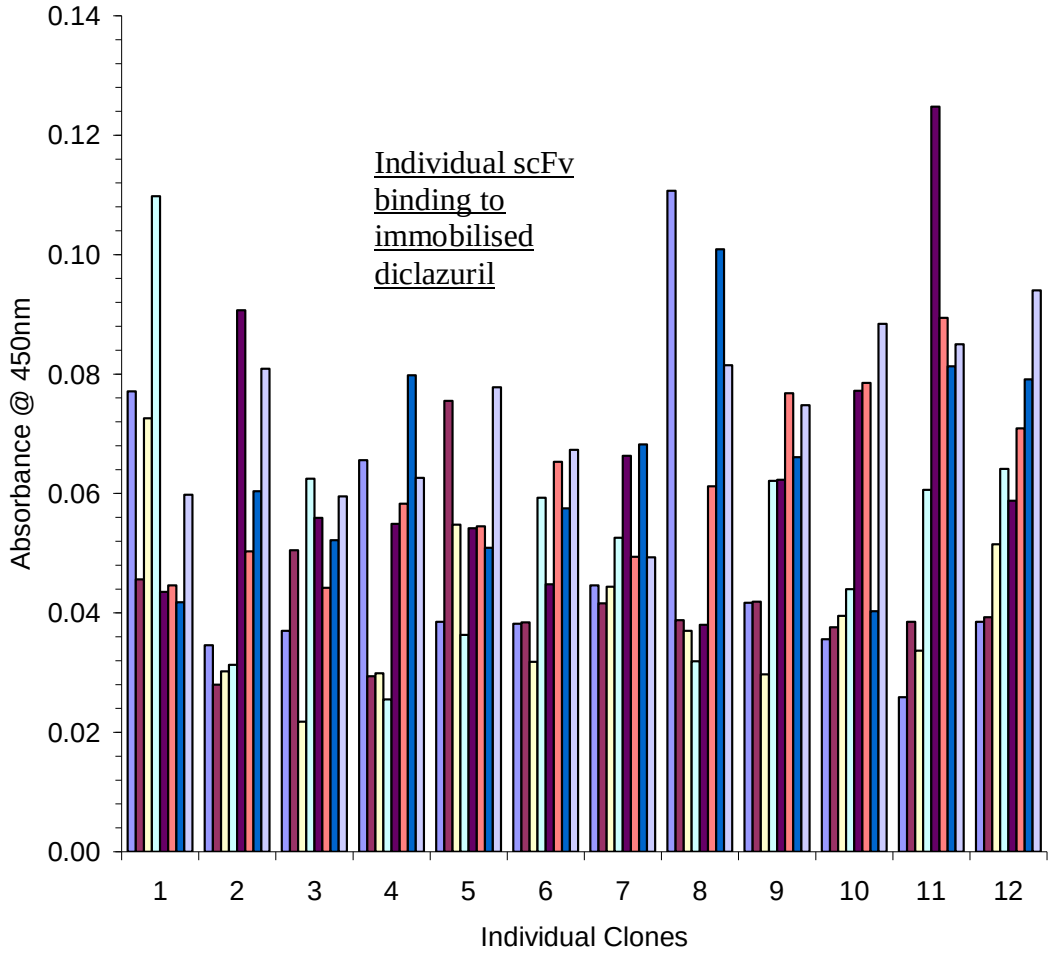


Figure 4.21 *Monoclonal ELISA to select for anti-diclazuril scFvs from randomly selected clones in round four of the repeated biopanning selection procedure. The clones were solubly expressed in Top10F cells. Nunc MaxiSorp 96-well ELISA plates were coated with 1 μg/mL of 'diclazuril'-HSA and blocked with an appropriate blocking agent. ScFv were detected using a HRP-labelled anti-HA antibody.*

4.3 Discussion and Conclusion

This chapter focuses on the development of a recombinant antibody library to isolate diclazuril-specific scFvs, for the subsequent incorporation into a validated diclazuril-detection assay. The first step involved evoking an immune response to diclazuril in a chicken. A chicken was administered with a diclazuril-derived immunogen ('diclazuril'-HSA) to stimulate the production of antibodies towards diclazuril. However, after several subsequent boosts, the avian serum was analysed for diclazuril-binding antibodies but no response was observed. It should be noted that the chicken was responding to a halofuginone immunogen that was administered at the same time as the 'diclazuril' immunogen, hence the problem was believed to be with the 'diclazuril' conjugate. A direct ELISA was performed to determine whether the 'diclazuril' entity was still present on the conjugate, or if the 'diclazuril'-HSA conjugate was unstable. A commercial diclazuril-binding antibody (Randox Laboratories) was used to detect 'diclazuril' on the conjugates. The results indicated that the 'diclazuril' entity was still present on the conjugate, and that the conjugate was working adequately.

Three mice were immunised with the 'diclazuril'-HSA conjugate and a full immunisation schedule followed. After five subsequent boosts, sera isolated from tail bleeds of the mice was analysed by ELISA for the presence of diclazuril-binding antibodies. The presence of an adequate titre of anti-diclazuril antibodies was confirmed and the mice were sacrificed. RNA isolated from the spleens of the mice was converted to cDNA and the scFv library was constructed using PCR assembly methods. Following extensive optimisation attempts the scFv was successfully assembled. However, the level of expression of this full-length scFv product was very poor. Hence, it was decided to prepare fresh cDNA once again. Using the re-synthesised cDNA preparation, the full length scFv product was re-assembled following further optimisation steps. This was produced on a large-scale and ligated into the pComb3XSS vector for the display of diclazuril-specific antibodies on the surface of phage. Enrichment of the library was performed by affinity selection against a 'diclazuril'-BTG conjugate. The polyclonal ELISA after biopanning indicated the presence of 'diclazuril'-specific phage in the final round of panning. This round was solubly expressed and it was discovered that no diclazuril-binding scFv were present in the library. It was decided that the library would be screened once more, using less stringent conditions. This method would allow antibodies with

very low affinities for diclazuril the opportunity to be enriched during the selection process. This second attempt was also unsuccessful in isolating anti-diclazuril scFvs.

The amplification problems that arose during the construction of the library led to the opinion that inefficient variable gene assembly could potentially be responsible for the production of a poor quality scFv library. During murine immunoglobulin gene assembly the heavy chain is generated by a combination of one of several hundred V_H genes with one of about thirty D segments and one of six J segments (Barbas *et al.*, 2004). Due to the copious amounts of variable genes involved, the generation of a murine antibody repertoire requires many V-specific oligonucleotide primers (Barbas *et al.*, 2004). This in turn, can require extensive optimisation to amplify the various heavy chain sequences and capture a good representation of the repertoire (Barbas *et al.*, 2004). The incorporation of all the appropriate heavy and light chain variable regions into the library using the Barbas series of primers (Barbas *et al.*, 2004) would involve amplification using a total of 166 separate primer combinations. To circumvent this copious workload, a 'master-mix' of primers was used. However, the use of this master mix has the potential to cause a loss of diversity from the immune repertoire. Certain primer combinations may amplify efficiently, whereas, other combinations may need thorough optimisation. This occurrence may lead to bias towards abundant genes over more conserved genes, which may be integral to the development of a highly specific scFv library. Furthermore, if the overlap PCR product of the variable heavy and variable light chains is not highly efficient, sequence frame shifts may occur resulting in poor quality of the library (Koohapitagtam *et al.*, 2010). These factors may have contributed to the production of an inferior scFv library, with minimal specificity for diclazuril.

4.4 Future Work

Hayhurst and colleagues (2003) previously noted the disadvantages of using murine scFvs. These include poor expression levels with low soluble product, cell culture arrest and lysis, misfolding of expressed antibody and protein aggregation. It is recommended that if the construction of a recombinant antibody library for diclazuril is required, immunisations should be carried out using commercial conjugates and avian immune models. The concern with conjugates produced 'in-house' is that no definitive method is in place for their characterisation. Once the exact quality of the conjugate is defined, chicken immunisations can proceed for the development of a

recombinant antibody for diclazuril. (The advantages of avian models are discussed in greater detail in Chapter 3.) A commercial anti-diclazuril antibody was obtained from Randox Ltd., which was incorporated into a multi-analyte detection assay alongside an anti-halofuginone and an anti-toltrazuril antibody described in detail in *Section 5.8.3*. Future library development for the construction and isolation of an anti-diclazuril scFv would prove highly advantageous in a range of applications for the detection of diclazuril.

Chapter 5

Polyclonal antibody-based assays for the detection of toltrazuril

Chapter outline

The aim of this work was to develop a highly sensitive immunoassay for the detection of the anti-protozoan drug, toltrazuril. This chapter describes the generation of toltrazuril-specific polyclonal antibodies and optimisation of their subsequent use in an ELISA. Trifluoraminoether (TFME), an antigenic mimic for toltrazuril, was coupled to bovine serum albumin (BSA) and used to immunise rabbits, for the production of polyclonal antibodies. Concurrently, a commercially obtained toltrazuril-BSA conjugate was also used for immunisation. Polyclonal antibodies specific to toltrazuril were purified from the rabbit sera and employed in an immunoassay for the detection of toltrazuril. The performance of the antibody preparation was assessed by competitive ELISA, and a validated assay was developed. The antibody was subsequently incorporated into Biacore-based and lateral flow-based assay formats. The efficiency of all three methods was directly compared. The development of a multi-analyte lateral-flow detection method for the simultaneous detection of halofuginone, diclazuril, and toltrazuril is also described.

5.1 Introduction

The quality of an immunoassay is primarily dependant on the quality of the incorporated antibody. Polyclonal antibody production was the method of choice for the generation of toltrazuril-specific antibodies. In contrast to the expense and expertise required for traditional hybridoma and recombinant antibody production, polyclonal antibodies can be raised quickly and at relatively low cost. Animals such as rabbits, guinea pigs, goats, sheep, donkeys and fowl are frequently used for the generation of polyclonal antisera (Hanly *et al.*, 1995).

One attractive feature of the immune system of rabbits, for the generation of antibodies, is the utilisation of both gene conversion and somatic hypermutation to diversify rearranged heavy and light chain genes. The antibody diversity generated by V_HDJ_H rearrangements in rabbits is more limited than in mice and humans. This is due to the fact that of about 50 functional VH gene segments, only one, V_H1, is predominantly used. In contrast to the limited V_HDJ_H rearrangements, $V_\kappa J_\kappa$ rearrangements in rabbits are much more diverse and, thus, the resulting rearranged kappa light chain genes may compensate for the limited diversity of rearranged heavy chain genes (Popkov *et al.*, 2003). Rabbits also have two kappa light chain isotypes,

K1 and K2, compared to only one in mice or humans. Further diversity to the immune repertoire may also be contributed from the kappa light chain regions due to the unusual germline VK-encoded variability in the length of the complementarity-determining region 3. Hence, the kappa light chain appears to be a major contributor towards the generation of the antibody diversity in the rabbit immune repertoire (Popkov *et al.,* 2003).

The first steps in polyclonal antibody production for haptens are to select the target molecule, to identify the appropriate functional groups for conjugation to a carrier protein, which can ensure antibody recognition of key structural elements, and then to design the hapten-protein conjugation strategy for suitable immunisation. Previously, Connolly and colleagues (Connolly *et al.,* 2002) identified an antigenic mimic which shared a common substructure for both toltrazuril and its metabolite, ponazuril. Using ChemOffice™ software they were able to search the database for drugs that contain a similar sub-structure to ponazuril. Trifluoraminoether was identified as having structural elements common to toltrazuril and ponazuril structures. In addition, it possessed a free amine group suitable for conjugation to a carrier protein. After immunising six rabbits with this antigenic mimic, the most sensitive polyclonal antibody produced was identified and characterised. Following 13 immunisations of the rabbit designated 'R609' with a TFME conjugate, 86% displacement of antibody occurred in a competitive ELISA. This occurred when the antibody was incubated with 50 ng/mL of ponazuril. The IC50 of this 'R609' antibody was 18 ng/mL. The sensitivity of these toltrazuril-specific antibodies encouraged the production of toltrazuril-specific antibodies in the same manner.

The TFME hapten, with a molecular weight of only 299.31 Da, is too small to induce an immune response and, therefore, requires conjugation to a carrier protein to render it immunogenic. TFME was conjugated to BSA via diazo-coupling (**Figure 5.0**).

Reaction mechanism for the preparation of the diazonium salt of TFAE-NH_2

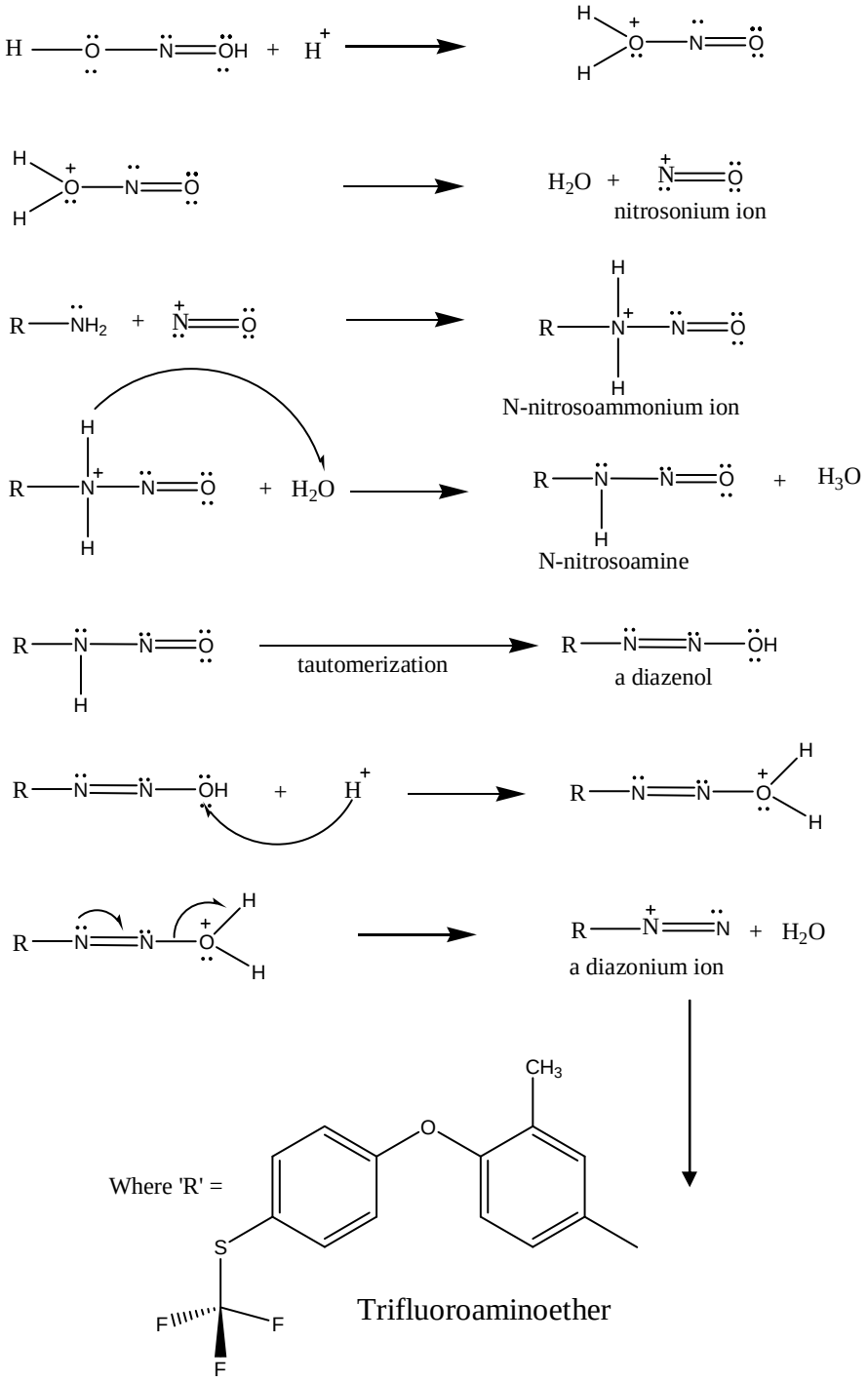


Figure 5.0 *Reaction mechanism for the preparation of trifluoraminoether for subsequent conjugation to a carrier protein (Section 2.14.1). Trifluoraminoether in sulphuric acid (H^+) is treated with sodium nitrite, to form a diazotized derivative. This derivative can then be used for conjugation to carrier proteins* (Szele and Zollinger, 1983). *Mechanism was modified from Wieder, (clem.mscd.edu/~wiederm/oc2ppt /aminesphenols.ppt, 2004).*

A commercial toltrazuril-BSA conjugate was obtained from Randox Laboratories. Researchers at Randox had discovered a method that allowed the conjugation of toltrazuril directly to a carrier protein. This was performed by linking the carrier protein to one of the nitrogen rings on the right-hand side of toltrazuril, away from the trifluoromethyl group. This conjugate was purchased and subsequently used for immunisations.

Rabbit immunisations were performed externally by the CER Groupe (Belgium). The specific antibody titre of the serum received from CER Groupe was estimated by direct ELISA.

5.2 Results

5.2.1 Serum titres of New Zealand white rabbits (Numbers 71-80)

Following an extensive immunisation regime, an antibody serum titre was performed from the final bleeds obtained from the five TFME-BSA immunised rabbits and the five toltrazuril-BSA immunised rabbits. As illustrated in **Figure 5.1**, serum from each of the immunised rabbits was diluted (1/50 to 1/156,250) in PBS containing 1% (w/v) BSA and analysed for binding to the toltrazuril-BSA conjugate. Bound antibody was detected by the addition of a HRP-labelled anti-rabbit IgG secondary antibody. Except for three rabbits, antibodies from all of the other polyclonal sera displayed binding to the immobilised toltrazuril conjugate.

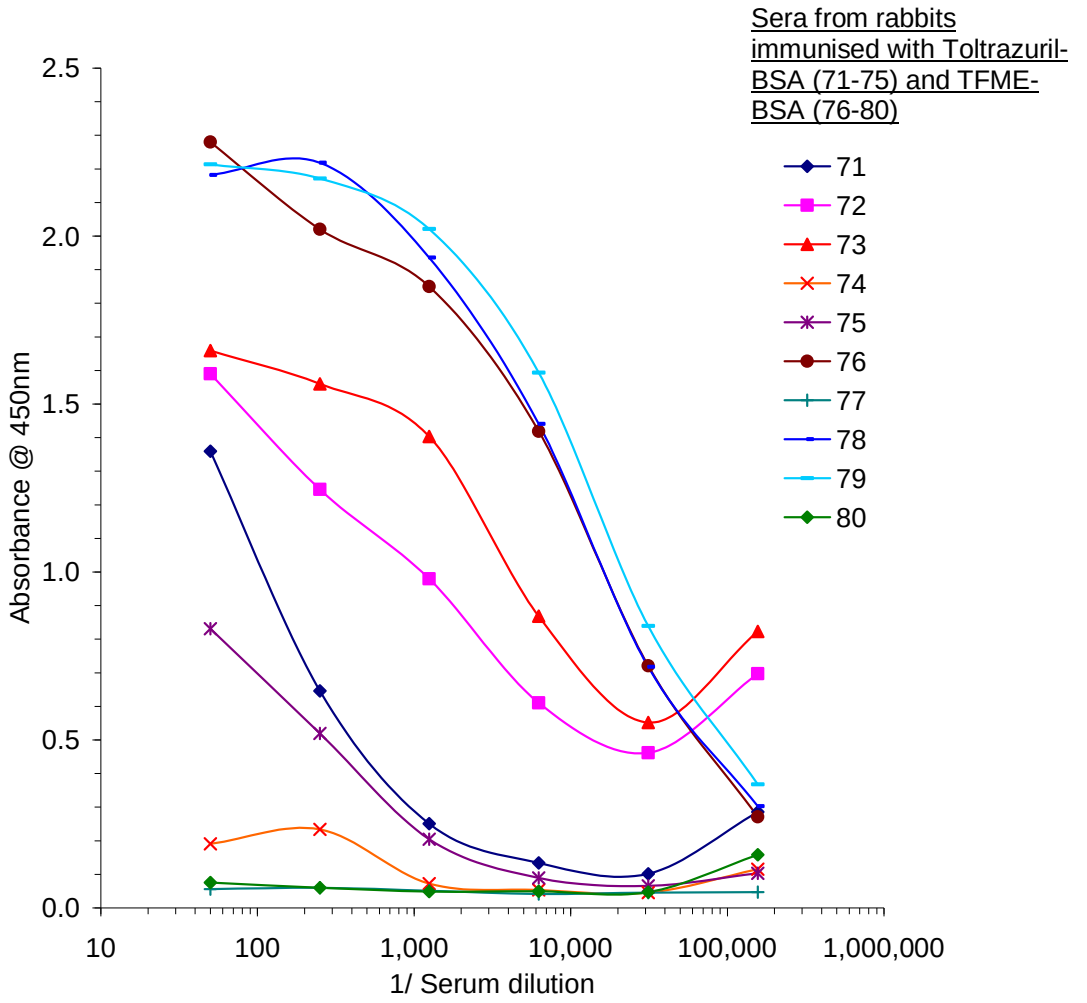


Figure 5.1 *Antibody titre of ten rabbits immunised with TLZ-BSA (71-75) and TFME-BSA (76-80) conjugates. The antibody response for toltrazuril from the sera was determined by performing a direct ELISA on Nunc MaxiSorp plates coated with TLZ-BSA. The immunoplates were blocked with Milk Marvel and serial dilutions of the serum from each rabbit were added to the plate. Bound polyclonal antibody was detected using a HRP-labelled anti-rabbit IgG secondary antibody.*

5.2.2 Competitive ELISA using serum from toltrazuril/trifluoraminoether immunised rabbits

A competitive ELISA was performed with ponazuril concentrations of 10 µg/mL, 2 µg/mL, 400 ng/mL, 80 ng/mL, 16 ng/mL and 0 ng/mL. It can be observed in **Figure 5.2** that only three polyclonal sera batches, namely 72, 73 and 75, displayed binding to free ponazuril. These three sera batches were taken from toltrazuril-BSA immunised rabbits

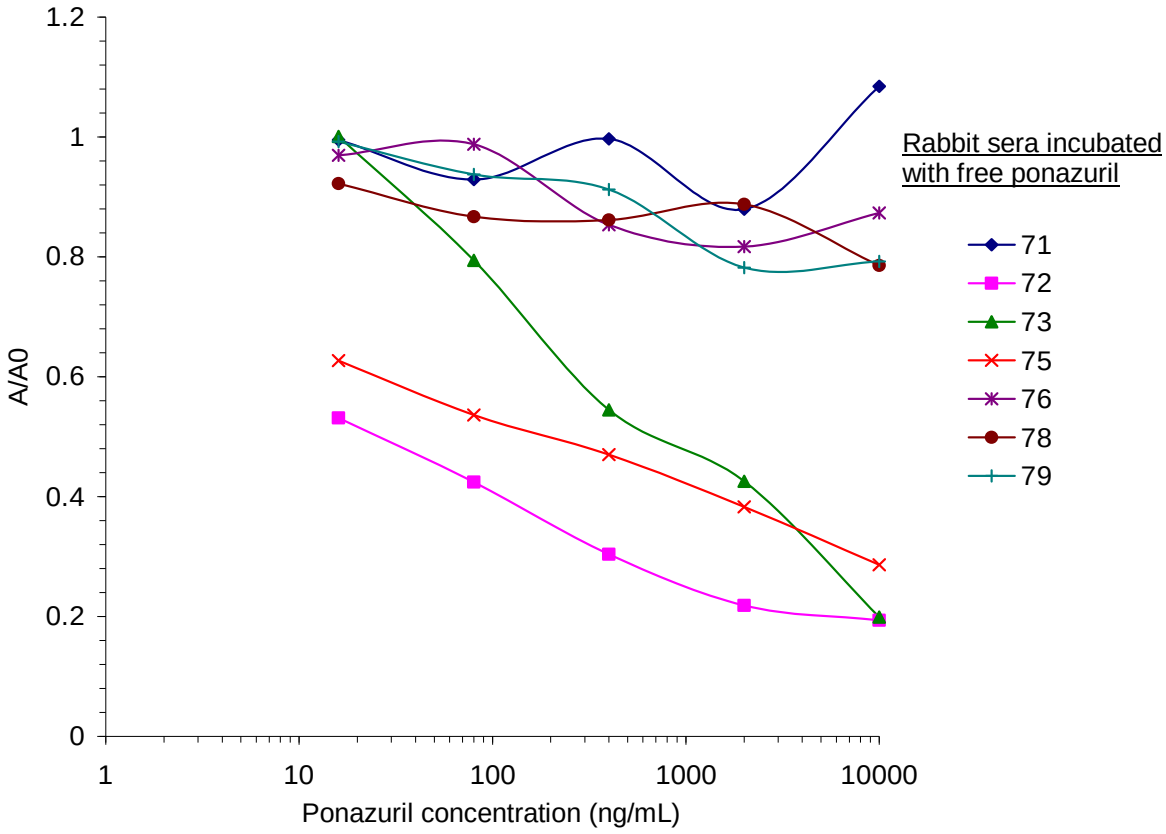


Figure 5.2 *Competitive ELISA analysing rabbit sera for toltrazuril-specific antibodies. The sera from 7 rabbits, which showed specific binding to the toltrazuril-BSA conjugate in a direct ELISA, were screened in a competitive assay format. One µg/mL TLZ-BSA was coated on the surface of a Nunc MaxiSorp ELISA plate. The sera were incubated with final concentrations of ponazuril at 10 µg/mL, 2 µg/mL, 400 ng/mL, 80 ng/mL, 16 ng/mL and zero ng/mL. The dilution of sera used in the competitive ELISA was estimated from the direct ELISA (**Figure 5.1**). Rabbit sera '71' and '75' were used at a final 1/250 dilution, sera '72' and '73' at a 1/1,000 dilution and sera ' 76', '78' and '79' at a 1/5,000 dilution. The results are shown as A/A0, where the absorbance values of the evaluated samples (A), are normalised by expressing them as a function of the blank standard (A0).*

5.2.3 Competitive ELISA analysis for toltrazuril detection

Another competitive ELISA was performed using a wider concentration range of ponazuril, using the three sera samples exhibiting toltrazuril-binding. These sera were isolated from rabbits designated '72', '73' and '75' (**Figure 5.3**). The three sera samples were pooled and purified by Protein G affinity chromatography.

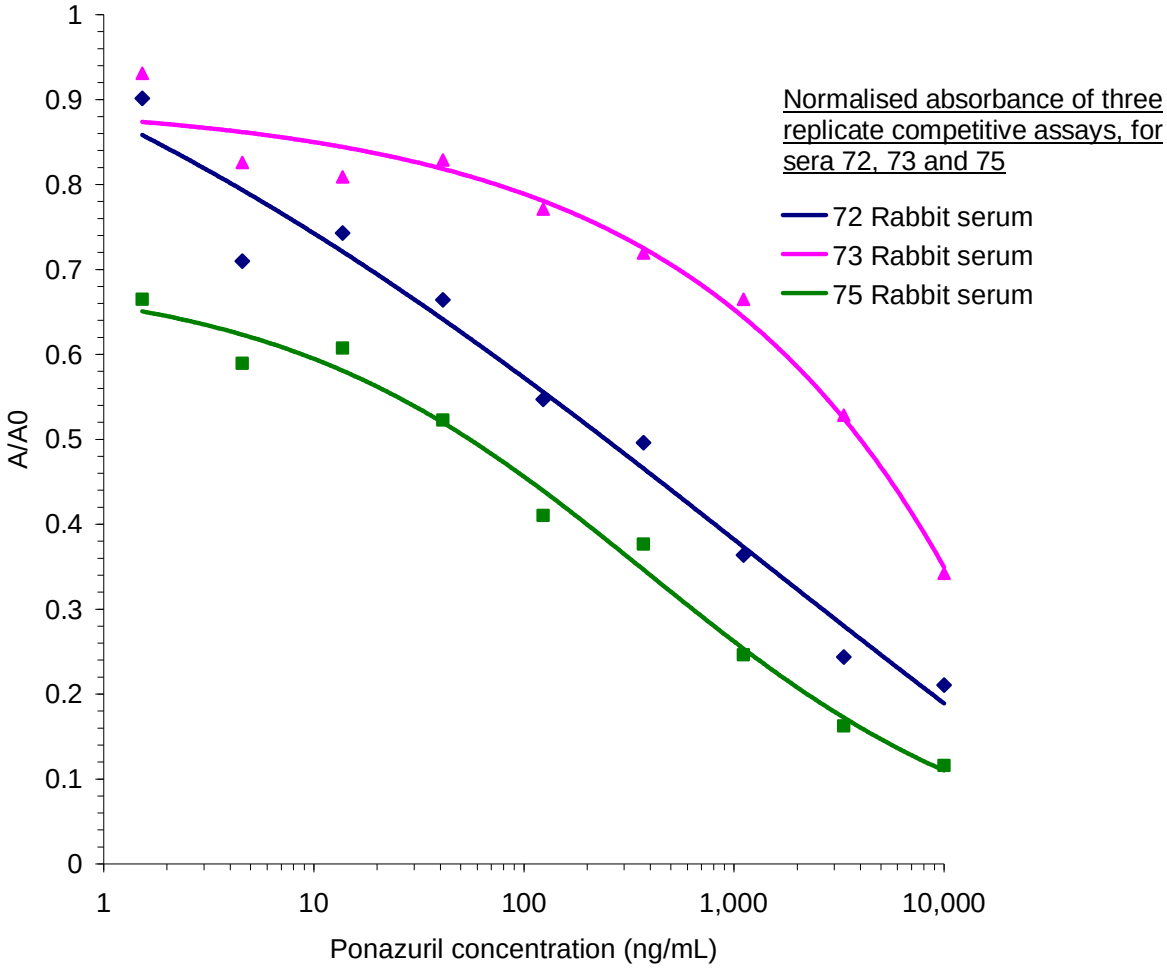


Figure 5.3 C*ompetitive ELISA using toltrazuril-specific polyclonal sera. TLZ-BSA was coated on a Nunc MaxiSorp ELISA plate. Three different serum batches, exhibiting displacement by free ponazuril (as determined in* ***Figure 5.2***), *were analysed in a broad range competitive assay. Serum '75' was used at a final 1/250 dilution, and '72' and '73' at a 1/1,000 dilution. The antibodies were incubated with final concentrations of ponazuril at 10 µg/mL, 3.3 µg/mL, 1.1 µg/mL, 370 ng/mL, 123 ng/mL, 41 ng/mL, 13.7 ng/mL, 4.5 ng/mL, 1.5 ng/mL and 0 ng/mL. The results are shown as A/A0, where the absorbance values of the evaluated samples (A), are normalised by expressing them as a function of the blank standard (A0).*

5.2.4 Purification of polyclonal antibodies from rabbit sera by Protein G affinity chromatography

Sera from rabbits designated '72', '73' and '75' were pooled. All three batches isolated from the rabbits were competitive with free ponazuril. Antibodies were purified by immunoaffinity chromatography using protein G-sepharose. Protein G (SpG) from *Streptococci* groups C and G is a surface IgG binding protein with specific affinity for the Fc region. However, it also associates with the constant heavy chain (C_H1) domain of the antibody binding fragment (Fab) portion through a β-zipper interaction (Roque *et al*., 2004). All eluted fractions, obtained from the Protein G column, were analysed by SDS-PAGE. Eluted fractions were approximately 3.2 mg/mL following concentration.

5.2.5 Characterisation of purified polyclonal antibodies by SDS-PAGE and Western Blot analysis

The level of purity of the antibodies was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). **Figure 5.4** shows the resulting gel and the increase in the level of purity from the crude serum to the affinity-purified fractions. To confirm the presence of rabbit IgG antibody molecules western blotting was performed on an SDS gel of purified antibody (**Figure 5.5**).

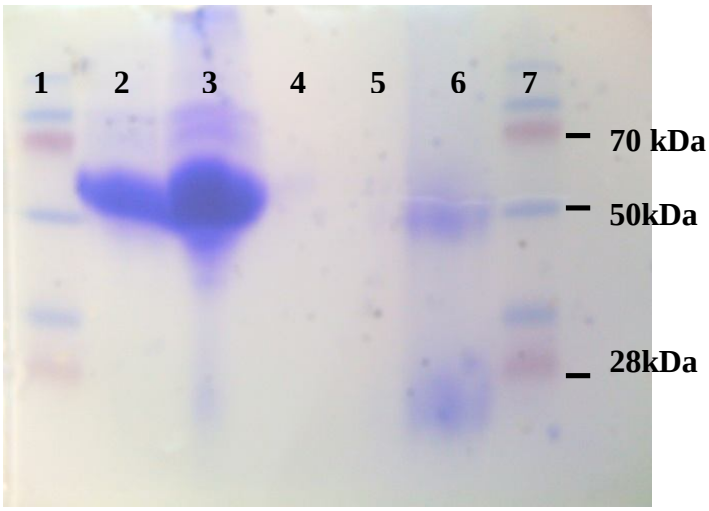


Figure 5.4 *SDS-PAGE analyses on the fractions obtained during protein G affinity chromatography purification of polyclonal antibody fractions, from toltrazuril-specific rabbit serum. Lanes 1 and 7 = Colour Burst molecular weight markers (Fermentas); Lane 2 = Serum samples diluted 10-fold in PBS; Lane 3 = 'Flow-through' mixture of 1 and 2 diluted 10-fold after passage of serum through a protein G column; Lane 4 = Wash 'A'; Lane 5 = Wash 'B'; Lane 6 = Eluted purified antibody pooled fractions used undiluted. Two distinct bands are visible at approximately 50 kDa and 25 kDa in the purified fractions, representing the heavy and light chains, respectively.*

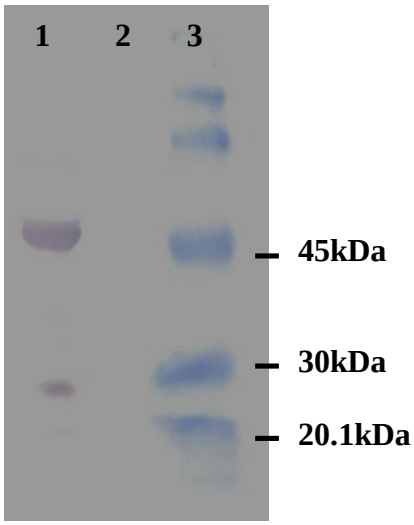


Figure 5.5 *Western blot analysis of the purified anti-toltrazuril antibodies. Lane 1 = Purified anti-toltrazuril polyclonal antibody; Lane 2 = HFG-specific avian scFv used as a control and Lane 3 = High Molecular Weight Marker (Sigma). HRP-labelled anti-rabbit IgG was used to probe for rabbit antibodies. Two distinct bands are visible in Lane 1 at approximately 50 kDa and 25 kDa. These bands indicate the heavy and light chains.*

5.3 Optimisation of an ELISA for the detection of toltrazuril using the purified anti-toltrazuril polyclonal antibody

Following purification of the polyclonal antibody preparation, its use in a competitive immunoassay for the detection of toltrazuril was investigated. In order to maximise the performance of the assay, several parameters were varied to determine the optimal conditions. These included the coating concentration of the conjugate, the buffer used for coating and the concentration of antibody. Once these parameters were determined, intra/inter-day assays were performed to establish the reproducibility of the assay.

5.3.1 Selection of conjugate coating buffer for the competitive ELISA for toltrazuril

To investigate the optimal pH for coating the TLZ-BSA conjugate onto the Nunc MaxiSorp plates, five different coating buffers were employed at five different pH levels. The buffers investigated were: 0.1 M acetate buffer, pH 4.8; 0.01 M TBS buffer, pH 7.4; 0.01 M TBS buffer, pH 4.2; 0.1 M carbonate buffer, pH 10.3 and 0.01

M PBS, pH 7.4. The results are illustrated in Figure 5.6. The results show that the amount of antigen bound to the plate was greatest when using PBS at a pH of 7.4, TBS at a pH of 7.4, or carbonate buffer at a pH of 10.3. PBS at a pH of 7.4 was chosen as the conjugate coating buffer for use in subsequent assays.

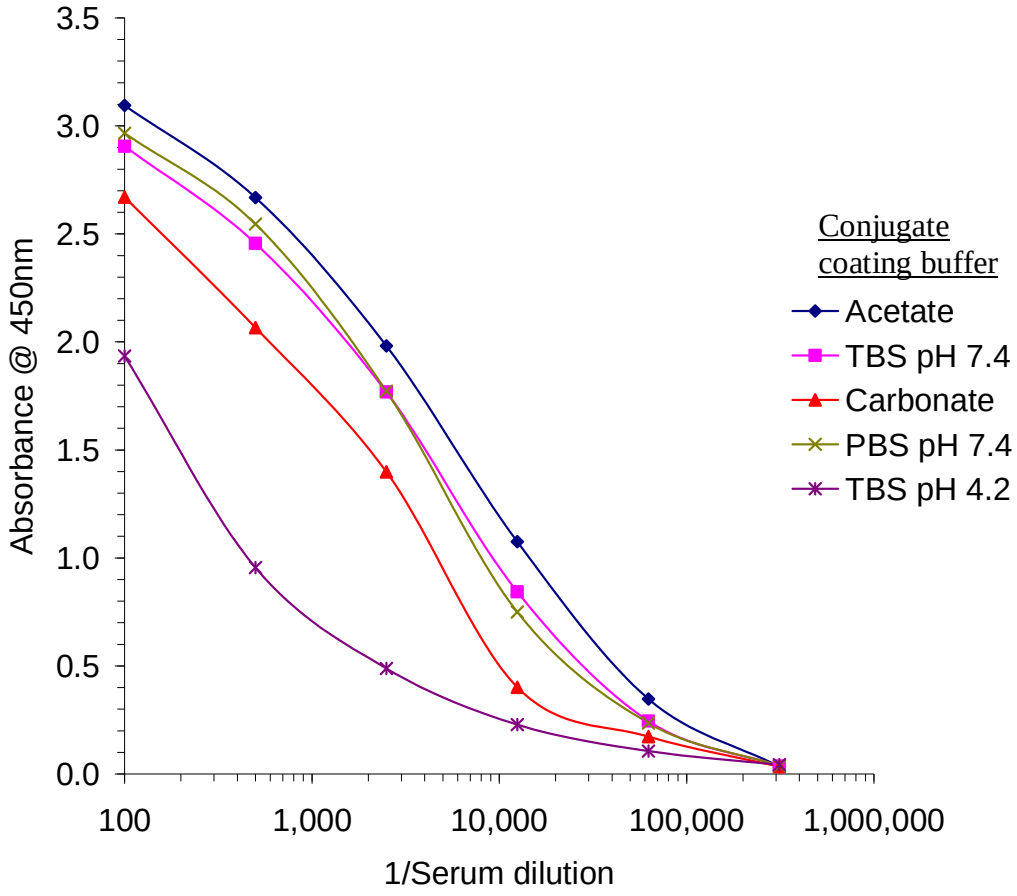


Figure 5.6 *Selection of optimal coating buffer for use in ELISA for detection of toltrazuril. One µg/mL of toltrazuril-BSA conjugate was used. Coating buffers tested were; acetate buffer, pH 4.8; TBS buffer, pH 7.4; carbonate buffer, pH 10.3; PBS, pH 7.4 and TBS buffer, pH 4.2. The anti-toltrazuril antibody preparation was serially diluted in buffer and added to the coated/blocked wells. Bound polyclonal antibody was then detected using HRP-labelled anti-rabbit IgG secondary antibody.*

5.3.2 Checkerboard ELISA for the determination of the optimum polyclonal antibody dilution and optimum coating concentration for toltrazuril conjugates

It was essential to optimise both the coating concentration of the conjugate and the optimal dilution of antibody in a competitive ELISA. Nunc MaxiSorp ELISA plates

were coated with varying concentrations of the TLZ-BSA conjugate in the optimal conjugate coating buffer as previously established. To determine the conjugate concentrations which would give the best assay sensitivity, a checkerboard ELISA was performed with varying dilutions of the affinity-purified antibodies. The results shown in **Figure 5.7** indicate that an optimum coating concentration of 2 µg/mL TLZ-BSA gave the highest absorbance using the most economical conjugate concentration. The optimal antibody dilution chosen, was a 1/3,000 dilution.

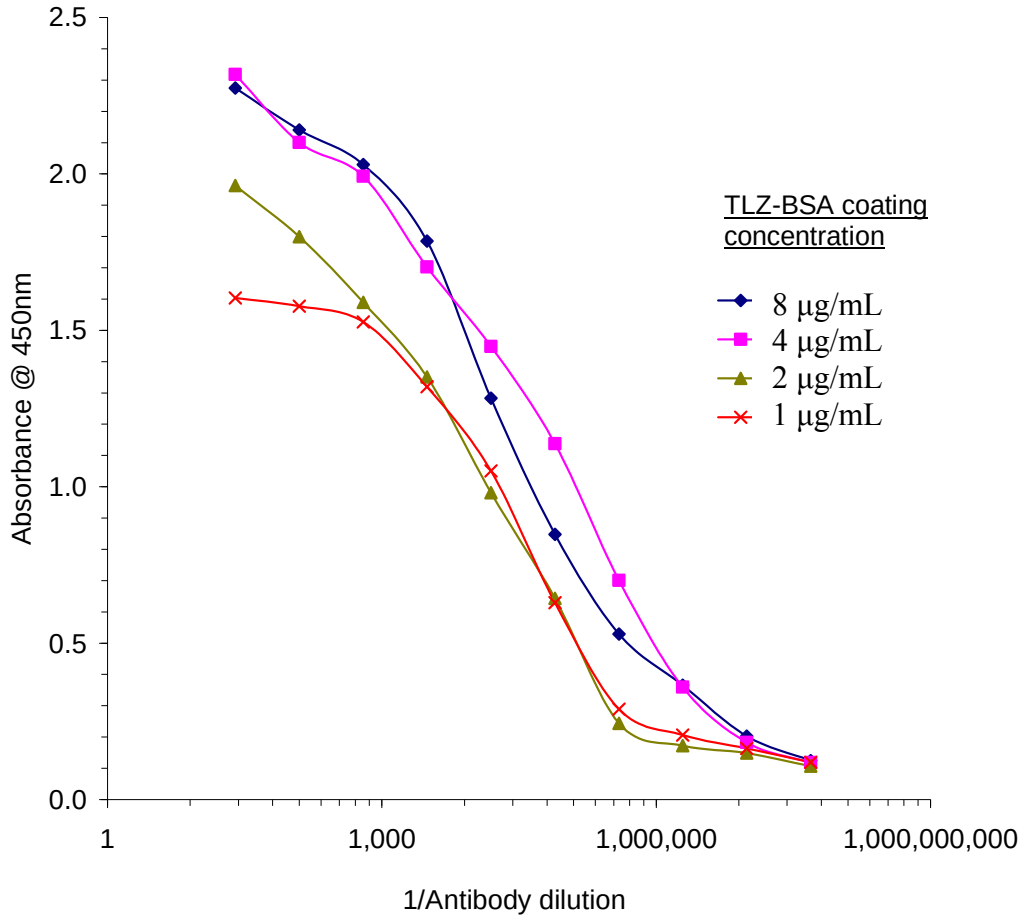


Figure 5.7 *Checkerboard ELISA for the determination of the optimal conjugate coating concentration and polyclonal antibody dilution for use in a competitive ELISA. TLZ-BSA concentrations of 8, 4, 2 and 1 µg/mL were used in a direct ELISA with varying dilutions of antibody ranging from 1/25 to 1/ 48,828,125. A 2 µg/mL TLZ-BSA concentration and a 1/3,000 dilution of the antibody preparation were chosen for use in a competitive assay format.*

5.3.3 Intra and inter-assay variability studies of the competitive ELISA for toltrazuril

Using the optimised parameters established, a competitive assay (free ponazuril in solution competing against immobilised conjugate for binding to the antibody), was performed in PBS to determine the range of detection of the ELISA. Standards of ponazuril were prepared in 0.01 M PBS, pH 7.4, and were diluted with equal volumes of the antibody at a 1/3,000 (final) dilution. The final concentrations of ponazuril ranged from 5 µg/mL down to 254 pg/mL. This antibody-ponazuril preparation was added to an immunoplate coated with TLZ-BSA in 0.01M PBS, pH 7.4, and blocked with 5% (w/v) Milk Marvel. HRP-labelled goat-anti-rabbit IgG was then used to detect toltrazuril-bound antibody. The assay format is shown in **Figure 5.8** below.

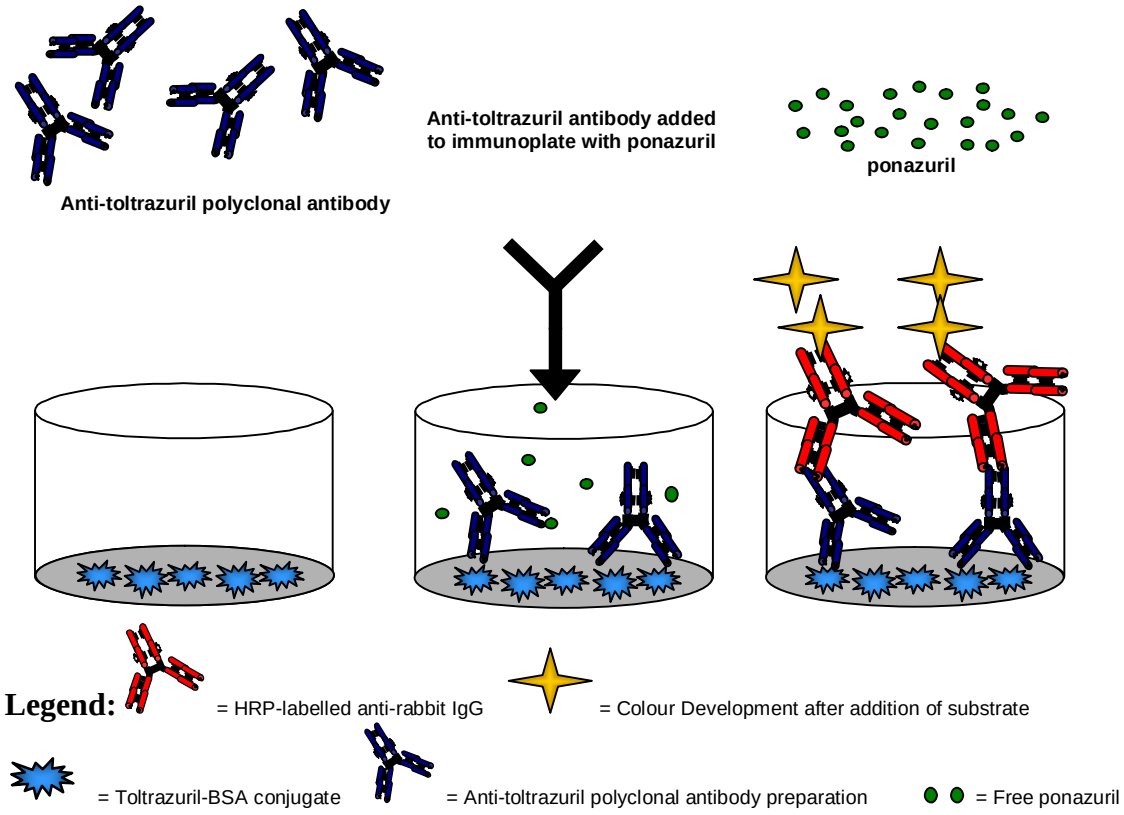


Figure 5.8 *Assay format used in the competitive ELISA for the detection of toltrazuril. TLZ-BSA conjugate was coated on the wells of the plate. Varying concentrations of free ponazuril (the primary metabolite of toltrazuril) were incubated with the anti-toltrazuril antibody preparation. This free antigen-antibody mix was added to the toltrazuril-coated immunoplate. The free ponazuril in solution competes with the immobilised toltrazuril conjugate for binding to the toltrazuril antibodies. Bound antibodies are detected following the addition of a HRP-labelled anti-rabbit IgG.*

Repeatability of an assay is defined as the precision of repeated measurements within the same analytical run under the same operating conditions, over a short period of time. This is also known as the intra day-assay precision (Mire-Sluis *et al.*, 2004). The intra-assay repeatability was calculated by comparing the variation between six measurements within the same assay on the same day. The reproducibility of the assay was determined by comparing triplicate measurements of the levels of antigen within three separate assays. This is also known as the inter-assay precision (Mire-Sluis *et al.*, 2004). The precision of an analytical method is usually described by coefficients of variation (CV) between and within assays (Forkman, 2005). The coefficients of variation (CV) for an assay are defined as the standard deviation divided by the mean (Forkman, 2005). Hence, to calculate the precision of an assay the formula given below is used:

$$\text{Precision (\%)} = \text{CV (\%)} = (\text{Standard Deviation} \div \text{Mean}) \text{ X } 100$$

This formula was employed to determine the precision of the competitive ELISA for toltrazuril detection. All calculated CV values for the inter-assay study are shown in **Table 5.0**. The highest intra-day CV was recorded at 17.7%. The highest inter-day CVs were calculated at 19%.

Table 5.0 *Inter-assay co-efficient of variation (CVs), for the detection of toltrazuril in PBS, in a competitive ELISA format. Each standard was analysed in triplicate over three separate days and the CVs were calculated.*

Ponazuril Conc. (pg/mL)	**Inter-day (% C.V)**	**A/A_0**
250,000	10.8	0.1
125,000	19.0	0.2
62,500	5.0	0.3
31,250	11.2	0.3
15,625	2.9	0.4
7812	3.4	0.5
3906	7.7	0.6
1953	3.6	0.7
976	7.1	0.7
488	12.0	1.0

Calibration curves were generated for both the inter/intra-day-assays. Using BIAevaluation 4.0.1 software, a four-parameter equation was fitted to the data to generate a calibration curve. **Figure 5.9** illustrates the calibration curve established for the inter-day assay using the anti-toltrazuril polyclonal antibody and free ponazuril in concentrations ranging from 500,000 pg/mL down to 254 pg/mL in PBS. Ponazuril standards were prepared singly and analysed in triplicate. The mean absorbance (A) obtained for each standard between the assays was divided by the absorbance value determined when no competitor (free ponazuril) was present (A0). Normalised absorbance values (A/A0) were plotted against the logarithm of the ponazuril concentration. The limit of detection of this assay, when performed in PBS, was calculated as approximately 600 pg/mL, when extrapolated from the calibration curve. The red dashed line shown in **Figure 5.9** indicates the lowest concentration of ponazuril that the antibody can detect.

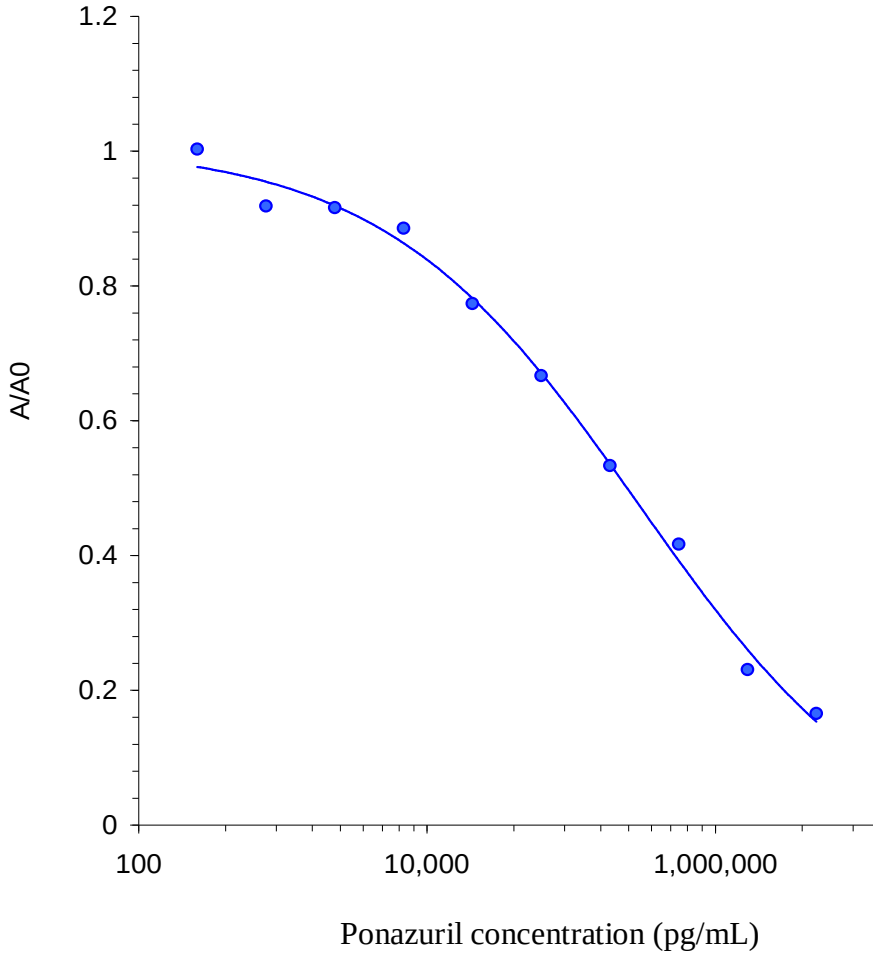


Figure 5.9 *Calibration curve for the determination of free ponazuril in solution. Ponazuril standards used in the assay were 500,000, 166,667, 555,555, 185,185, 61,728, 20,576, 6,858, 2,286, 762 and 254 pg/mL. A plate was coated with 2 µg/mL toltrazuril-BSA conjugate and blocked with 5% (w/v) Milk Marvel. The ponazuril standards were incubated with the anti-toltrazuril antibody preparation and added to the immunoplate. Bound anti-toltrazuril antibody was detected using a HRP-labelled goat-anti-rabbit IgG secondary antibody. The data is the result of triplicate measurements in three different assays performed over a three day period.*

5.3.3.1 Development of an ELISA for the detection of toltrazuril in egg samples

Organic eggs were spiked with known concentrations of ponazuril ranging from 488 pg/mL to 2.5 µg/mL. Following the extraction procedure described in *Section 2.12*, the extracted residues were incubated with an equal volume of anti-toltrazuril polyclonal antibody on a toltrazuril-BSA-coated ELISA plate. Bound toltrazuril antibody was detected following the addition of a HRP-labelled anti-rabbit IgG. The

overlaid graphs in **Figure 5.10** illustrate the effect that the egg matrix had on the calibration curve when directly compared to the curve prepared in PBS. The decrease in sensitivity of the assay can be observed by a shift in the calibration curve when the assay is performed in extracted egg samples. However, this loss of sensitivity is not statistically significant, as when the overall extraction efficiency is calculated, average losses of 14 % were incurred. This loss level is below the 20 % loss allowable for an efficient extraction procedure by E.U standards. **Table 5.1** lists the ponazuril concentrations used in the inter-day assay, and the corresponding variability and efficiency data calculated for each standard in the assay. The 'back calculated concentrations' are extrapolated from the calibration curve for each respective absorbance value, determined for the different ponazuril concentrations. This 'back calculated concentration' is subsequently expressed as a percentage of the actual ponazuril concentration to determine the 'estimated recovery'. Finally the 'actual recovery' is calculated by expressing the absorbance values obtained from the egg-extracted calibration curve as a percentage of the non-extracted PBS curve.

Table 5.1 *Inter-assay co-efficient of variation (CVs) for the detection of free ponazuril, from spiked egg samples, using the purified anti-toltrazuril antibody in a competitive ELISA format. Each standard was analysed in triplicate over three separate days and the CVs were calculated.*

Ponazuril Conc. (pg/mL)	**Inter-day (% C.V)**	**$A/A_0 \pm$ S.D**	**Back Calculated Concentration (pg/mL)**	**Estimated Recovery (%)**	**Actual Recovery (%)**
5,000,000	5.9	0.3	3,826,471	77	45
1,666,667	2.1	0.3	2,193,832	131	69
555,555	3.8	0.5	559,487	100	92
185,185	3.7	0.6	180,379	97	91
61,728	3.1	0.8	55,055	89	91
20,576	1.9	0.9	20,077	98	92
6,858	0.9	0.9	8,560	125	97
2,286	1.4	1.0	4,033	176	96
762	1.3	1.03	183	24	91
254	1.3	1.0	126	50	98

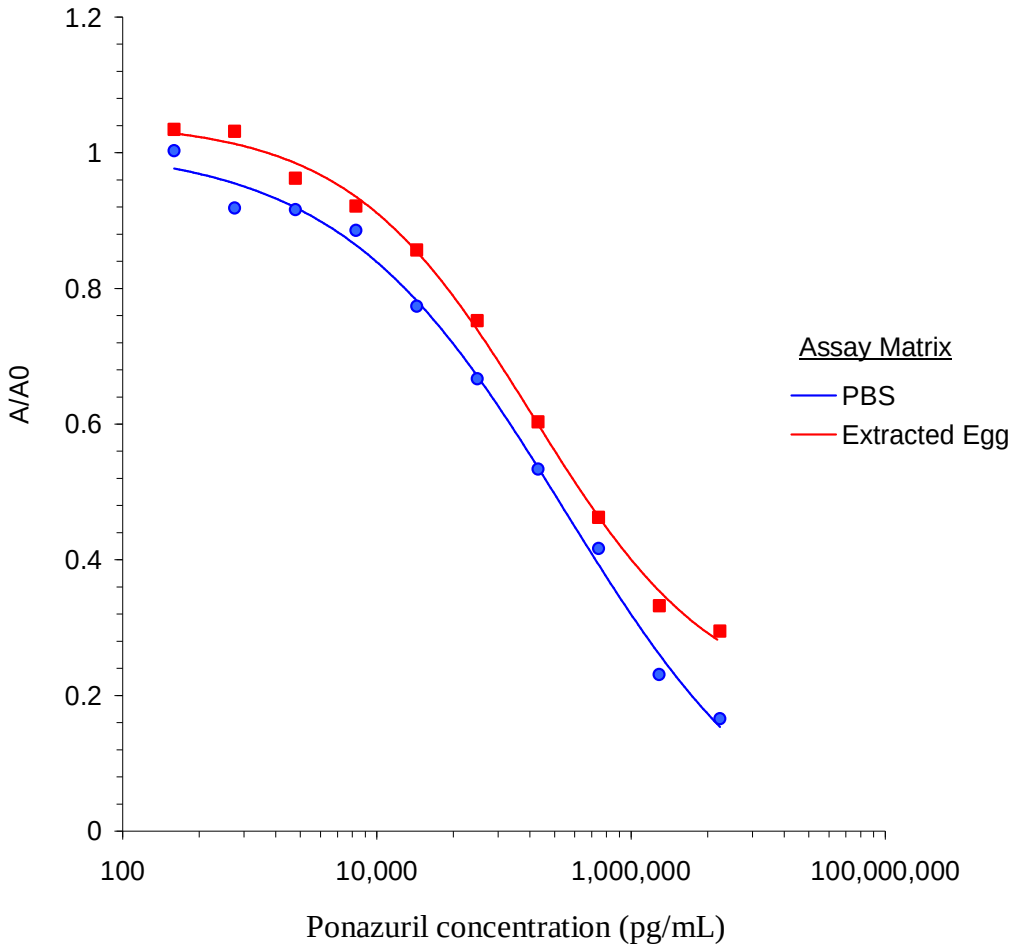


Figure 5.10 *Inter-day calibration curves comparing the determination of free ponazuril in both PBS and extracted egg samples. Ponazuril standards used were 250,000, 125,000, 62,500, 31,250, 156,25, 7812, 3906, 1953, 976 and 488 pg/mL. A plate was coated with 2 µg/mL toltrazuril-BSA conjugate and blocked with 5% (w/v) Milk Marvel. The ponazuril standards were either extracted from spiked egg samples or spiked directly into PBS and incubated with the anti-toltrazuril antibody preparation (at the optimal working dilution). Bound anti-toltrazuril antibody was detected using a HRP-labelled anti-rabbit IgG secondary antibody. This data is the result of triplicate measurements in three different assays performed over a three day period. The absorbance readings were normalised whereby the absorbance for each ponazuril concentration incubated with antibody (A) was divided by the maximum absorbance for each selected antibody without antigen (A0).*

5.3.4 Determination of analytical limits

The limit of detection (LOD) is the smallest concentration of an analyte that produces a signal which can be significantly distinguished from zero for a given sample matrix (Armbruster *et al.*, 1994). This value was determined by selecting the mean normalised absorbance minus three standard deviations for the blank standard. When extrapolated from **Figure 5.10** this was found to be approximately 900 pg/mL. The LOD can also be experimentally verified using the precision assay. This is a more accurate method to determine the LOD within a smaller range of concentrations. This is also known as the CCβ or the detection capability. It involves determining the lowest concentration which can be reliably differentiated with 95% confidence from the blank standard. The CCβ determined using this precision assay was confirmed to be 900 pg/mL. This was established by analysing 20 positive samples of concentrations flanking each side of this concentration. Twenty samples of 1,200 pg/mL, 900 pg/mL and 700 pg/mL were assayed in a competitive ELISA format, alongside 20 negative blank samples to investigate the accuracy of the assay. It can be observed in **Figure 5.11** that 900 pg/mL could be distinguished from the blank replicates in 100% of the samples. In contrast, 700pg/mL could only be distinguished from the blank 55% of the time (11/20). This assay sensitivity is far below the lowest allowable E.U. MRL for toltrazuril of 100 µg/kg (corresponding to approximately 100 ng/mL). Hence, this assay does not need to be modified further to improve sensitivity. The assay could now be successfully applied for the detection of toltrazuril in 'real' food samples.

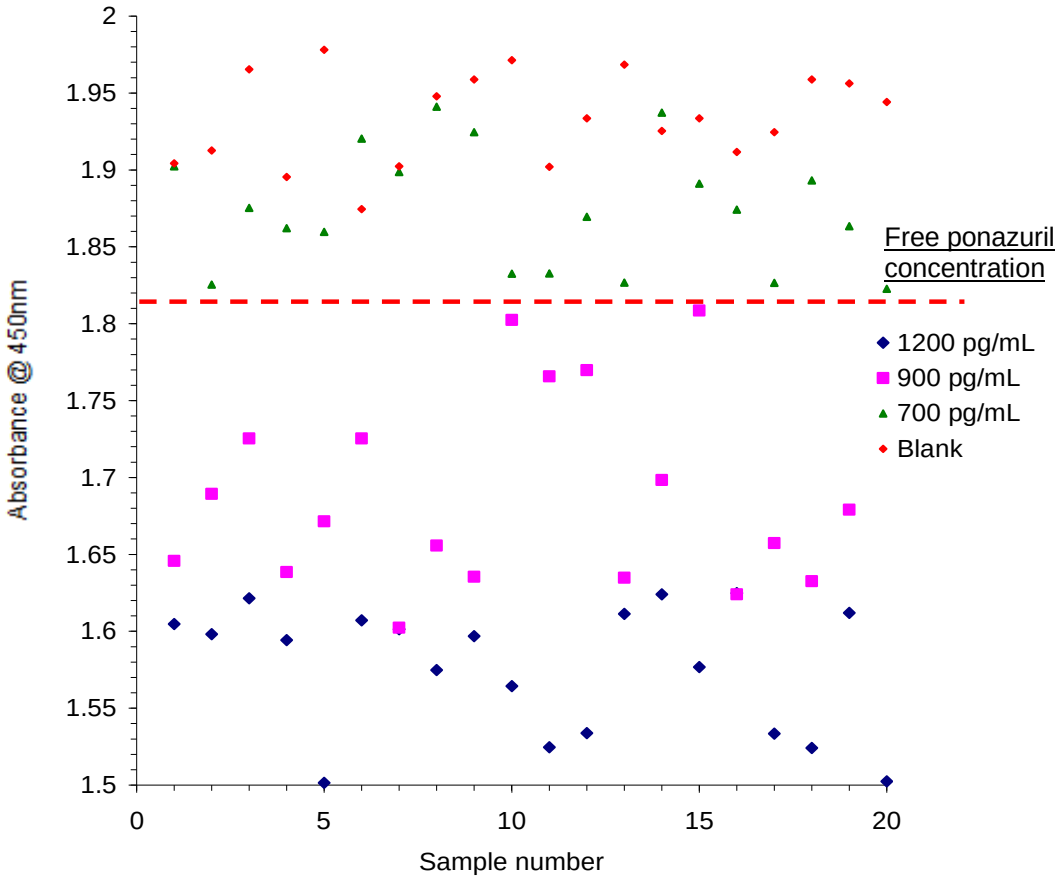


Figure 5.11 *Precision assay to experimentally verify the detection capability of the toltrazuril ELISA. Twenty replicates of 1,200 pg/mL, 900 pg/mL, and 700 pg/mL ponazuril were analysed with twenty blank samples to determine the lowest concentration of analyte that could be detected with 95% confidence. This was performed in a competitive ELISA format using anti-toltrazuril polyclonal antibodies. Bound anti-toltrazuril antibody was detected using a HRP-labelled anti-rabbit IgG secondary antibody.*

5.4 Development of a polyclonal antibody-based Biacore assay for the detection of toltrazuril

Using the polyclonal antibody previously described, a surface plasmon resonance-based assay, using the Biacore 3000^{TM} system, was optimised to detect toltrazuril.

5.4.1 Preconcentration studies for the immobilisation of toltrazuril-BSA onto the CM5 sensor chip surface

Maximizing the electrostatic attractive force between the proteins and the carboxyl surface of a CM5 Biacore chip leads to an accumulation of the proteins close to the surface. This is also known as preconcentration (Bonroy *et al.*, 2006). Preconcentration studies were performed to determine the optimum pH at which the electrostatic interactions between the toltrazuril-BSA and a CM5 sensor chip surface are most favourable for maximum binding to occur. A broad pH range was investigated using buffers with different buffering capacities. The conjugate was passed over the activated chip surface in different buffers with pH below the isoelectric point (pI) of the protein. Under these conditions, the conjugate acquires a positive charge and is effectively preconcentrated onto the negatively charged carboxymethyl dextran matrix (Jason-Moller *et al*., 2006). **Figure 5.12** demonstrates that the amount of conjugate electrostatically absorbed onto the CM dextran surface is dependant on the pH of the buffer. The pH allowing the optimum amount of conjugate to be immobilised on the surface was 4.0. This buffer was selected for the immobilisation of the conjugate onto the CM5 sensor chip.

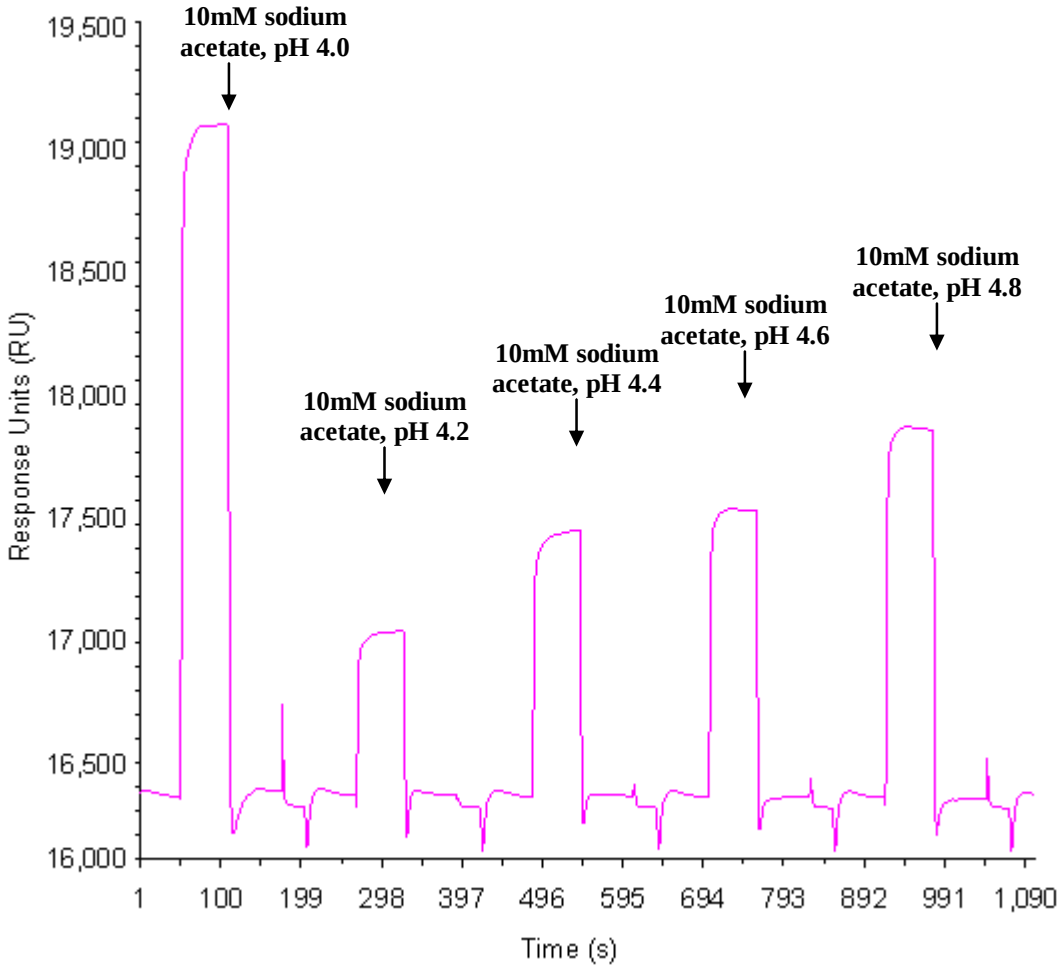


Figure 5.13 *The toltrazuril-BSA conjugate in 10mM solutions of sodium acetate, at pH levels of 4.0, 4.2, 4.4, 4.6, and 4.8, was passed over the surface of a CM5 dextran chip at a flowrate of 10 µl/minute. The optimal pH for the immobilisation of the toltrazuril-BSA conjugate to the CM5 dextran surface was pH 4.0 with the highest levels of apparent binding observed.*

5.4.2 Production of a CM5 sensor chip surface with directly immobilised toltrazuril-BSA

Toltrazuril-BSA was directly immobilised onto the surface of a CM5 chip, as described in *Section 2.11.4***,** by coupling free primary amine groups, such as lysine residues, on the conjugate. This immobilisation process can be observed in **Figure 5.14**. The carboxymethyl groups on the chip are activated by passing a mixture of EDC/NHS over the surface. In the presence of NHS, EDC converts carboxyl groups to amine-reactive sulfo-NHS esters. The generated NHS esters react with nucleophiles to release the NHS or sulfo-NHS group and to create stable amide and imide bonds

with primary or secondary amines, such as the free N-terminus and groups in lysine side chains of proteins (BSA in this case) (Kalkhof and Sinz, 2008).

In the last step, the remaining esters are transformed into amides by reaction with ethanolamine, essentially 'capping' any unreacted sites. A final level of 2,203.9 RU of covalently attached toltrazuril-BSA was achieved.

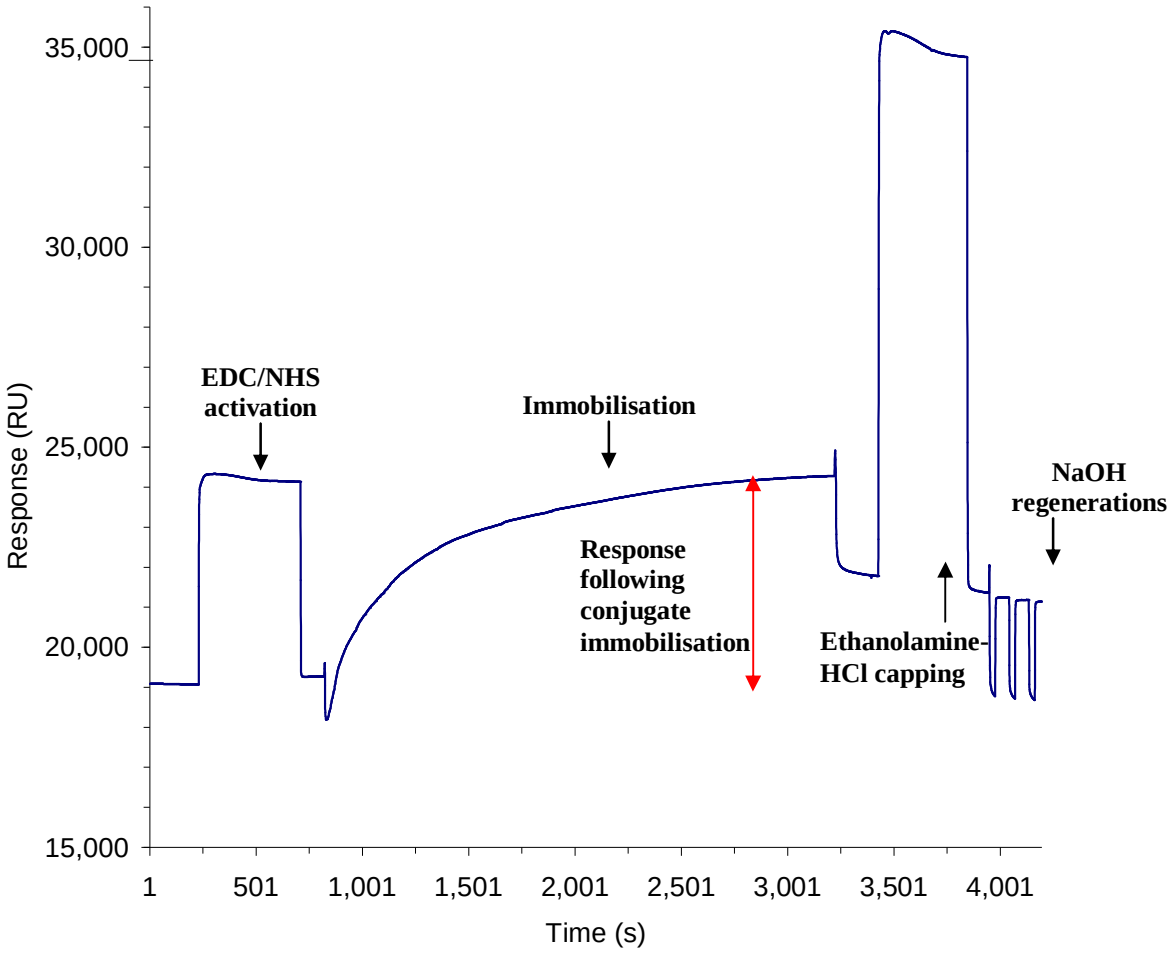


Figure 5.14 *Sensorgram illustrating the direct coupling of toltrazuril-BSA to a CM5 sensor chip surface. The surface was activated with an EDC/NHS solution, and the toltrazuril-BSA was passed over the activated carboxyl surface. Deactivation of any unbound or free functional ester groups was achieved by treating the chip surface with ethanolamine-HCl, resulting in the capping of any reactive groups. The surface was then regenerated with a solution of NaOH. A final level of 2,204 RU of covalently attached toltrazuril-BSA was achieved as illustrated by the red arrow.*

5.4.3 Binding studies on the directly immobilised toltrazuril-BSA surface.

Different concentrations of polyclonal anti-toltrazuril antibody ranging from 20 to 100 µg/mL were prepared in egg matrix (as described in *Section 2.12*) and tested for binding to the surface of the toltrazuril-BSA-immobilised chip. These solutions were each injected over the chip for 2 minutes at 10 µL/minute. The specific binding was recorded, and the optimal antibody concentration was chosen by comparing response units with antibody concentration. It can be observed in **Figure 5.15** that the antibody concentration of 100 µg/mL was the optimal concentration of antibody for use in subsequent assays, with the highest RU observed when analysed for binding in egg matrix. This 250 RU level is high enough to be able to distinguish small decreases in binding when a competitor is present.

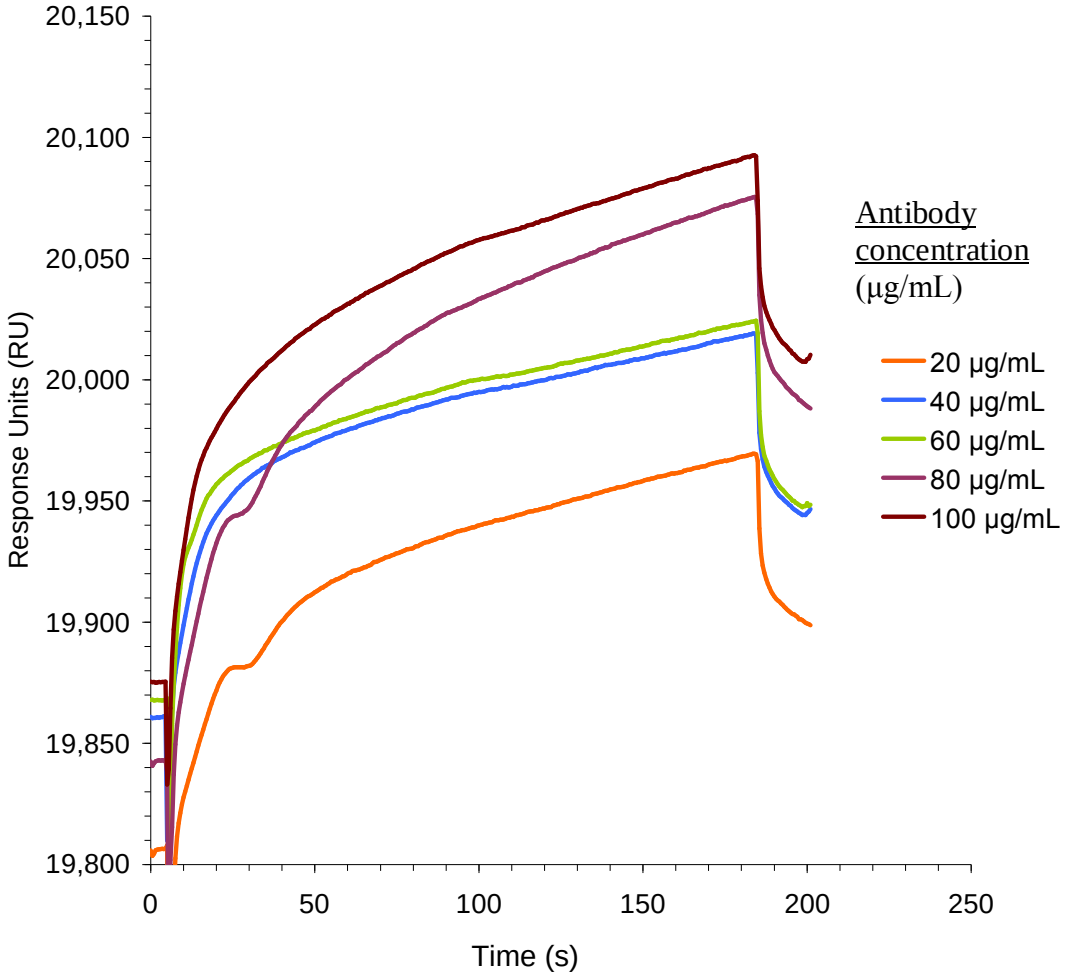


Figure 5.15 *Overlay sensorgram plot illustrating the binding of the polyclonal anti-toltrazuril antibody to the immobilised toltrazuril-BSA surface in an egg matrix. Concentrations of 20, 40, 60, 80 and 100 µg/mL of anti-toltrazuril antibody were passed over the surface of the toltrazuril-immobilised CM5 sensor chip, for 2 minutes at a flowrate of 10 µL/minute and specific binding was recorded.*

5.5 Preliminary analysis of a Biacore inhibition assay format for ponazuril-binding in PBS

A preliminary competitive assay was carried out in PBS to determine the range of toltrazuril-detection of the polyclonal anti-toltrazuril antibody in a Biacore competitive assay format. Concentrations of ponazuril from 254 pg/mL to 5 µg/mL were mixed with an equal volume of antibody (100 µg/mL) and passed over the toltrazuril-BSA immobilised chip-surface. Inter- and intra-assay variation studies (as outlined previously in *Section 5.3.3*) were performed with each standard injected in

triplicate at a flow-rate of 10 µL/minute for 2 minutes. The amount of response units observed was recorded after each injection. To assess the assay precision, CV values for each standard were also determined. These are summarized in Table 5.2. The mean response obtained at each ponazuril concentration (R) was divided by the response determined in the presence of zero drug (R0). The normalised responses (R/R0) were plotted against the logarithm of ponazuril concentration using BIAevaluation 4.0.1 software and a four-parameter equation fitted to the data. **Figure 5.16** shows the inter-assay (mean results of three separate assays) calibration curve using the anti-toltrazuril-polyclonal antibody. The LOD (as outlined in *Section 5.3.4*) of the assay was found to be 890 pg/mL, when extrapolated from the calibration curve. The red dashed line shown in **Figure 5.16** indicates the lowest distinguishable concentration of ponazuril the antibody can detect in the Biacore assay format. However, for this assay to be a 'validated' toltrazuril detection assay, it has to be performed in an egg matrix.

Table 5.2 *Inter-assay co-efficient of variation (CVs) for the detection of free ponazuril in PBS using the purified anti-toltrazuril antibody in a Biacore assay format. Each standard was analysed in triplicate over three separate days and the CVs calculated.*

Ponazuril Conc. (pg/mL)	**Inter-day (% C.V)**	**R/R_0**	**Back Calculated Concentration (pg/mL)**	**% Recovery**
5,000,000	13	26	5,762,608	115
1,666,667	14	37	1,737,825	104
555,555	10	58	504,765	91
185,185	3	86	178,085	96
61,728	5	119	65,213	106
20,576	6	155	21,660	105
6,858	5	184	7,091	103
2,286	3	208	1,517	66
762	1	216	590	77
254	1	216	595	234

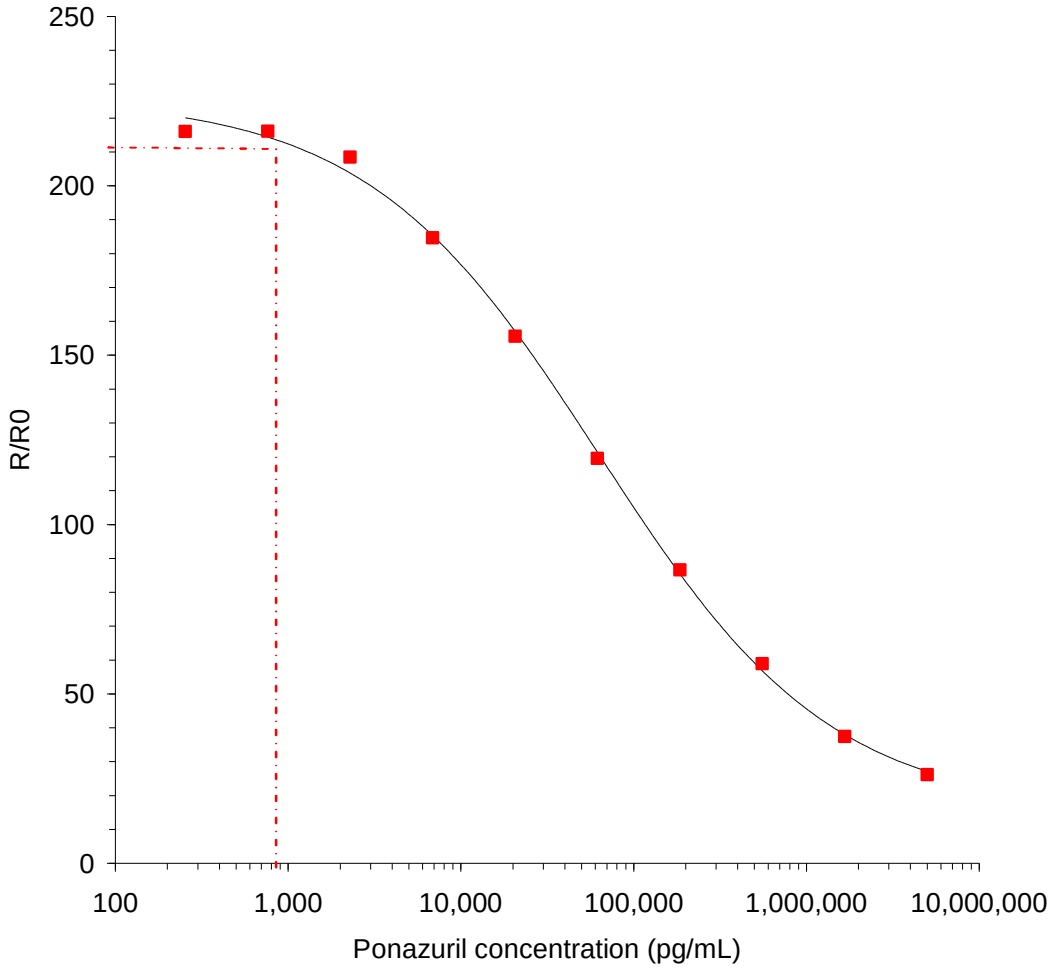


Figure 5.16 *Calibration curves for the detection of ponazuril in PBS. Three replicates of each standard were analysed in three separate assays. Toltrazuril-BSA was directly immobilised onto the surface of a CM5 sensor-chip. The binding response at each ponazuril concentration was divided by the antibody response in the absence of free ponazuril, to give the normalised response. The detection limit of the assay was determined to be approximately 890 pg/mL, as extrapolated from the calibration curve, and indicated by the red dashed line.*

5.5.1 Stability of the immobilised toltrazuril-BSA surface after multiple sample/regeneration cycles.

Repeated use of the immobilised-sensor chip surface is a key aspect of a successful biosensor immunoassay. The sensor chip surface requires re-conditioning after each sample analysis, hence, it is imperative that the antigen-reactivity and conformation remains unaltered (Mason *et al.*, 2003). To study the integrity of the biosensor surface, the baseline stability was monitored following 50 consecutive binding/regeneration cycles. Egg matrix was extracted as previously outlined in *Section 2.12*. Antibody (100 µg/mL) was added to the egg matrix and passed over the surface of the toltrazuril-BSA-immobilised chip, at a flow rate of 10 µL/minute for 2 minutes. The chip surface was regenerated with two 30-second pulses of 100 mM NaOH. Following fifty consecutive binding/regeneration cycles, no significant decrease in the baseline stability occurred (**Figure 5.17**) with only a 3.6 % variation in binding response over all 50 cycles.

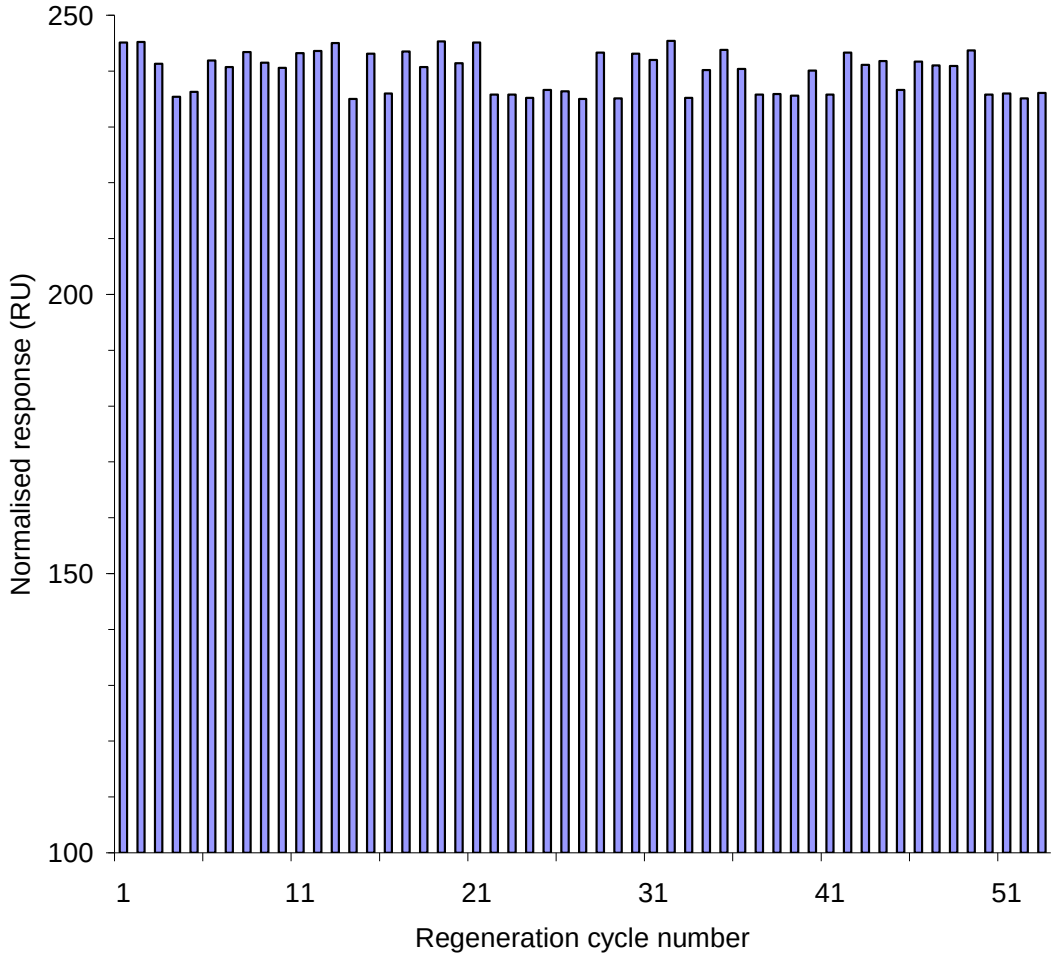


Figure 5.17 *Typical regeneration profile for a CM5 chip immobilised with toltrazuril-BSA. For 50 consecutive binding-regeneration cycles, a 2 minute pulse was performed, passing purified polyclonal antibody, in egg extract, over the chip surface. Antibody, at a concentration of 100 μg/mL, was used and the surface regenerated with two 30 second pulses of 100 mM NaOH after binding. The binding response stayed constant after 50 cycles with a 3.6 % variation in binding response, indicating high stability and reproducibility.*

5.6 Analysis of Biacore-based toltrazuril-detection assay performed in egg matrices.

Egg samples were spiked with known concentrations of ponazuril ranging from 5 μg/mL to 254 pg/mL and subsequently extracted. These standards were mixed with an equal volume of antibody at the final optimal concentration of 100 μg/mL and passed over the toltrazuril-BSA immobilised chip-surface. Samples were incubated for 30 minutes at room temperature before they were each injected over the toltrazuril-BSA

immobilised surface. Each standard was injected in triplicate at a flow-rate of 10 µL/minute for 2 minutes. The quantity of response units observed was recorded after each injection. Inter- and Intra-assay variation studies were performed and CV values for each standard were determined (**Table 5.3**). The normalised responses were calculated and a four-parameter equation was fitted to the data curve. **Figure 5.18** shows the inter-assay mean calibration curve in extracted egg matrix using the toltrazuril-polyclonal antibody. The red dashed line in **Figure 5.18** indicates the lowest level of ponazuril detectable in this matrix. The LOD of the assay in egg samples was found to be approximately 1,920 pg/mL as extrapolated from the calibration curve.

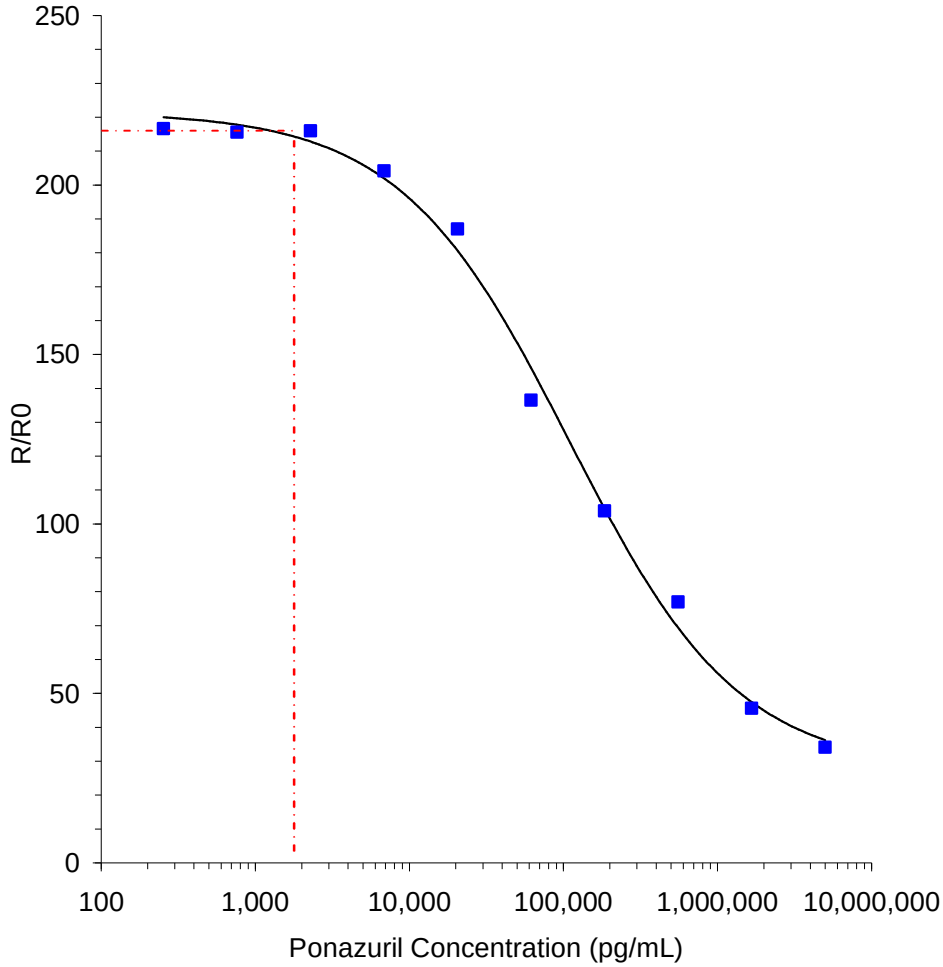


Figure 5.18 *Calibration curves for the detection of ponazuril in extracted egg samples. Replicates (3) of each standard were analysed in three separate assays. The binding response at each ponazuril concentration was divided by the antibody response in the absence of free ponazuril, to give the normalised response. The LOD was determined to be 1,920 pg/mL. The results are shown as R/R0, where the*

response units of the evaluated samples (R), are normalised by expressing them as a function of the blank standard (R0).

5.6.1 Evaluation of extraction efficiency from egg samples

Eggs from organically treated hens were spiked with ponazuril post-extraction (i.e following recovery of residues from the egg matrix) and compared to standards spiked in eggs prior to extraction, to assess the efficiency of the procedure. **Figure 5.19** shows that the average extraction efficiency from the eggs was quite high at 93 %. The actual recoveries, outlined in **Table 5.3,** correspond to the percentage of the total ponazuril concentration efficiently extracted from each of the spiked standards. Recoveries can also be estimated from the calibration curve for each standard. Using this method, the mean percentage recovery was calculated as 139%, as shown in the recoveries listed in **Table 5.3.** This 'estimated' recovery from the calibration curve suggests that a higher amount of ponazuril was present in the samples than that which was actually spiked, as the value is greater than 100%. The reason the curve indicates the presence of higher concentrations of ponazuril, is that fewer antibodies are binding to the surface of the chip, which indicates that more free drug is displacing the antibody than is actually present. However, as can be observed from **Table 5.2,** when the samples are analysed is PBS, the average recovery calculated from the calibration curve is at 110%. This indicates only a 10% overall deviation from the actual concentrations. Taking into account the 7% loss observed during extraction, when analysis is performed in standards extracted from egg matrix, there is a 32% deviation from the apparent concentrations. This is assumed to be due to matrix effects from the extracted egg samples interfering with the assay.

When comparing the calibration curve of the spiked ponazuril in extracted egg samples (**Figure 5.19**) to the calibration curve in PBS, it can be observed that a 2.2-fold loss in sensitivity of the assay can be attributed to matrix interference and losses incurred during the extraction procedure. Indyk and Filonzi (2004) observed that it is common practice to generate calibration curves for the detection of contaminants in analyte-free matrix. This can be observed in **Figure 5.19,** whereby a calibration curve performed on egg samples spiked with ponazuril post extraction is directly compared to the calibration curve in PBS. The influence of the matrix and the extraction effects can also be seen in **Figure 5.19.**

Table 5.3 *Inter-assay co-efficient of variation (CVs) for the detection of free ponazuril in PBS, using the purified anti-toltrazuril antibody, in a Biacore assay format. Each standard was analysed in triplicate over three separate days and the CVs calculated.*

Ponazuril Conc. (pg/mL)	**Inter-day (% C.V)**	**R/R_0**	**Back Calculated Concentration (pg/mL)**	**Estimated Recovery (%)**	**Actual Recovery (%)**
5,000,000	18	34	7,129,648	143	99
1,666,667	6	46	1,899,391	114	100
555,555	7	77	421,740	76	99
185,185	2	104	188,097	102	93
61,728	5	137	79,537	129	89
20,576	1	187	15,718	76	93
6,858	2	204	5,800	85	85
2,286	2	216	1,261	55	90
762	2	217	428	56	89
254	2	216	162	63	88

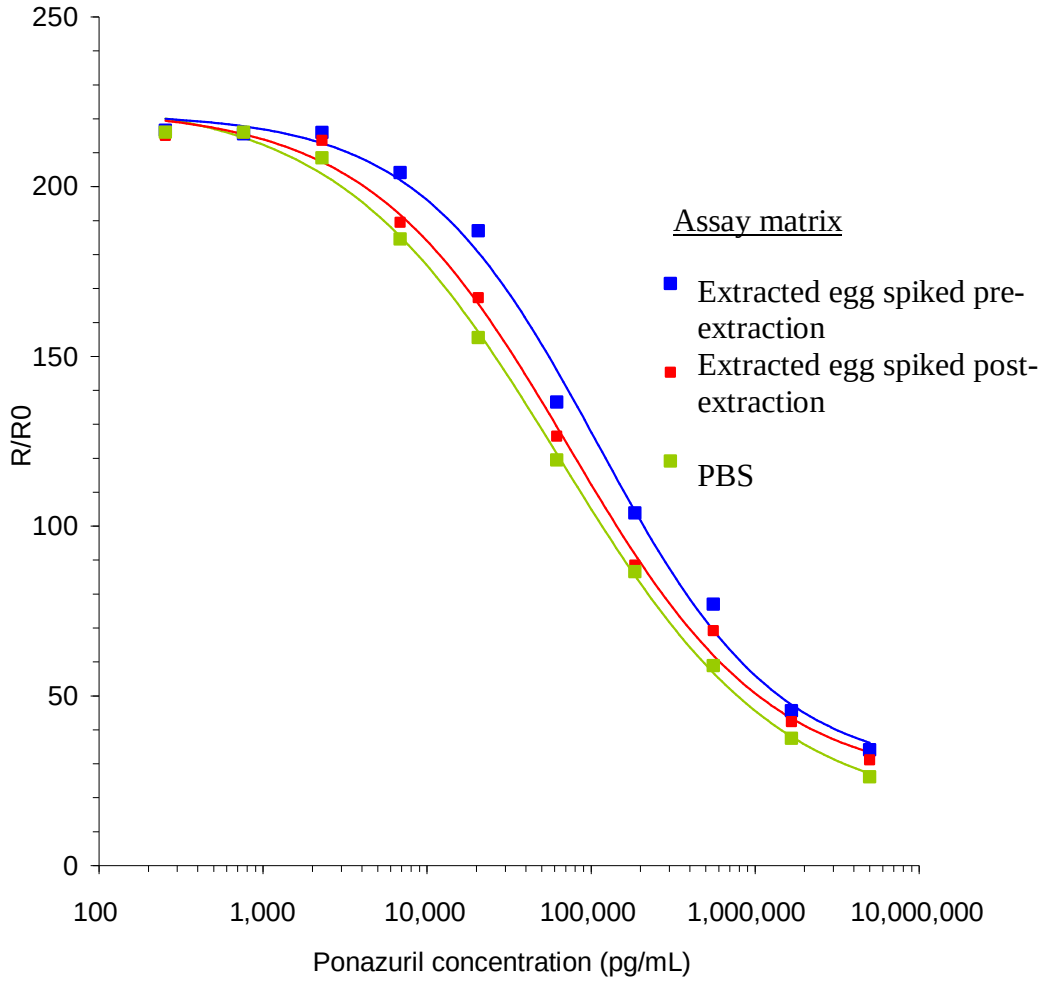


Figure 5.19 *Comparison of the inter-assay calibration curves for the detection of ponazuril in PBS, extracted egg spiked with concentrations of ponazuril prior to extraction, and extracted egg spiked with concentrations of ponazuril post-extraction. The influence the matrix has on the sensitivity of the assay can be observed by the shift of the curve between the 'green' PBS calibration curve, and the 'red' egg matrix calibration curve. The efficiency of the extraction procedure is shown by comparing both the pre-spiked 'blue' and post-spiked 'red' calibration curves.*

In order to experimentally verify the detection capability (CCβ) of the Biacore assay from extracted egg samples, a precision assay was performed. Twenty samples of concentrations, 2,000, 1,900, 1,500, 1,000, 500, and 0 pg/mL were spiked into egg samples and extracted, as previously described in *Section 2.12*. Each of the samples was incubated with an equal volume of (100 µg/mL) anti-toltrazuril polyclonal antibody and passed over the surface the toltrazuril-BSA-immobilised CM5 chip. The response units observed were recorded for each replicate. It can be deduced from

Figure 5.20 that 1,500 pg/mL of ponazuril can be successfully distinguished from blank samples. This concentration of 1,500 pg/mL of ponazuril represents the lowest concentration of analyte capable of being detected with a 95% confidence level using the anti-toltrazuril antibody in a Biacore-based assay format.

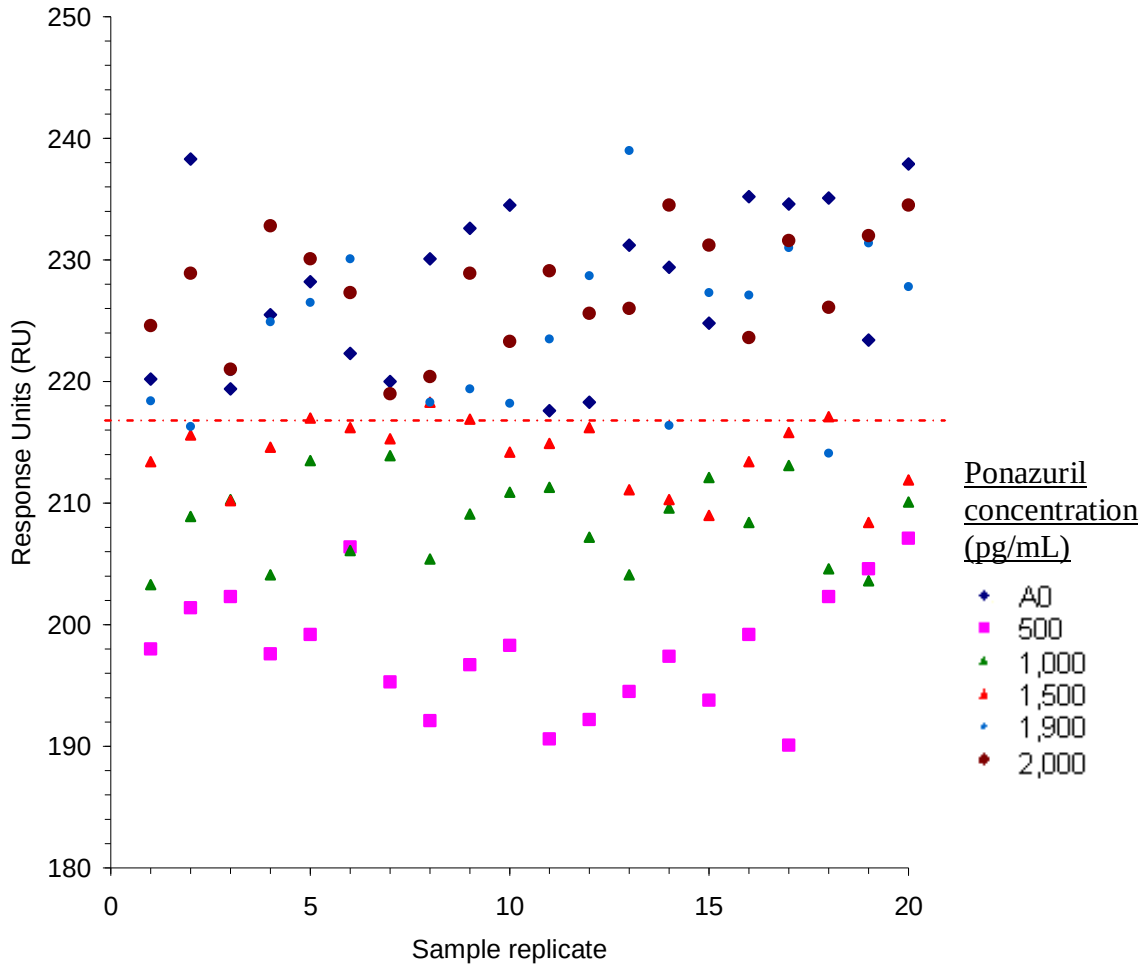


Figure 5.20 *Precision assays to experimentally verify the functional limit of detection of toltrazuril in a Biacore assay format. Twenty replicates of 2,000, 1,900, 1,500, 1,000, 500 and 0 pg/mL of ponazuril were analysed with twenty blank samples to determine the lowest concentration of analyte that can be detected with 95% confidence level. This was performed in a competitive Biacore format using the anti-toltrazuril polyclonal antibody. Bound anti-toltrazuril antibody was recorded in response units (RU).*

5.7 Development of a lateral-flow based immunoassay using the anti-toltrazuril polyclonal antibody for the detection of toltrazuril

In an effort to reduce the assay time for the detection of ponazuril and circumvent the time-consuming washing and incubation steps of the ELISA-based approach, a lateral-flow immunoassay was developed for the rapid detection of ponazuril using the anti-toltrazuril polyclonal antibody. This method also has several field advantages when comparing to ELISA and Biacore-based methods, in that no specially trained personnel are required to perform the assay and the assay can be easily performed outside of the laboratory.

Lateral-flow immunoassays (LFIAs) have been employed for the detection of several medicinal veterinary contaminants, including nicarbazin in poultry feed (Campbell *et al.*, 2007) and sulfonamides in eggs and chicken muscle (Kandimalla *et al.*, 2007; Guo *et al.*, 2010).

In lateral flow devices, the principal element is the movement of a liquid sample, or an extract containing the analyte of interest, along a strip of polymeric material where it can interact with immobilised molecules (Posthuma-Trumpie *et al.*, 2009). A typical lateral flow format (**Figure 5.21**) consists of a surface layer to carry the sample from the sample application pad through the conjugate release pad, along the strip and the detection zone up to the absorbent pad (Posthuma-Trumpie *et al.*, 2009). The chosen format for a lateral flow immunoassay depends on the analyte being detected. For the detection of toltrazuril, the format is restricted to a competitive assay due to the small size and the lack of more than one epitope on the hapten. The optimal assay format for use with small haptens would contain either a labelled-antigen or labelled-antibody, whereby sample analyte competes with immobilised or labelled antigen for binding to the antibody. Generally, labels used in lateral-flow are made of coloured or fluorescent labels or, more recently, nanoparticles with sizes of 15–800 nm, allowing an unobstructed flow through the membrane (Posthuma-Trumpie *et al.*, 2009). Following preliminary studies and assay optimisation using a HRP-labelled secondary antibody and a colorimetric substrate, the assay was performed with coloured polystyrene beads.

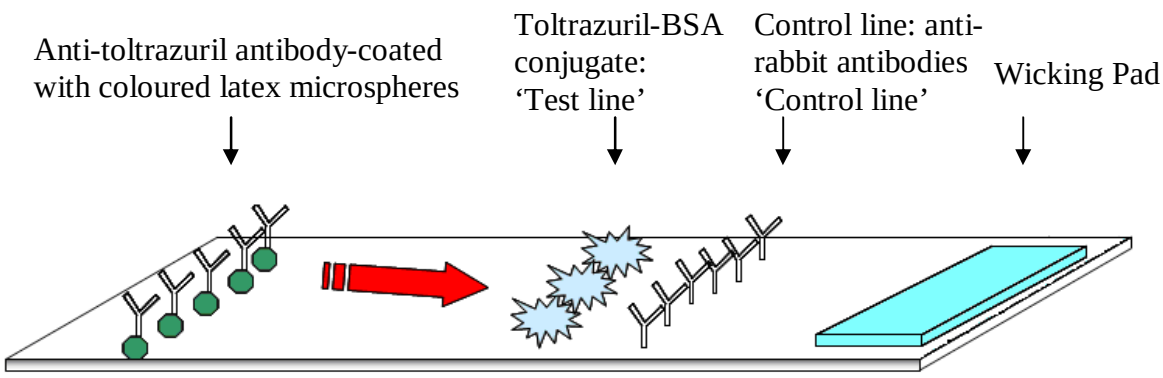


Figure 5.21. *Schematic of a potential lateral flow assay for the detection of a hapten, e.g. toltrazuril. Using anti-toltrazuril-coated-coloured latex microspheres, visualisation of bound antibody occurs at the 'test'-Toltrazuril-BSA conjugate-coated line, or the 'control'- anti-rabbit IgG-coated line. Sample travels through the nitrocellulose membrane with the assistance of an absorbent 'wicking pad' positioned at the top of the membrane.*

5.7.1 Determination of the range of detection of toltrazuril in PBS using a LFIA inhibition assay format

Initially a preliminary lateral-flow assay was performed for toltrazuril detection to determine the optimal concentration of toltrazuril-BSA for use in the assay. A strip of Hi-Flow plus nitrocellulose membrane was cut to specific dimensions (4cm x 1cm). Anti-rabbit IgG was used as a control line. Varying concentrations of toltrazuril-BSA were sprayed as the test lines. The buffers used for spotting samples onto the test strips and running the assay were; 50 mM sodium phosphate, pH 7.5/ 1% (w/v) trehalose, and 50mM sodium phosphate, pH 7.5/0.5% (v/v) Tween20. Anti-toltrazuril antibody was diluted in running buffer and allowed to travel up the strip by capillary action. The antibody concentration for use in the assay was estimated from previous ELISA results. The strip was washed by the addition of running buffer and HRP-labelled anti-rabbit IgG was used to probe for bound toltrazuril-antibody. Following the addition of the chromogenic substrate, TMB, a colour develops at the test line, where anti-toltrazuril antibody is bound. The completed assay can be observed in **Figure 5.22**. A slight colour is observed in the test line in A (Blank), which may be attributed to non-specific binding of the antibodies when travelling up the membrane

as no conjugate was sprayed onto the strip. Kaur *et al.* (2007) previously noted that because of the hydrophobicity of the nitrocellulose membrane, non-specific interaction of a hydrophobic analyte with the membrane can generate significant non-specific signals. To circumvent this, 0.1% (w/v) Milk Marvel was included in all subsequent assays. In **Figure 5.22** the intensity of the test line is only visible between the concentrations of 10 µg/mL and 100 µg/mL of ponazuril. From this, 50 µg/mL of toltrazuril-BSA was chosen as the optimal test-line spotting concentration for all further assays. Validation of the assay was performed through inter-assay studies with 'real' samples. This would enable the detection limit of the assay to be verified.

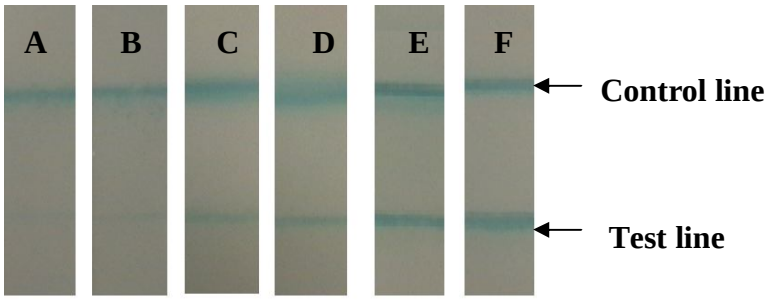
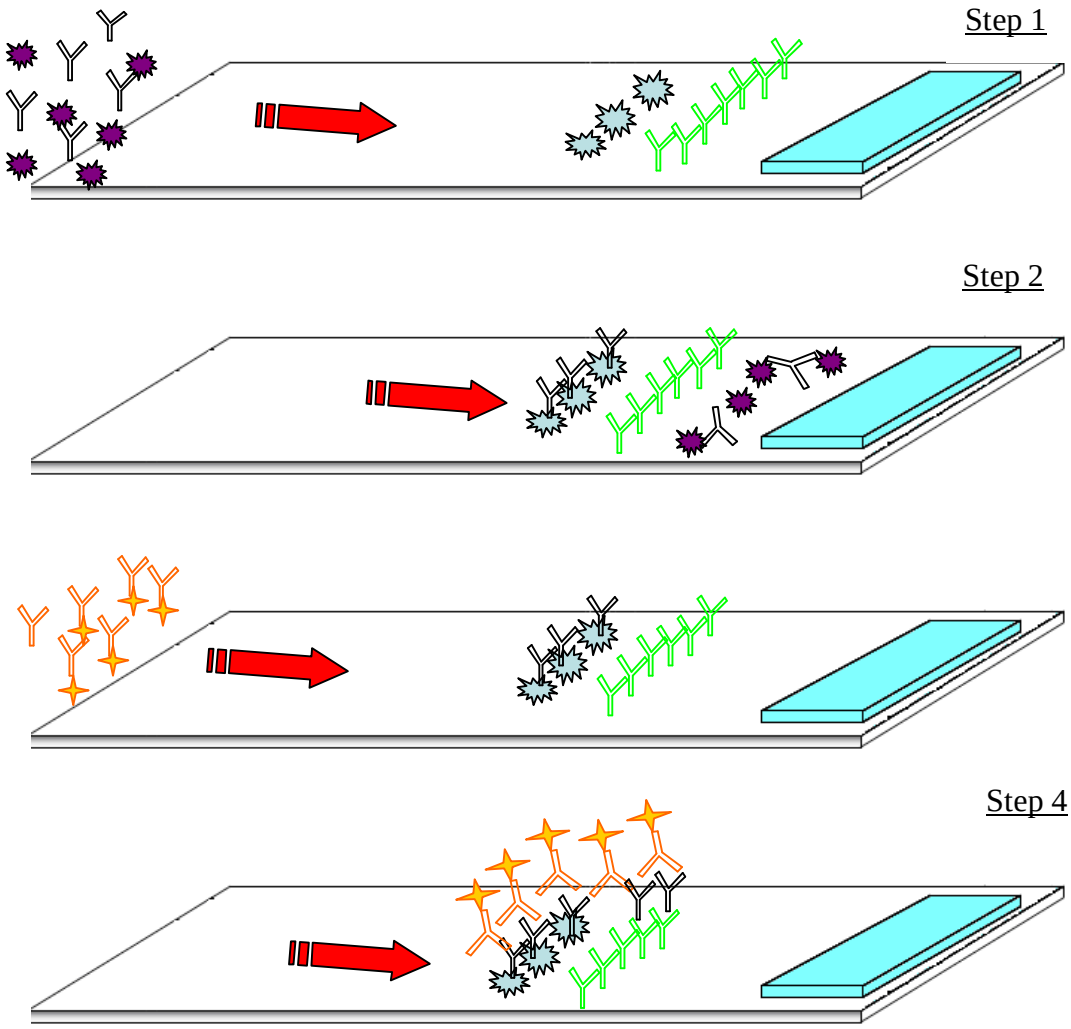


Figure 5.22 *TMB-developed nitrocellulose membrane strips from the toltrazuril-lateral-flow detection assay. Anti-rabbit IgG was used as the control line and toltrazuril-BSA as the test line, at concentrations of 0, 5, 10, 20, 50 and 100 µg/mL. Anti-toltrazuril antibody was allowed to travel up the strip. Bound antibody was detected following the addition of a HRP-labelled anti-rabbit IgG. Toltrazuril-BSA concentrations A-F listed above are as follows; A= 0 µg/mL; B= 5 µg/mL; C= 10 µg/mL; D= 20 µg/mL; E= 50 µg/mL and F= 100 µg/mL.*

5.7.2 Analysis of lateral-flow based assay for the detection of toltrazuril in an egg matrix.

In order to validate the lateral-flow assay and determine the detection capability (CCβ) for toltrazuril, a validated assay was prepared and tested in egg samples. Egg samples were spiked with 100, 80, 40, 20, 10, 5 and 0 ng/mL of ponazuril and subsequently extracted as previously outlined in *Section 2.12*. It was initially believed that methanol in the extraction buffer would interfere with the nitrocellulose membrane. However, 5% (v/v) methanol had no apparent effect on the nitrocellulose. It was previously determined by Wang and colleagues (2005) that standard nitrocellulose can tolerate up to ten percent methanol without damage.

Inter-assay studies were performed in triplicate over three days. A schematic of the lateral flow immunoassay set-up can be observed in **Figure 5.23**.



Key: Anti-toltrazuril antibody, Free toltrazuril, Toltrazuril-BSA conjugate, Anti-rabbit polyclonal antibody, HRP-labelled anti-rabbit Ig antibody

Figure 5.23 *Schematic of a lateral flow immunoassay for the detection of toltrazuril using HRP-labelled anti-rabbit IgG secondary antibody and TMB substrate for visualisation of binding. Step 1: Toltrazuril-BSA conjugate is sprayed onto a nitrocellulose membrane as the 'test-line', anti-rabbit antibody was sprayed onto the nitrocellulose as the 'control line'. Toltrazuril-specific antibody was incubated with free toltrazuril and the mixture was allowed to travel up the length of the nitrocellulose membrane. Step 2: Free ponazuril in the sample will occupy the*

binding sites of the antibody leaving it unable to react with the toltrazuril on the nitrocellulose; hence the amount of ponazuril in the sample is inversely proportional to the amount of binding that should be visible. Step 3: Toltrazuril-specific antibody bound on the nitrocellulose membrane is detected by the addition of a HRP-labelled anti-rabbit IgG secondary antibody. Step 4: TMB substrate is then added to the strip which reacts with the HRP-conjugated anti-rabbit IgG antibody, thereby allowing visualisation of bound antibody at both the test and control lines.

Goat-Anti-rabbit IgG was sprayed on the nitrocellulose membrane denoting the 'control' line and toltrazuril-BSA was sprayed as the test line. Anti-toltrazuril antibody was incubated with equal volumes of each of the spiked egg samples. Each of the mixtures was allowed to travel up specified strips of nitrocellulose. Any toltrazuril-specific antibodies that were not displaced by free ponazuril would bind to the test line, and the free drug-antibody complex would bind to the control line. The membrane was washed 3 times with running buffer prior to probing the strip with HRP-labelled anti-rabbit IgG antibody. Following this, the washing cycle was repeated and TMB was allowed to migrate up the strip to visualize the presence of bound-HRP-labelled anti-rabbit IgG antibody. The three inter-assay LFIAs can be observed in **Figure 5.24.** Detection of ponazuril is clearly evident down to a concentration of approximately 40 ng/mL across all of the inter-assay results. However, as this assay is purely qualitative, a positive result can only be confirmed with 100% confidence by the complete absence of a test line. Hence, it can only be stated that the LFIA has a detection capability of 100 ng/mL, as this is the only ponazuril concentration whereby a positive test-line is eliminated, due to the high concentration of ponazuril occupying the antibody binding sites and inhibiting binding on the surface of the nitrocellulose.

The detection capability of the previous lateral flow assays was determined solely by judging visually the control intensity of bands generated (Wang *et al.*, 2005). When excess ponazuril is present, the ponazuril occupies any free antibody binding sites and inhibits the accumulation of the antibody at the toltrazuril-BSA test line. This ponazuril concentration was determined from the inter-assay LFIAs to be 100 ng/mL. Any ponazuril concentration lower than 100 ng/mL would only occupy a certain limited amount of binding sites on the anti-toltrazuril antibodies, leaving other binding sites free to bind to the 'test line'. This results in a visible coloured band after

the addition of the HRP-labelled anti-rabbit IgG and TMB substrate. It can be concluded that the detection capability of the anti-toltrazuril polyclonal antibody in an LFIA is 100 ng/mL of ponazuril. If a hand-held reader was available to determine the exact colour intensity of the bands, a more quantitative method of determining the analytical range of the assay would be achievable. However, as no such instrument was available, a visual evaluation of the detection limits of the assay had to suffice.

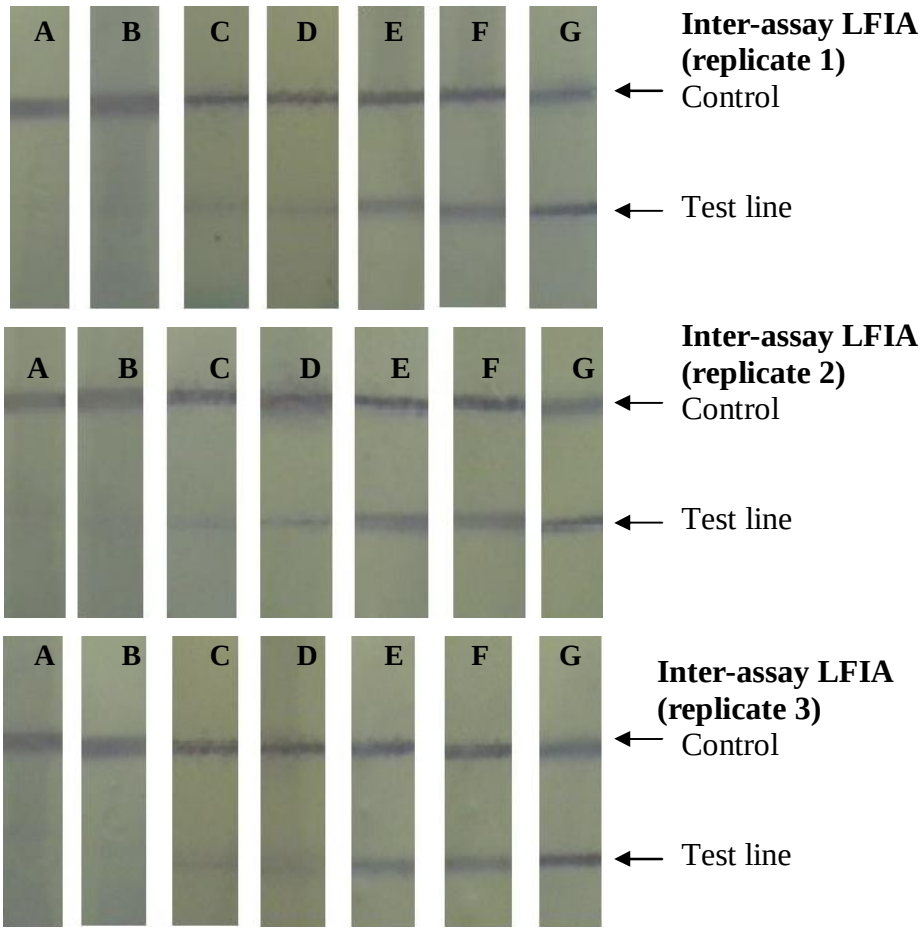


Figure 5.24 *Inter-assay LFIAs for the detection of ponazuril from spiked egg samples using the anti-toltrazuril polyclonal antibody. Anti-rabbit IgG was used as a control line and toltrazuril-BSA conjugate was used as the test line. Ponazuril concentrations of 0, 5, 10, 20, 40, 80, 100 ng/mL were spiked into egg samples prior to extraction. An equal volume of 10 μg/mL of anti-toltrazuril antibody was mixed with the ponazuril samples following extraction. The free ponazuril-toltrazuril antibody mix was applied to the bottom of the nitrocellulose strip and allowed to travel up to the detection zones. Bound antibody was detected by probing with HRP-labelled anti-rabbit IgG antibody. Following the addition of TMB substrate, a colour develops at the control/test lines. Ponazuril concentrations A-G listed above are as follows; A= 100*

ng/mL; B= 80 ng/mL; C= 40 ng/mL; D= 20 ng/mL; E= 10 ng/mL; F= 5 ng/mL and G= 0 ng/mL.

5.8 Generation of a multi-analyte assay for the detection of halofuginone, toltrazuril and diclazuril

Effective assays for the rapid detection of multiple contaminants are in great demand for food quality monitoring.

In order to generate a quick and efficient assay, amenable to the simultaneous detection of halofuginone, toltrazuril and diclazuril, a lateral-flow based platform was developed. This one-step, qualitative, lateral flow-immunoassay offers several advantages over other multi-analyte detection systems, including a simple yes/no response which can be interpreted visually and reduce time and cost per analysis. The simple assay protocol also means that the final assay procedure can be completed in less than 5 minutes.

When applied to spiked egg samples, the assay gives accurate and reproducible results for detecting all three anti-protozoan drug residues. Hence, this assay is a highly attractive 'on-site' screening technique for the simultaneous detection of anti-protozoan drug residues.

5.8.1 Covalent coupling of anti-toltrazuril/halofuginone/diclazuril antibodies to polybead-coloured carboxylated polystyrene microspheres, for use in lateral-flow immunoassays

In order to visualise antibody binding on the nitrocellulose membrane and facilitate a one-step assay, coloured carboxylated polystyrene microspheres were coupled to the antibodies. The beads are 0.2 μm in diameter, which allows them to easily traverse through the nitrocellulose membrane. In this format, the assay time is accelerated as there is no requirement for the addition of substrate. The protocol used for covalent coupling is outlined in *Section 2.15.3.2.* The coupling was performed overnight using carbodiimide hydrochloride (EDC). Any remaining unreacted carbodiimide sites were blocked by the addition of ethanolamine. To determine whether the antibodies were coupled to the polystyrene microparticles, and ensure functionality of the assay in this format, preliminary lateral-flow test assays were performed.

Individual secondary antibodies specific to each of the anti-halofuginone/diclazuril and toltrazuril primary antibodies, were employed to capture the primary antibody-

carboxylated microsphere complexes. Anti-HA mAb was used to capture the anti-halofuginone-microsphere complex, goat-anti-rabbit IgG to capture the anti-toltrazuril complex and rabbit-anti-goat IgG to capture the anti-diclazuril complex. Each of the secondary antibodies was sprayed on separate nitrocellulose strips and each of the antibody-microsphere beads were sprayed along the bottom of the strip. The antibody-microsphere mixture was then sprayed on the bottom of each of the corresponding 'secondary antibody-embedded' strips i.e. the anti-toltrazuril antibody-microsphere complex was sprayed on the strip embedded with anti-rabbit IgG. The strips were then dipped in a borate buffer (the same as the storage buffer used for the microsphere complexes) with 0.05% (v/v) Tween, which is a routinely used buffer in lateral-flow assays. The buffer could be observed travelling up the strip via capillary action. However, no movement of the green microspheres could be observed up the strip. In order to overcome this, several components of the assay procedure were optimised focusing on the spotting and running buffers utilised in the assay.

5.8.1.1 Running and spotting buffer optimisation for use in a multi-analyte lateral-flow assay

Prior to optimising the final multi-analyte assay, each of the antibody-microsphere complexes were sprayed on separate strips with each corresponding conjugate embedded on the strip. To facilitate the flow of the antibody-microsphere up the membrane, several different spotting and running buffers were used. The buffers studied for spotting of the antibody complex onto the membrane were; 2 mM borate buffer, pH 7.0, (0.1% (w/v) BSA, 0.05% (v/v) Tween, 0.01% (v/v) Triton X), 50 mM sodium phosphate, pH 7.5, with 1% (w/v) trehalose and 15 mM sodium citrate, pH 7.0, with 0.01% (v/v) Triton X. These three spotting buffers were then used in conjunction with three different running buffers (2 mM borate buffer, pH 7.0, with 0.05% (v/v) Tween, 50 mM sodium phosphate, pH 7.5, with 0.05% (v/v) Tween and 15 mM sodium citrate, pH 7.0, with 0.05% (v/v) Tween), to determine which buffer combination gave the greatest resolution of the antibody-microsphere complex through the membrane, and the least amount of non-specific interactions. The results of this are not shown because only one buffer combination induced any movement of the antibody-bead complex up the membrane. When 15 mM sodium citrate, pH 7.0, with 0.01% (v/v) Triton X was employed alongside the 15 mM sodium citrate, pH 7.0, with 0.05% (v/v) Tween running buffer, partial movement of the antibody

complex was observed. Similarly, Carter *et al.,* (2007) found that microsphere aggregation was reduced when using this running buffer. However, at least half of the antibody complex did not travel through the membrane. Hence, the problem did not appear to be in using the optimal buffer combinations, but in the actual antibody-microsphere complexes themselves. The complexes were characterised by dynamic light scattering to determine if coupling occurred successfully and also to identify if any particle aggregation had occurred.

5.8.1.2 Dynamic Light scattering analysis of anti-halofuginone, anti-toltrazuril, and anti-diclazuril-microsphere covalently bound complexes.

To further characterise the antibody-bead complexes and to determine the exact size of the covalently coupled particles, dynamic light scattering (DLS) was used. Dynamic light scattering is a non-invasive technique that can easily detect the presence of small aggregates. Hence, this technique would help to elucidate the issue with the hindered mobility of the complexes. The DLS analysis was performed as described in *Section 2.15.3.6.* Each analysis was performed in triplicate, with data generated using the Delsa Nano UI 2.21 software. From this data, polydispersity was calculated. This is used to describe the width of the particle size distribution (Arzenšek, 2010). The higher the polydispersity index (PDI), the greater the size variation of the particles. When the beads were analysed on their own (**Figure 5.25**), the average size was found to be approximately 172 nm. The monodispersity of the solution is evident by having a PDI of lower than 0.1 (Nobbmann *et al.*, 2007). The PDI for the polystyrene beads alone was calculated as 0.033 indicating a highly homogeneous solution. The anti-diclazuril-bead complexes were analysed in triplicate and the average diameter (nm) was plotted as a function of the differential intensity (%) as shown in **Figure 5.26** .The z-average size (hydrodynamic diameter) can be observed at 5,670 nm with a PDI of 0.628. This PDI is quite high, indicating that the solution is not very homogenous. The sizes of the polystyrene beads are approximately 200 nm, and the conjugated antibody has no greater size than approximately 20 nm, implying that a large degree of aggregation of the particles was occurring. This can also be observed in **Figures 5.27** and **5.28** for the anti-toltrazuril/anti-halofuginone-bead complexes, respectively. The anti-toltrazuril bead complex has an average hydrodynamic diameter of 5,433 nm with a PDI of 0.599

(**Figure 5.27**). The anti-halofuginone bead complex has an average diameter of 7,291 nm with a PDI of 0.859 (**Figure 5.28**).

The Hi-Flow 135 membrane (Millipore) used in the lateral flow assay has an average capillary flowrate of 135 seconds/4cm of strip. However, this flowrate is significantly slower when using particles greater than 3 μm according to the manufacturer's specifications. F**igures 5.26-5.28** indicate that particle aggregates are at their highest between sizes of 1.6 – 25 μm. This increased amount of aggregation appears to be the contributing factor to the diminished mobility of the complexes. Lucas *et al*. (2006) reported how they found that highly carboxylated microspheres, such as those used in this assay, have a very strong electronegative polarity, providing polar repulsion betweens the particles, subsequently preventing aggregation. Therefore, it is believed that aggregation is being caused by the coupling method employed. Aggregates caused by chemical bonding are quite stable and not susceptible to breaking up under shear force (Pease *et al.*, 2008). Hence, an alternative method for the conjugation of the antibodies onto the beads was sought.

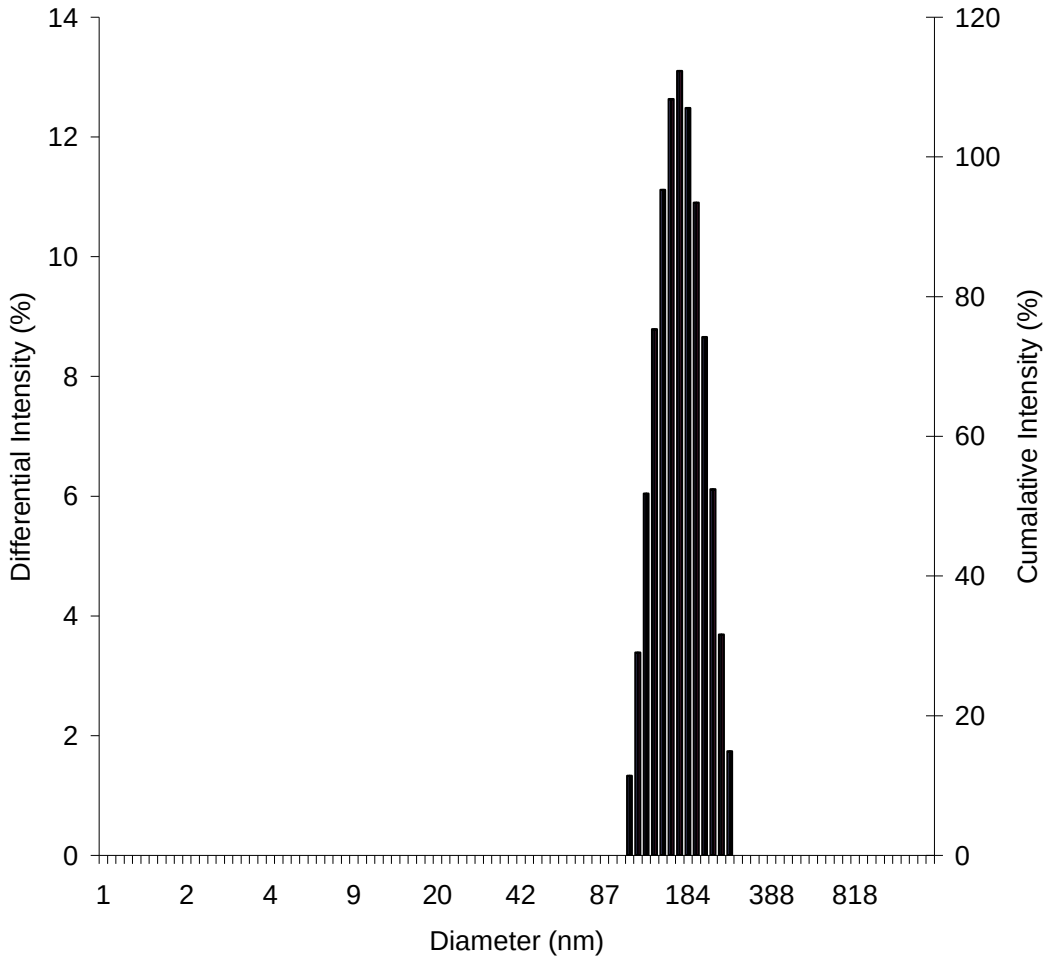


Figure 5.25 *Size distribution profile of the green polystyrene carboxylated beads used for conjugation to the anti-diclazuril, anti-halofuginone and anti-toltrazuril antibodies. Data was obtained from DLS analysis of three different samples.*

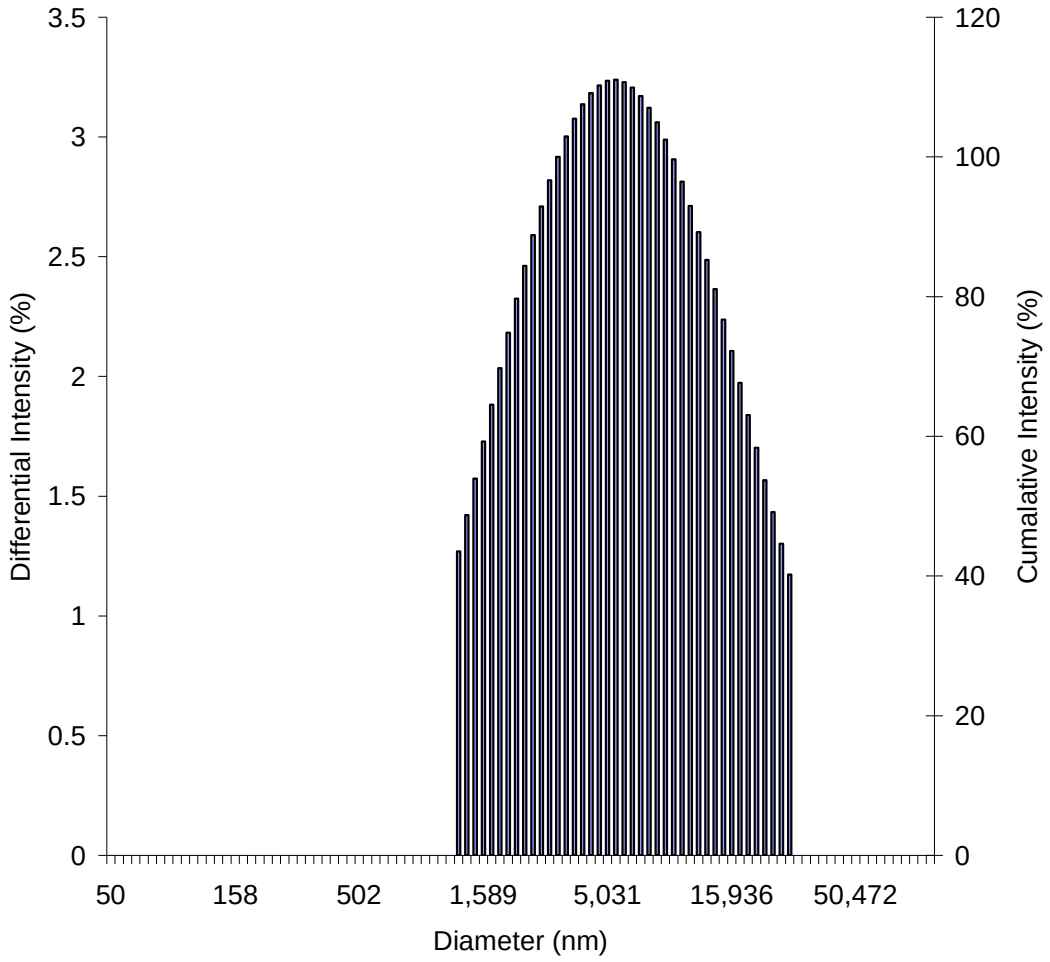


Figure 5.26 *Size distribution profile by DLS analysis of the anti-diclazuril antibodies conjugated to carboxylated polystyrene beads via covalent coupling. The average diameter of the particles in the solution was 5,760 nm, with particles ranging in size from 1,178 nm to 29,985 nm. The distribution profile shows many differential intensity peaks indicating a heterogeneous population in solution. The data was analysed using the Delsa Nano UI 2.21 software.*

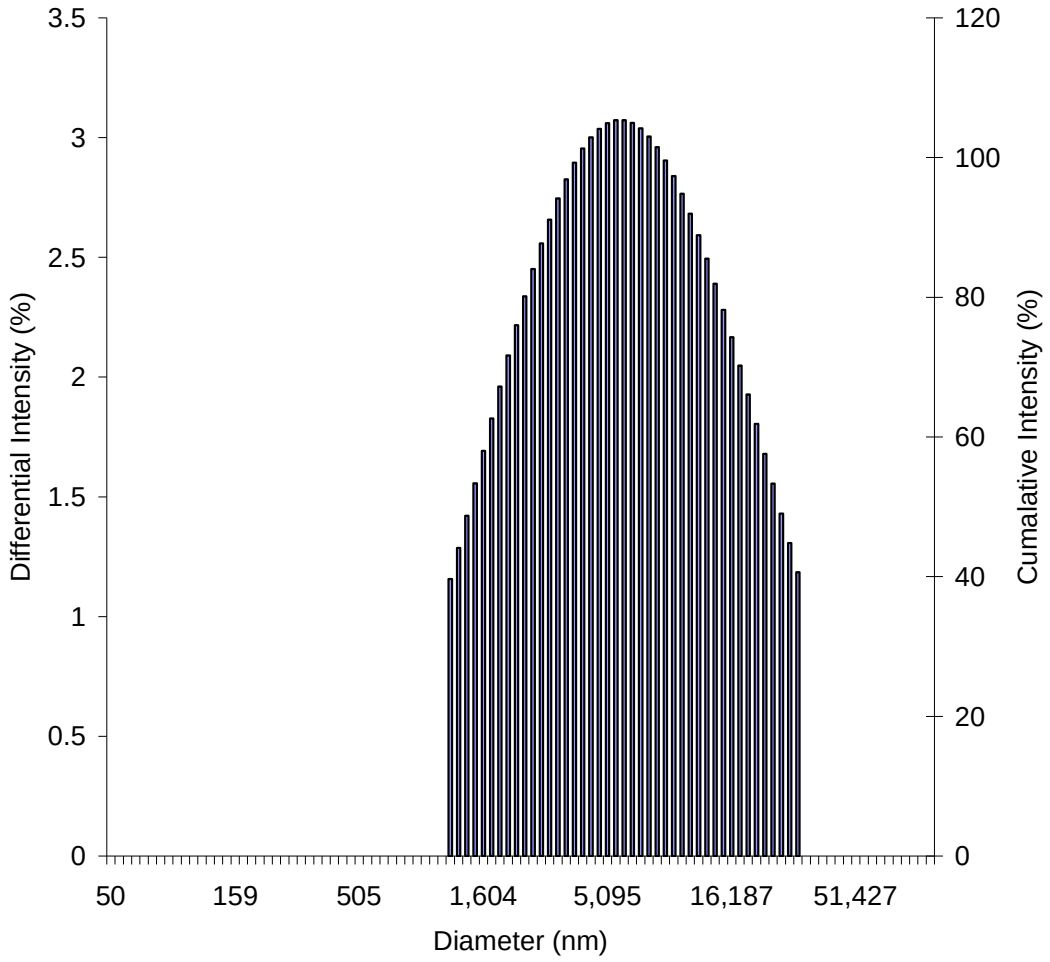


Figure 5.27 *Size distribution profile by DLS analysis of the anti-toltrazuril antibodies conjugated to carboxylated polystyrene beads via covalent coupling. The average diameter of the particles in the solution was 5,433 nm, with particles ranging from 1,261 nm to 27,291 nm. The data was analysed using the Delsa Nano UI 2.21 software.*

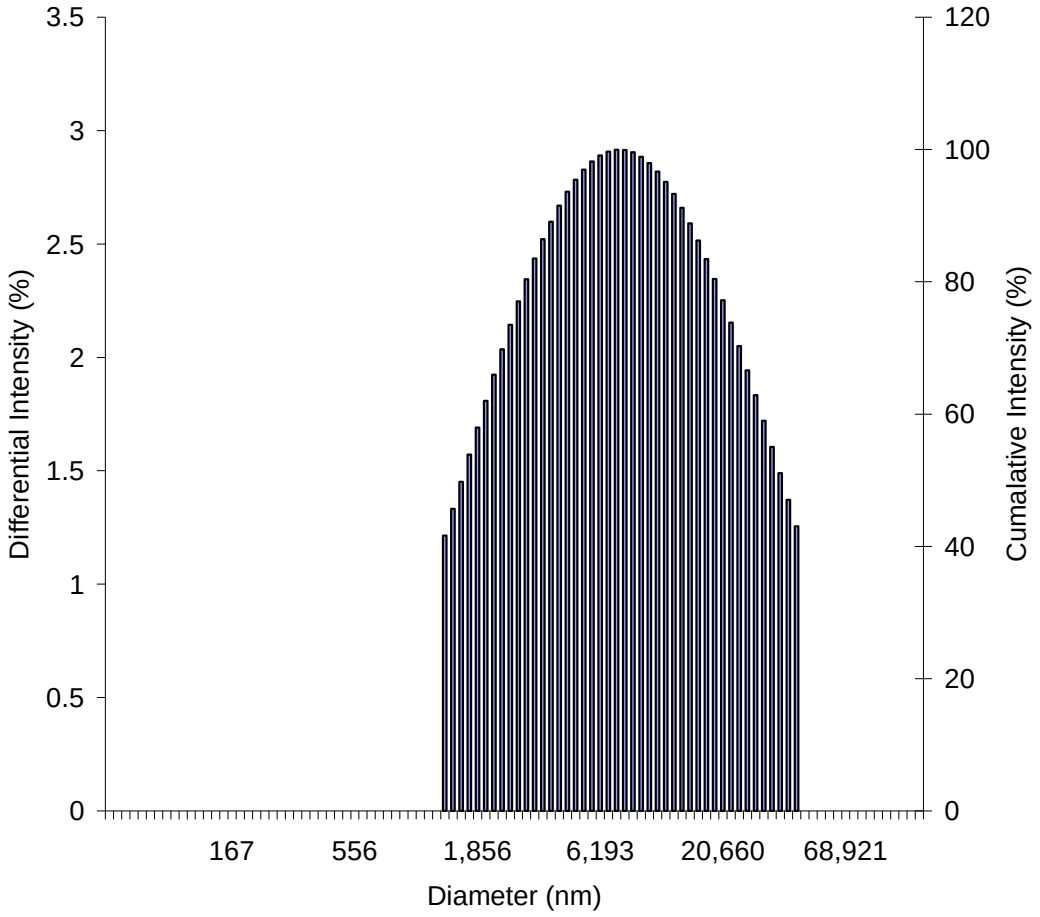


Figure 5.28 *Size distribution profile by DLS analysis of the anti-halofuginone antibodies conjugated to carboxylated polystyrene beads via covalent coupling. The average diameter of the particles in the solution was 7,291 nm, with particles ranging in size from 1,346 nm to 42,565 nm. The data was analysed using the Delsa Nano UI 2.21 software.*

5.8.2 Passive adsorbtion of the anti-diclazuril, anti-halofuginone and anti-toltrazuril antibodies to green carboxylated microspheres.

A substantial degree of aggregation occurred when the antibodies were coupled to the microspheres via chemical coupling. Antibodies can be bound to microspheres passively through hydrophobic interactions, hydrogen bonding or electrostatic interactions (Lucas, *et al*., 2006). Passive adsorption was used to conjugate the microspheres to the antibody with the expectation that fewer aggregates would develop when compared to covalent coupling. Passive adsorption was carried out as described in *Section 2.15.3.5.* To determine if the antibodies were coupled to the

polystyrene microparticles, and ensure functionality of the assay in this format, preliminary lateral-flow test assays were performed. Lateral flow strips were coated with specific secondary antibodies, corresponding to each of the anti-halofuginone, anti-diclazuril and anti-toltrazuril antibodies, as described previously to test for successful conjugation (*Section 5.8.1*). The antibody-microsphere complexes were sprayed onto the end of each of the strips, and allowed to travel upwards, following contact of the end of the strip with running buffer. The antibody-microsphere complex travelled the length of the strip towards the test-line to interact with the corresponding secondary antibodies (i.e. anti-rabbit IgG for the anti-toltrazuril-microsphere complex, anti-goat IgG for the anti-diclazuril-microsphere complex, and anti-HA mAb for the anti-halofuginone-microsphere complex).

Using the passive adsorption coupling method, it was apparent that the antibody-bead complexes could easily traverse up the membrane indicating no significant aggregation had occurred. The antibodies appeared to be successfully coupled to the beads, as green lines were clearly visible at each of the test lines. This binding was characterised further by performing competitive titration lateral-flow assays, with each of the anti-protozoan drugs in the free form (See *Section 5.8.2.2*).

5.8.2.1 Dynamic light scattering analysis of anti-halofuginone, anti-toltrazuril, and anti-diclazuril-microsphere, passively adsorbed bound complexes

Following passive adsorption coupling, characterisation of the antibody-microsphere complexes was performed using DLS analysis. It is apparent from **Figures 5.29- 5.31** that a different size distribution profile is obtained when compared to chemical coupling. **Figure 5.29** shows the size distribution profile for the anti-diclazuril-bead complexes, with an average particle size of 490 nm. This average particle size implies that coupling has occurred efficiently as the size is larger than the 172 nm average for uncoupled beads. It also suggests that a small degree of aggregation was still occuring amongst the particles, with the largest aggregate particle size determined as 1,758 nm. This is also the case for the anti-toltrazuril-bead complexes as shown in **Figure 5.30** where the average particle size is 403 nm, with the largest aggregated particle size reaching 1,191 nm. **Figure 5.31** shows the size distribution profile for the anti-halofuginone-bead complex. These complexes have a higher average diameter of 505 nm. A higher overall complex size was also observed at 2,093 nm, when compared to the other two full size IgG antibodies. This may be because scFv dimerise more

readily than IgG, causing a greater degree of aggregation than IgG. **Table 5.4** compares the polydispersity index and average diameter of the antibody-microsphere complexes with the two different coupling methods. The percentage aggregation shown was calculated as a function of the maximum diameter of particle size observed. The maximum particle size was denoted with 100% aggregation, which occurred when the anti-halofuginone scFv was used. It can also be noted, that when coupling was performed using covalent methods, at least 12 times more aggregation occurred when compared to the passively adsorbed coupling.

Table 5.4 *Comparison of the polydispersity index and particle size between antibodies conjugated to carboxylated microspheres via both covalent and adsorption coupling methods.*

Sample analysed by Dynamic Light Scattering Analysis (All results are average of triplicate assays)	**Polydispersity Index**	**Diameter 50% (nm)**	**Diameter 100% (nm)**	**Percentage Aggregation (%)**
Polybead carboxylate beads 0.2 μm	0.033	173	225	0.5
Covalently coupled anti-diclazuril Ab-beads	0.628	5670	29,985	70.4
Covalently coupled anti-toltrazuril Ab-beads	0.599	5433	27,291	64.1
Covalently coupled anti-halofuginone Ab-beads	0.859	7291	42,565	100
Passively adsorbed Anti-diclazuril Ab-beads	0.201	490	1,758	4.1
Passively adsorbed Anti- toltrazuril Ab-beads	0.195	403	1,191	2.8
Passively adsorbed anti-halofuginone Ab-beads	0.225	505	2,093	4.9

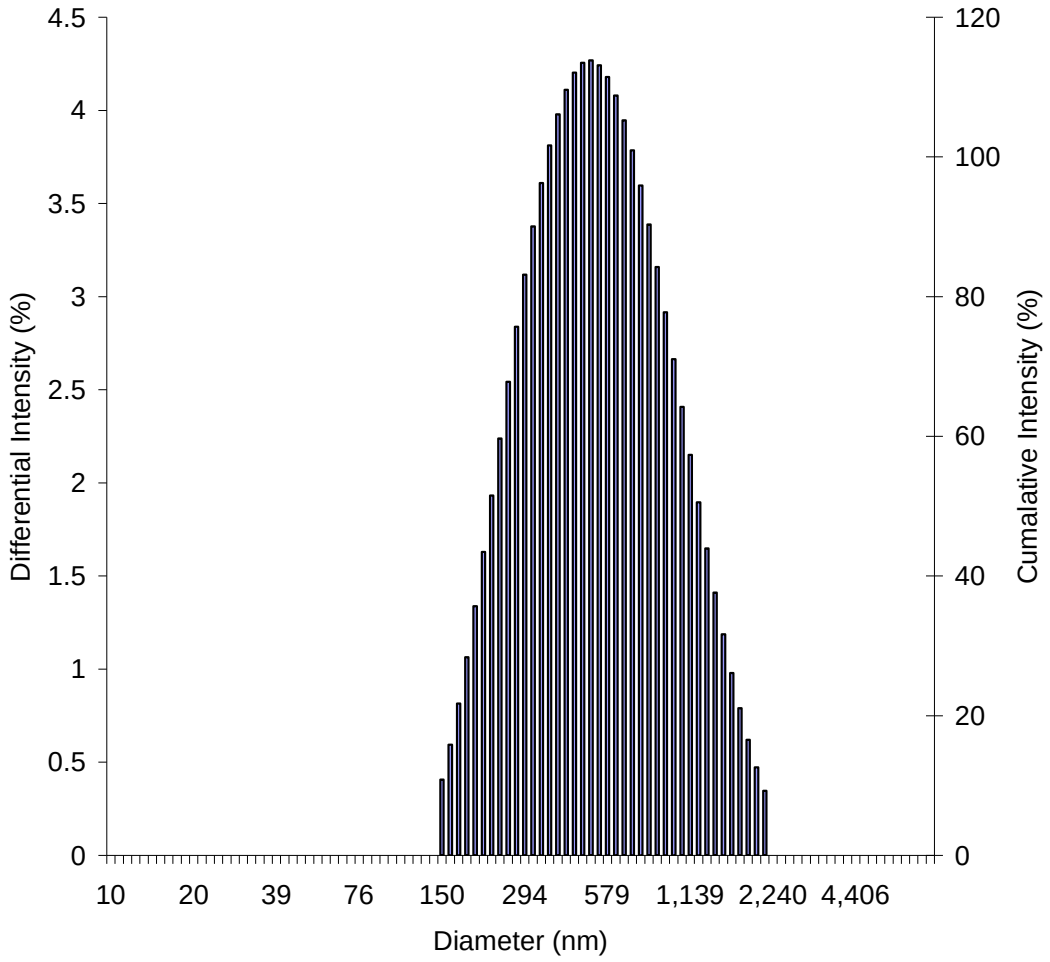


Figure 5.29 *Size distribution profile by DLS analysis of the anti-diclazuril antibodies conjugated to carboxylated polystyrene beads via passive adsorption. The average diameter of the particles in the solution was 490 nm, with particles ranging in size from 168 nm to 1,758 nm. The data was analysed using the Delsa Nano UI 2.21 software.*

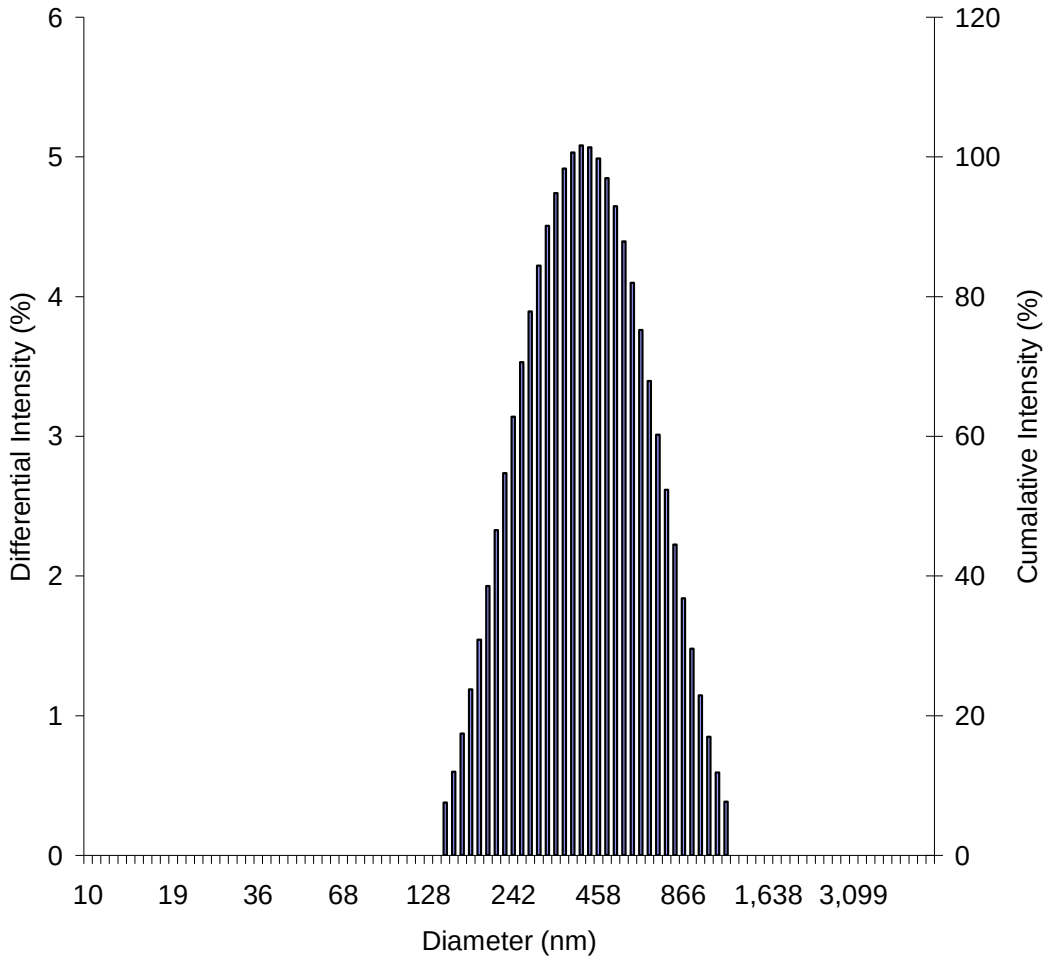


Figure 5.30 *Size distribution profile by DLS analysis of the anti-toltrazuril antibodies conjugated to carboxylated polystyrene beads via passive adsorption. The average diameter of the particles in the solution was 403 nm, with particles ranging in size from 145 nm to 1,191 nm. The data was analysed using the Delsa Nano UI 2.21 software.*

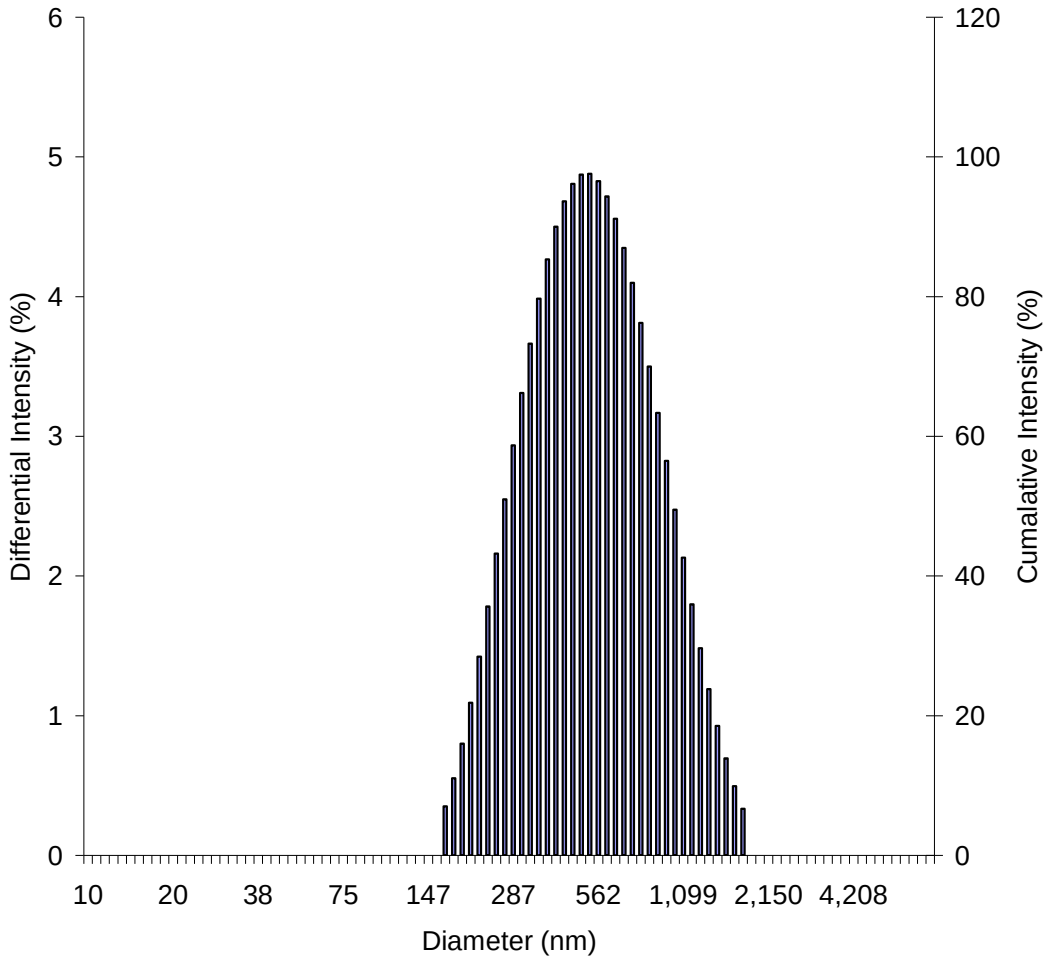


Figure 5.31 *Size distribution profile by DLS analysis of the anti-halofuginone antibodies conjugated to carboxylated polystyrene beads via passive adsorption. The average diameter of the particles in the solution was 505 nm, with particles ranging in size from 150 nm to 2093 nm. The data was analysed using the Delsa Nano UI 2.21 software.*

5.8.2.2 Characterisation of anti-halofuginone, anti-diclazuril and anti-toltrazuril Ab-microsphere complexes via a competitive lateral flow immunoassay to determine the detection range of each antibody-coated microsphere complex from extracted egg residues

The competitive lateral-flow immunoassay was performed as described in *Section 2.15.3.7.* Varying concentrations of free antiprotozoan drugs were spiked into the running buffer for each separate LFIA strip. The free drug competes with the conjugate drug embedded on the LFIA at the test-zone. A positive result would yield no visible test-line. The amount of colour present at the test line is inversely proportional to the amount of free drug present. A broad range of each free drug was used to observe full saturation of antibody at the test line to complete competition of binding at the test line. The concentrations of halofuginone, diclazuril and toltrazuril extracted from egg samples were; 10 µg, 1 µg, 100 ng, 10 ng, 1 ng and 0 ng/mL in the optimal running buffer (15mM sodium citrate, pH 7.0, with 0.05% (v/v) Tween and 5 % (v/v) methanol for resuspending the extracted residues) from *Section 5.8.1.2.* It can be observed from **Figure 5.32,** that the detection limit of the anti-toltrazuril-bead complex in the LFIA is at approximately 100 ng/mL. The anti-diclazuril-microsphere complex showed a similar result with a detection limit of 100 ng/mL also. The halofuginone scFv-bound microsphere has a lower limit of detection for halofuginone at approximately 10 ng/mL. This assay was performed in triplicate. Once the limit of detection of each antibody-complex was clearly established, a multi-analyte assay was performed in extracted egg samples.

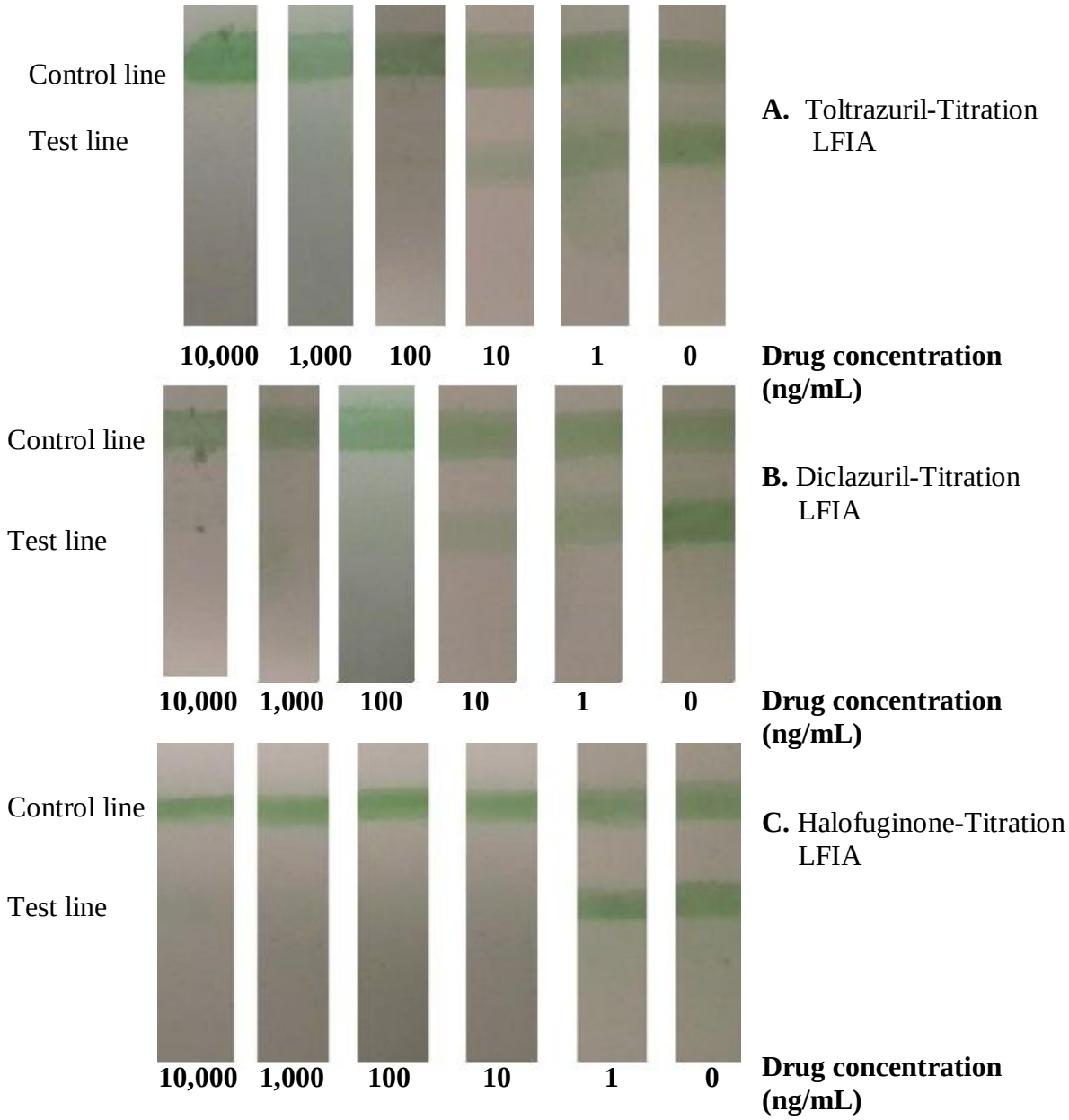


Figure 5.32 *Lateral-flow immunoassays for the determination for the limit of detection of the anti-halofuginone, anti-toltrazuril and anti-diclazuril Ab-microsphere coupled complexes in a competitive assay format. The concentrations of each of the free anti-protozoan drugs spiked into each of the running buffers are denoted below the lateral flow strips. These concentrations were 10 µg, 1 µg, 100 ng, 10 ng, 1 ng and 0 ng/mL. No visible line at the test line indicates a high concentration of drug in the sample analysed. The limit of detection of the anti-toltrazuril/anti-diclazuril Ab complexes appears to be at approximately 100 ng/mL with the anti-halofuginone Ab complex detecting slightly lower concentrations at 10 ng/mL.*

5.8.3 Multi-analyte detection of halofuginone, diclazuril and toltrazuril in a competitive lateral flow immunoassay using antibody conjugated coloured microspheres.

In the field of veterinary residue monitoring, numerous anti-protozoan drugs may be used at any given time. Hence surveillance of all residues in food can be costly and time consuming, if several different assays are required. To circumvent this, and ensure a cost-efficient and rapid screening method could be made available for the detection of anti-protozoan drug residues, a multi-analyte assay was developed. This multi-analyte assay allows the concurrent detection of toltrazuril, halofuginone, and diclazuril residues below the MRL's set for each, in one easy-to-use lateral-flow immunoassay. The multi-analyte assay method is outlined in *Section 2.15.3.7*. This method is a proof-of-principle assay for the simultaneous detection of all three anti-protozoan residues. If the method was to be validated fully 20 samples at each of the varying concentrations of the drugs would be spiked and extracted from real matrices and analysed in triplicate on separate days, If this assay was going to be commercialised, use of mathematical models for competitive lateral-flow assays, such as those outlined by Qian and Bau (2004), could be used for the optimisation of the parameters of the competitive assay, thus reducing the time for laboratory optimisation. A digital scanner may also be used to determine the signal intensity and to record the results quantitatively. To establish if any cross reactivity or non-specific binding would occur between the antibodies and all three of the test zones, assays were prepared for each of the drugs using the lowest concentration of each of the anti-protozoan drugs detectable by the antibodies. Each set of assays included one of the drugs at the lowest concentration detectable by the corresponding assay, and neither of the other drugs. Hence, binding was inhibited at one test zone, but not at the others. **Figure 5.33** illustrates the binding of the antibodies, when varying concentrations of each of the target drugs were assessed, in the lateral-flow immunoassay. It is apparent from the lateral-flow strips that no significant cross-reactivity of the antibody-bead complexes can be observed. At the toltrazuril test zone in assay (**A**) from **Figure 5.33** a coloured line was observed when it was not observed in the other two test-zones. This indicates the presence of diclazuril and halofuginone at quantities greater than 100 ng/mL and 10 ng/mL, respectively. This can also be observed in assays (**B**) and (**C**) for diclazuril and halofuginone respectively. An apparent test line in (**B**) in the diclazuril test-zone indicates the absence of diclazuril. Similarly, in assay (**C**) a test

line is present at the halofuginone test-zone but not at the other two, indicating no halofuginone is present but the other two drugs are present in quantities at or greater than the limit of detection. **Figure 5.34** illustrates the assay run in the presence of no competitors, whereby all three test lines and control line are visible. In **Figure 5.35** the assays were run with only one of the competitors present. Assay (**A**) shows the assay performed with 100 ng/mL of toltrazuril present, (**B**) shows the assay with 100ng/mL of diclazuril present and (**C**) shows the assay with 10 ng/mL of halofuginone present. It can be observed that the only test-line not visible is at the test-zone corresponding to the specific drug that was present in the sample mix. This proves that the multi-analyte assay is functional for simultaneous detection.

When comparing the lateral flow set-up to other formats for simultaneous detection, the lateral-flow multi-analyte detection method appears to be 'fit-for-purpose' and feasible for high throughput screening in an 'on-line' monitoring environment.

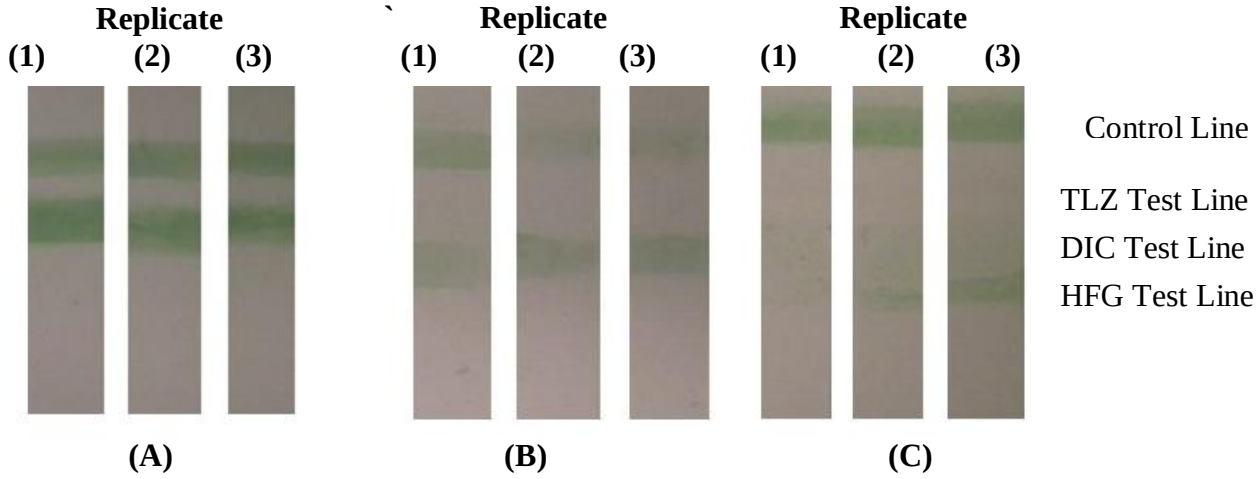


Figure 5.33 *Multi-analyte lateral-flow immunoassays for the simultaneous detection of halofuginone, toltrazuril and diclazuril in a competitive assay format (in triplicate). In (A) no toltrazuril was present in the spiked samples, in (B) no diclazuril was present in the spiked samples and in (C) no halofuginone was present. It can be observed in each of the assays that when no competitor is present, a visible line can be observed in the relevant specific test-zone. The required control-line is also evident in all of the above assays.*

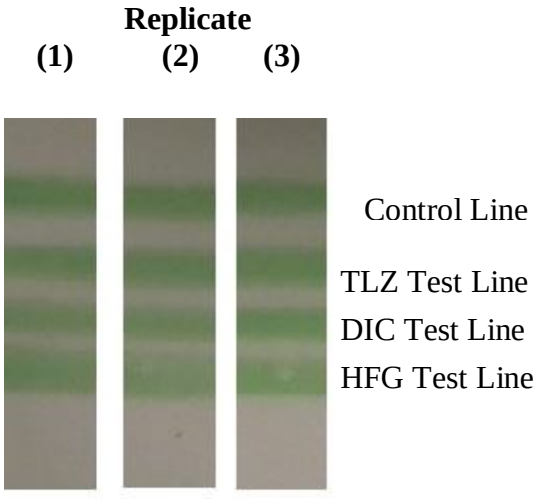


Figure 5.34 *Multi-analyte lateral-flow immunoassays performed in the presence of no anti-protozoan drugs. The control line is clearly visible at the top ot the strip along with three lines observed at the toltrazuril, diclazuril and halofuginone test-zones.*

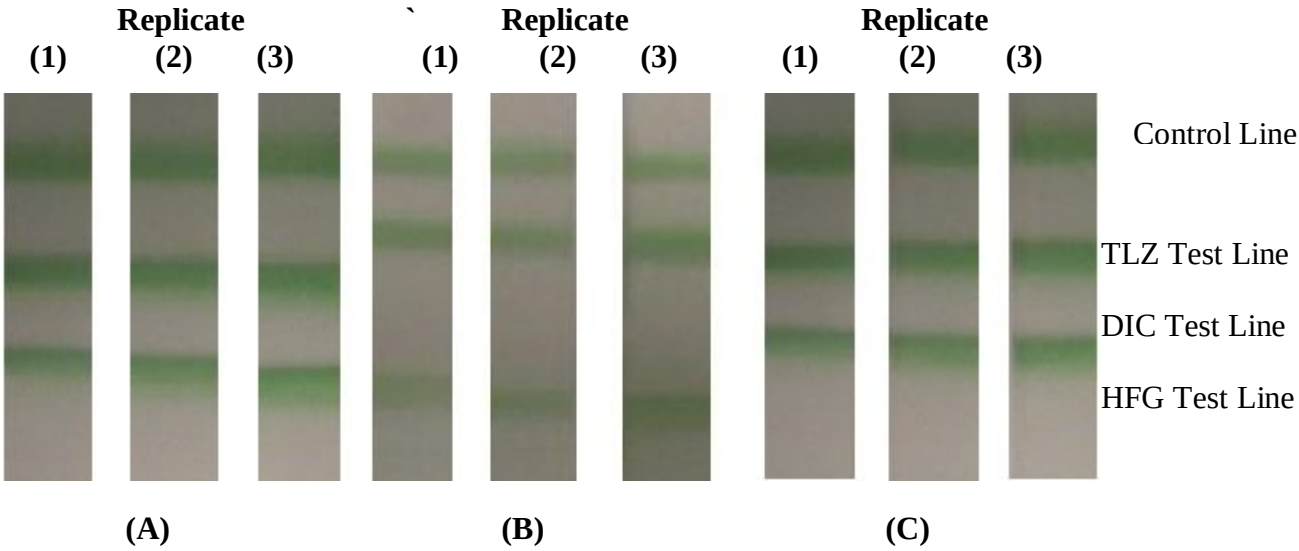


Figure 5.35 *Multi-analyte lateral-flow immunoassay. The assay was performed in the presence of only one of the anti-protozoan drugs. The control line is clearly visible at the top of each of the strips. Assay set (A) was performed in the presence of 100 ng/mL of toltrazuril, assay set (B) with 100ng/mL of diclazuril, and assay set (C) with 10ng/mL of halofuginone.*

5.9 Discussion and Conclusions

The production and characterisation of polyclonal antibodies to toltrazuril, and their subsequent incorporation into ELISA, Biacore and lateral-flow-based assays for the detection of toltrazuril residues was described in this chapter. Toltrazuril-BSA and TFME-BSA conjugates were used for the immunisation of ten rabbits. The presence of toltrazuril-specific antibody from sera samples was determined by competitive ELISA using the primary toltrazuril metabolite 'ponazuril'. The toltrazuril-BSA conjugate induced a high antibody response to toltrazuril in three of the rabbits. Subsequently, the anti-toltrazuril antibodies were purified from the sera by immunoaffinity chromatography using Protein G-immobilised sepharose. The purification process was assessed by SDS-PAGE and the presence of rabbit IgG molecules was confirmed by Western blotting.

Initially, the polyclonal antibodies were used in a competitive ELISA format for the detection of toltrazuril in PBS. The optimal conditions for the assay were investigated by analysing different conjugate coating buffers, conjugate concentrations and amounts of antibody used. Once these were optimised, the resulting ELISA proved reproducible, with inter-assay CVs less than 19%. The assay was then validated by performing the inter-assay evaluations in egg-samples. The extraction efficiency was determined to be approximately 86%, which is above the mandatory recovery of 80%, for a method to be validated by E.U. standards. The LOD of the assay was determined to be 900 pg/mL by extrapolation from the inter-assay calibration curve. This ponazuril concentration for the LOD was also experimentally verified via a precision assay in egg matrix, confirming this detection capability of the assay. This assay sensitivity is significantly below the lowest EU MRL for toltrazuril, of 100 µg/kg, corresponding to approximately 100 ng/mL. This sensitivity makes the assay highly applicable for the detection of toltrazuril from certain matrices, where loss of residue may occur due to lengthy extraction procedures.

In an attempt to develop the most sensitive assay format for the detection of toltrazuril, both Biacore and lateral-flow assay formats were also investigated. A Biacore inhibition assay was produced, employing the anti-toltrazuril polyclonal antibody,a CM5 Biacore chip immobilised with the toltrazuril-BSA conjugate and free ponazuril. Following preliminary analysis a concentration of 100 µg/mL of polyclonal antibody to what proved optimal for use in the development of the toltrazuril-Biacore assay. A preliminary inhibition assay showed that using the

Biacore-based assay format, the anti-toltrazuril polyclonal antibody could detect down to 890 pg/mL of ponazuril in a PBS matrix. When the same analysis was performed in an egg matrix, the limit of detection of the assay was determined to be approximately 1920 pg/mL. The average extraction efficiency was calculated as 93%. When a precision assay was performed to verify the limit of detection, 1.5 ng/mL of ponazuril could be distinguished 100% of the time from the blank samples.

Previous studies comparing both ELISA and Biacore formats have often shown the advantages of using biosensors (Guidi *et al.*, 2001), particularly in environmental monitoring when high throughput sampling is required. The use of Biacore technology allows 'real-time' measurement, cutting out lengthy incubation stages. It requires low amounts of reagents without any special preparation and does not require antibody labelling or a second reagent for immunocomplex detection. However, greater or similar sensitivities to Biacore assays have also been found when using ELISA for monitoring trace residues. Fodey *et al.* (2007) reported greater sensitivity in ELISA over Biacore formats for monitoring chloramphenicol residues when the assay was performed in milk extracts. This was not the case when the assay was performed in buffer, and therefore the loss of sensitivity in the SPR assay was determined to be influenced by the matrix. He *et al.*, (2007), also noted how matrix interferences were observed during detection of sudan-dye residues from egg yolk, when extraction with acetonitrile was used. Hence, other methods such as extraction with *n*-hexane or toluene, may help in reducing effects caused by the egg matrix.

It is apparent from the assay results, when the Biacore detection assay is performed in buffer the LOD is actually lower than the LOD of the ELISA. However, according to the European Commission Decision 2002/657, for a method to be valid for monitoring residues in foodstuffs, analysis should be performed in the contaminated matrix. Hence, it can be concluded that for the analysis of toltrazuril residues in real-food samples, ELISA detection is marginally more sensitive than Biacore detection methods.

In an effort to ensure the assay will be compatible for field applications, the development of a dipstick-based lateral flow format was investigated. A simple method using anti-rabbit IgG as a control capture antibody and toltrazuril-BSA as a test zone was used. The strips were exposed to extracted egg samples spiked with varying concentrations of ponazuril. Equal volumes of anti-toltrazuril antibody were mixed with the ponazuril samples following extraction. Any anti-toltrazuril antibody

bound on the strip was detected following the addition of an HRP conjugated anti-rabbit IgG antibody. The results were visually determined from the lateral flow-strips, and after inter-assay analysis from real samples, it was clear that 100 ng/mL of toltrazuril could be distinguished from blank samples. This LOD is relatively poor in comparison to that achievable by ELISA and Biacore methods. However, it is still capable of detecting levels of toltrazuril at and above the MRL set by the E.U. The advantage of this assay is that it is an inexpensive assay suitable for 'on-site' high throughput screening.

The significant differences in sensitivity limits between the 900pg/mL LOD for the ELISA and the 100ng/mL LOD for the lateral-flow assay for the detection of toltrazuril could be justified by several factors. The higher binding capacity of the antibodies on polystyrene microtiter plate in comparison to that on nitrocellulose membranes and the interaction time of the antibody and the plate in the ELISA assay is greater when compared to the minimal interaction time available during the lateral flow assay. Also, the use of a sophisticated ELISA reader, for signal detection, compared to visually analysing results in the LFIA (Kaur *et al*., 2007) may also affect detection capability. All of these factors contribute to the higher sensitivity recorded with ELISA.

It is understood that there is currently only one reported antibody-based detection method for toltrazuril (Connolly *et al.,* 2002). That immunoassay uses a polyclonal antibody, produced from rabbits immunised with a trifluoraminoether conjugate. An LOD is not reported for this ELISA assay. However, they described an IC50 of 18 ng/mL, which is slightly less sensitive than the limits reported herein for the ELISA and Biacore detection methods. Even though the LFIA is not as sensitive as the previously reported method, all three of the immunoassay-based methods reported here have advantages over HPLC-based methods for detecting toltrazuril due to their rapidity, sensitivity and cost-effectiveness.

A multiplex assay for the detection of toltrazuril concurrently with other anti-protoazoan agents, diclazuril and halofuginone was developed. This assay would prove very advantageous in the simultaneous monitoring and control of these residues, by significantly reducing operator time and costs of analysis. The toltrazuril/diclazuril/halofuginone antibodies were immobilised on coloured polystyrene beads via chemical coupling. However, after substantial dynamic light scattering analysis, it was apparent that the chemical coupling was inducing

aggregation amongst the polystyrene beads which were hindering the antibody-bead complexes from passing up the lateral flow-strips. To circumvent this, passive adsorption methods were investigated. Passive adsorption of the antibody onto the beads was successful and following further analysis using dynamic light scattering, it was apparent that little aggregation had occured. Preliminary optimisation assays were performed. and, once the range of the assays was established, the multi-analyte assay was performed with real samples. It was observed from the LFIA that the LOD of the assay in this format was at 10 ng/mL for halofuginone, and at 100 ng/mL for both diclazuril and toltrazuril. This LOD of the assay is below the MRL set for both halofuginone and toltrazuril. However, the MRL for diclazuril is 1.5 ng/mL, therefore, this assay would not be suitable for diclazuril detection in foodstuffs. The antibody employed to detect diclazuril was a commercial antibody. This leads to the belief that had a recombinant diclazuril antibody been successfully developed, greater assay sensitivities would be achievable, and this recombinant antibody would be far more suitable for incorporation into this multi-analyte detection assay.

High-throughput screening for the semi-quantification of residues in food is proving to have increasingly more merit in food analysis as shown by Pang *et al.,* (2006). More samples can be analysed in a shorter time for a variety of different contaminants. The introduction of a two-tier system for residue analysis in food, consisting of an initial low-cost screening step, means that a fast, effective assay capable of detecting contaminating residues in vast quantities of samples is required. The potential health risks imposed from anti-protozoan residues in food, highlight the particular importance to provide easy to use, precise, reliable and accurate tests for the efficient monitoring of these residues, to ensure food safety throughout the EU. Most of the traditional analysis of coccidiostats is applied to the detection of a single component, making thorough screening processes expensive and arduous. Multi-analyte systems like the lateral-flow assay described herein are likely to totally transform environmental monitoring for the better in the foreseeable future. This multi-analyte assay not only proved that all three anti-protozoan drugs could be detected simultaneously from food samples with no cross reactivity of the antibodies occurring, but also, that each drug could be quantified independently of the other two.

Most lateral-flow assays previously reported for monitoring of food contaminants, only focus on the detection of one contaminant such as those reported by Laura *et al.*, (2011), Zhang *et al.*, (2008) and Wang *et al.*, (2005). Kranthi and colleagues (2009)

previously reported the development of a multi-analyte lateral flow strip for the determination of different insecticide residues. However, this method did not test the residues individually, as only one test line was used, therby making it impossible to determine which residue was present or quantify it. Xie *et al*., (2009) also reported a multi-residue detection lateral flow for cephems in milk. However, this method also did not allow for the individual detection of the different residues, instead a polyclonal antibody was employed with the capability of detecting several residues. These methods, however time-saving, would not be suitable for the quantification of several unknown contaminants in foodstuffs.

When comparing this lateral-flow assay method to other multi-analyte detection methods, such as those reported by Li and collegues in 2006 using LC-MS or by Peters *et al*., (2009) using TOF-MS, the LFIA reported herein appears advantageous due to the overall simplicity of the assay. No specially trained personnel, expensive laboratory equipment for quantification, or even a laboratory setting are required for use of this assay. This means on-site testing can be performed in a matter of minutes. Ideally the LFIA would be extended to include a panel of all actively used anti-protozoan drugs, whereby one assay-strip would be applicable to detect any potentially contaminating veterinary residues.

This assay would be useful for commercialisation if a scanner was incorporated to quantify the results and further validate the assay performance. This simple detection methodcould potentially revolutionise the field of environmental monitoring. Whereby, in the current climate an assay that is relatively inexpensive to use and yet highly efficient in multi-analyte detection, appears to have a significant and highly applicable niche.

Chapter 6

Overall Conclusions

6.1 Overall Conclusions

The aim of the work presented in this thesis was to develop immunoassays for the detection of anti-protozoan drug residues. The strategies employed involved the construction of a recombinant scFv library for diclazuril, the generation, characterisation and subsequent affinity maturation of recombinant antibody fragments (scFv) to halofuginone, and the production of polyclonal antibodies for toltrazuril. The antibodies generated have been characterised by ELISA, SDS-PAGE, Western blotting and Biacore. ELISA, lateral-flow and Biacore-based immunoassays were developed for the detection of these anti-protozoan drugs and/or their metabolites.

Chapter 3 describes the construction of a halofuginone-specific scFv library. Phage display was employed to facilitate the isolation of a halofuginone-specific scFv via biopanning. However, the resulting scFv was not sufficiently sensitive to detect HFG below the MRL set by the E.U. To overcome this problem, light-chain shuffling was employed to determine the optimal light: heavy chain pairings of the scFv to enhance its sensitivity. This led to the isolation of a scFv with 185-times greater sensitivity than the original scFv. This scFv was incorporated into an ELISA assay for the detection of HFG residues, which was then validated according to E.U. guidelines. This work was published in *Analytical Biochemistry* in March 2011.

A SPR-based detection method for the determination of HFG residues in eggs was optimised and validated. Initially a HFG conjugate was immobilised on a CM5 Biacore chip, and regeneration studies were performed, while using the halofuginone-specific scFv. Several different extraction procedures for spiked HFG in eggs were investigated. This was to determine which procedure gave the highest extraction efficiency from the homogenised egg matrix. Once this was optimised, egg samples were spiked with varying concentrations of HFG and subsequently extracted. The extracted yields were good and recoveries of up to 86.5% were observed. This assay was fully validated according to E.U. guidelines. The limits of the assay were subsequently determined, whereby, the CCβ or detection capability of the assay, was shown to be 2.8 ng/mL. This is the first SPR-based assay described for the detection of HFG.

The construction of a murine-derived scFv library for diclazuril is described in Chapter 4. Initially a chicken was immunised with a carboxy-diclazuril conjugate, but after several immunisations no response was observed. Mice were then immunised with the same conjugate. A response to diclazuril was observed in the mice and, after a full immunisation schedule, the RNA was extracted from the mice spleens and a scFv library was constructed. Individual antibody fragments were selected by biopanning but none of the selected clones showed binding to diclazuril in solution. Further clones were chosen in a bid to isolate any diclazuril-binding scFv. However, even after re-construction of the library from the RNA, no diclazuril-specific scFv were found. Due to time constraints, no further work could be performed in developing a recombinant antibody fragment. The problems encountered during the construction of this library were identified as issues which arose from the construction of the library from murine-derived cDNA.

The incorporation of an anti-toltrazuril polyclonal antibody into an ELISA is described in Chapter 5. The toltrazuril structure proved highly problematic for conjugation to another protein carrier (required for immunisation), due to the lack of available functional groups. Previously, a research group had discovered that a toltrazuril-mimic (with an identical functional group) could be used to elicit an immune response and produce antibodies that would recognise toltrazuril. For this reason, rabbits were immunised with both the antigen mimic-conjugate and a commercially available toltrazuril conjugate. The sera of the rabbits immunised, were screened by ELISA and toltrazuril-specific antibodies purified via Protein G affinity-chromatography. The purified antibody preparation was characterised by SDS and western blotting and was subsequently incorporated into ELISA, Biacore and lateral flow-based detection assays using ponazuril (toltrazuril metabolite) residues. The co-efficients of variation of the ELISA and Biacore assays and the limit of detection were determined. These were calculated by extrapolation from a four-parameter calibration curve plotted using BIAevaluation ^{TM} software.

For the lateral flow assay, the limit of detection was visually determined to be 100 ng/mL. To experimentally verify the detection capabilities of the Biacore and ELISA assays, precision assays were performed for both. These precision assays confirmed a limit of detection of 900 pg/mL for both the Biacore and the ELISA assays for

toltrazuril detection. The sensitivity of both the Biacore and ELISA assays were comparable, with the lateral flow assay being somewhat less sensitive. However, as methods for toltrazuril detection would be required in 'on-line' monitoring environments with a high throughput of samples, the lateral flow-assay may prove the easiest to use in such a lateral-flow application.

Chapter 5 also describes the development of a multi-analyte detection system for the simultaneous monitoring of halofuginone, toltrazuril and diclazuril residues. Coloured carboxylated microspheres were coated with each of the detection antibodies and used in a lateral-flow assay format. Initially problems with chemical coupling of the antibody-bead conjugation arose but these were circumvented by using passive adsorption methods to couple the antibodies to the beads. Dynamic light scattering analysis showed that the beads were the correct size when coupled, and no major aggregates were present. Thus the multi-analyte lateral flow assay format was feasible. Using this approach, halofuginone was detectable at a limit of 10 ng/mL or greater, toltrazuril at 100 ng/mL and, similarly, diclazuril had a detection limit of 100 ng/mL. This assay approach is highly advantageous in field applications where all three of these anti-protozoan drugs are used as prophylactic/preventative methods for treating protozoans. This assay could easily be adapted for commercial purposes by incorporating a hand-held reader to analyse results, and it could pave the way for more efficient monitoring of anti-protozoan drug residues in the future.

Chapter 7

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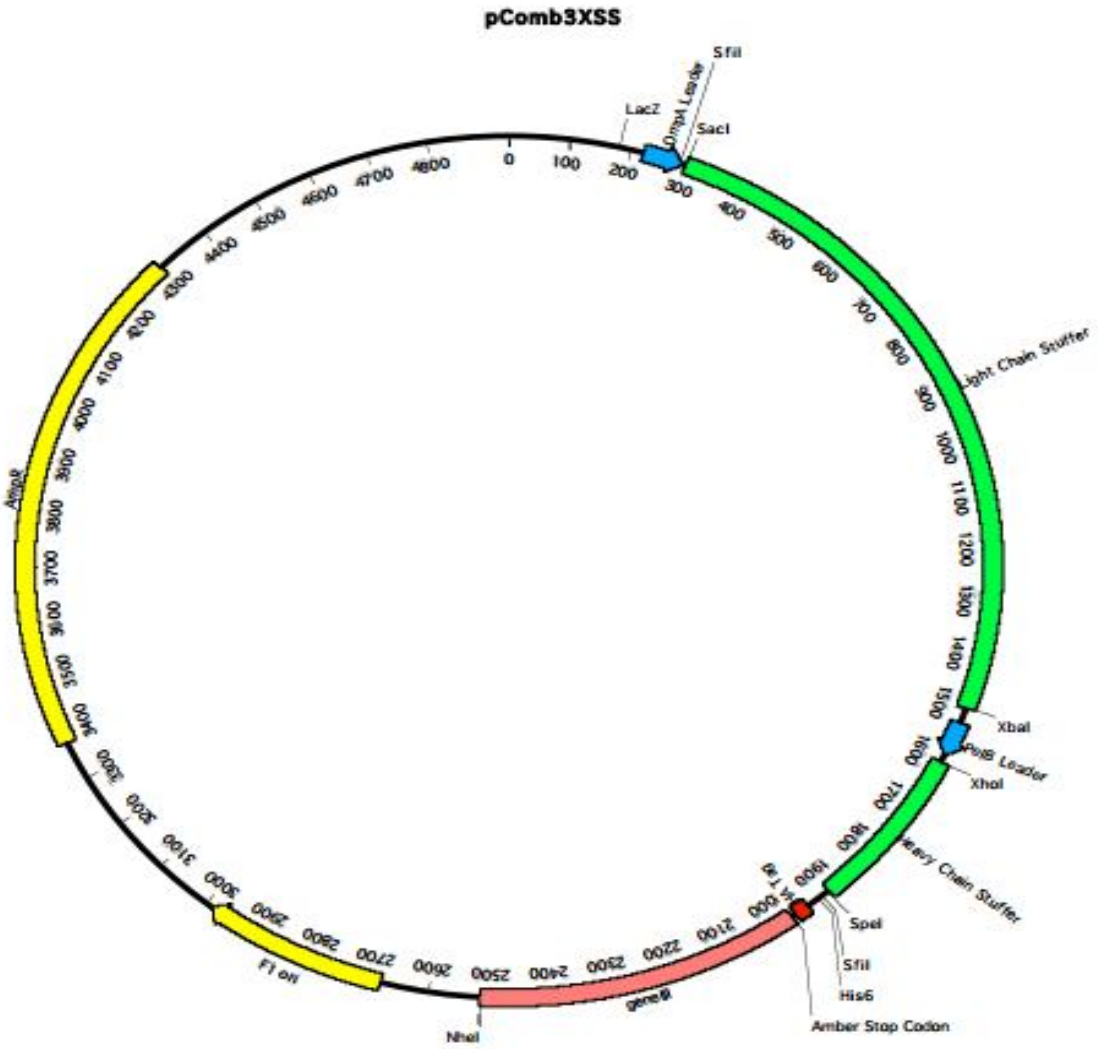
Chapter 8
Appendices

Please refer to *Section 2.6.3* regarding the use of the Pcomb3XSS vector for cloning of the scFv libraries. Taken directly from Barbas *et al*., 2001.

pComb3XSS, available for request by MTA
Contains nonsense SS stuffer in light chain and heavy chain cloning regions
Refer to Protocols 9.1-9.9 in Phage Display: A Laboratory Manual for detail on use



Appendix 8.1 *Map of pComb3XSS vector used for the construction of anti-halofuginone and anti-diclazuril antibody libraries. Amber stop codon, 6 x His tag, HA-tag, Sfi1 digestion sites and ampicillin resistant cassettes can all be observed.*

QIAquick Gel Extraction Kit Protocol

using a microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 8). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the MinElute Reaction Cleanup Kit.

Important points before starting

- The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.

Procedure

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**

   Minimize the size of the gel slice by removing extra agarose.

2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**

   For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**

   IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

   The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. **Add 1 gel volume of isopropanol to the sample and mix.**

   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**

   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. **Discard flow-through and place QIAquick column back in the same collection tube.**

   Collection tubes are reused to reduce plastic waste.
9. **Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**

   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.
10. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**

    Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
11. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x *g* (13,000 rpm).**

    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
13. **To elute DNA, add 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.**

    IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl.

    Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
14. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

Gel Extraction Spin Protocol

Appendix 8.2 *Qiagen gel purification kit protocol taken directly from the manufacturers handbook 'Qiagen Spin Handbook 03/2008'.*

Protocol: Plasmid or Cosmid DNA Purification Using QIAGEN Plasmid Mini Kit

This protocol is designed for preparation of up to 20 µg of high-copy plasmid or cosmid DNA using the QIAGEN Plasmid Mini Kit. For additional protocols, such as for cosmid, low-copy-number plasmid, BACs, PACs, P1s, and double-stranded M13 replicative form purification, see the recommendations at www.qiagen.com/goto/plasmidinfo .

Important notes before starting

- New users are advised to familiarize themselves with the detailed protocol provided in this handbook. In addition, extensive background information is provided on our plasmid resource page www.qiagen.com/goto/plasmidinfo .
- Optional: Remove samples at the steps indicated with the symbol ☞ in order to monitor the procedure on an analytical gel (see page 41)

Things to do before starting

- Add the provided RNase A solution to Buffer P1 before use. Use one vial of RNase A (centrifuge briefly before use) per bottle of Buffer P1, to give a final concentration of 100 µg/ml.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.
- Pre-chill Buffer P3 at 4°C.
- Optional: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use one vial LyseBlue (centrifuge briefly before use) per bottle of Buffer P1 to achieve a 1:1000 dilution. LyseBlue provides visual identification of optimum buffer mixing thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For more details see "Using LyseBlue reagent" on page 14.

Procedure

1. **Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approximately 8 h at 37°C with vigorous shaking (approx. 300 rpm).**

   Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. **Dilute the starter culture 1/500 to 1/1000 into 3 ml selective LB medium. Grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm).**

   Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3–4 x 10^9 cells per milliliter, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium.

3. **Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C.**

   ⊗ If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. **Resuspend the bacterial pellet in 0.3 ml of Buffer P1.**

   Ensure that RNase A has been added to Buffer P1.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle before use to ensure LyseBlue particles are completely resuspended. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. **Add 0.3 ml of Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min.**

   Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO_2 in the air.

   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

6. **Add 0.3 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for 5 min.**

   Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and KDS. The lysate should be mixed thoroughly to ensure even potassium dodecyl sulphate precipitation. If the mixture still appears viscous, more mixing is required to completely neutralize the solution.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

7. **Centrifuge at maximum speed in a microcentrifuge for 10 min. Remove supernatant containing plasmid DNA promptly.**

   Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed at maximum speed in 1.5 ml or 2 ml microcentrifuge tubes (e.g., 10,000–13,000 rpm in a microcentrifuge). Maximum speed corresponds to 14,000–18,000 x *g* for most microcentrifuges. After centrifugation, the supernatant should be clear. If the supernatant is not clear, a second, shorter centrifugation should be carried out to avoid applying any suspended or particulate material to the column. Suspended material (which causes the sample to appear turbid) will clog the column and reduce or eliminate flow.

   ☞ Remove a 50 µl sample from the cleared lysate and save it for an analytical gel (sample 1).

8. **Equilibrate a QIAGEN-tip 20 by applying 1 ml Buffer QBT, and allow the column to empty by gravity flow.**

   Place QIAGEN-tips into a QIAvack over the waste tray or use the tip holders provided with each kit (see "Setup of QIAGEN-tips" page 13). Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

9. **Apply the supernatant from step 7 to the QIAGEN-tip 20 and allow it to enter the resin by gravity flow.**

   The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again before loading to prevent clogging of the QIAGEN-tip.

   ☞ Remove a 50 µl sample of the flow-through and save for an analytical gel (sample 2).

10. **Wash the QIAGEN-tip 20 with 2 x 2 ml Buffer QC.**

    Allow Buffer QC to move through the QIAGEN-tip by gravity flow.

    ☞ Remove a 220 µl sample of the combined wash fractions and save for an analytical gel (sample 3).

11. **Elute DNA with 0.8 ml Buffer QF.**

    Collect the eluate in a 1.5 ml or 2 ml microcentrifuge tubes (not supplied).

    Note: For constructs larger than 45–50 kb, prewarming the elution buffer to 65°C may help to increase yield.

    ☞ Remove a 45 µl sample of the eluate and save for an analytical gel (sample 4).

12. **Precipitate DNA by adding 0.7 volumes (0.56 ml per 0.8 ml of elution volume) of room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥10,000 rpm for 30 min in a microcentrifuge. Carefully decant the supernatant.**

    All solutions should be at room temperature in order to minimize salt precipitation. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

13. **Wash DNA pellet with 1 ml of 70% ethanol and centrifuge at 10,000 rpm for 10 min. Carefully decant the supernatant without disturbing the pellet.**

    The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

14. **Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10mM Tris·Cl, pH 8.5)**

    Redissolve the DNA pellet by rinsing the walls to recover all the DNA. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm and quantitative analysis on an agarose gel. For reliable spectrophotometric DNA quantification, A_{260} readings should lie between 0.1 and 1.0.

Agarose gel analysis

We recommend removing and saving aliquots during the purification procedure (samples 1–4). If the plasmid DNA is of low yield or quality, the samples can be analyzed by agarose gel electrophoresis to determine at what stage of the purification procedure the problem occurred (see page 41).

Appendix 8.3 *Qiagen plasmid preparation kit protocol taken directly from the manufacturers handbook 'Qiagen Plasmid preparation handbook, Third Edition, Novermber 2005'.*

Invitrogen
life technologies

SuperScript™ III First-Strand Synthesis System for RT-PCR

Cat. No: 18080-051 **Size: 50 reactions**

Store at -20°C

Description

The SuperScript™ III First-Strand Synthesis System for RT-PCR is optimized to synthesize first-strand cDNA from purified poly(A)$^+$ or total RNA. RNA targets from 100 bp to >12 kb can be detected with this system. The amount of starting material can vary from 1 pg to 5 µg of total RNA. SuperScript™ III Reverse Transcriptase is a version of M-MLV RT that has been engineered to reduce RNase H activity and provide increased thermal stability. The enzyme is used to synthesize cDNA at a temperature range of 42–55°C, providing increased specificity, higher yields of cDNA, and more full-length product than other reverse transcriptases. Because SuperScript™ III RT is not significantly inhibited by ribosomal and transfer RNA, it may be used to synthesize first-strand cDNA from a total RNA preparation.

cDNA synthesis is performed in the first step using either total RNA or poly(A)$^+$-selected RNA primed with oligo(dT), random primers, or a gene-specific primer. In the second step, PCR is performed in a separate tube using primers specific for the gene of interest. For the PCR reaction, we recommend one of the following DNA polymerases: Platinum® *Taq* DNA Polymerase provides automatic hot-start conditions for increased specificity up to 4 kb, Platinum® *Taq* DNA Polymerase High Fidelity provides increased yield and high fidelity for targets up to 15 kb, and Platinum® *Pfx* DNA Polymerase provides maximum fidelity for targets up to 12 kb.

System Component	Amount
Oligo(dT)$_{20}$ (50 µM)	50 µl
Random hexamers (50 ng/µl)	250 µl
10X RT buffer*	1 ml
25 mM $MgCl_2$	500 µl
0.1 M DTT	250 µl
10 mM dNTP mix	250 µl
SuperScript™ III RT (200 U/µl)	50 µl
RNaseOUT™ (40 U/µl)	100 µl
E. coli RNase H (2 U/µl)	50 µl
DEPC-treated water	1.2 ml
Total HeLa RNA (10 ng/µl)	20 µl
Sense Control Primer (10 µM)	25 µl
Antisense Control Primer (10 µM)	25 µl

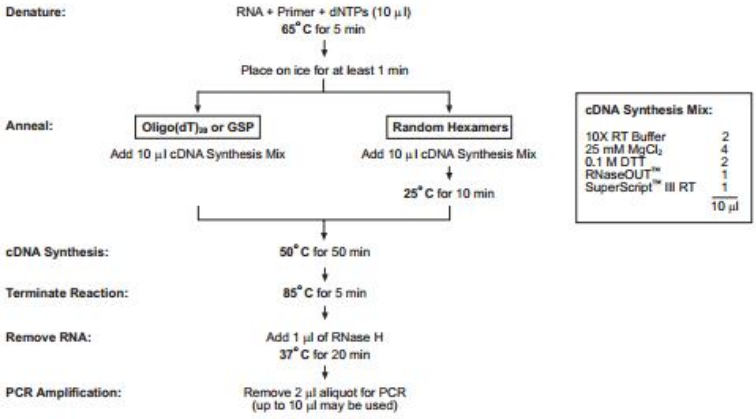
*200 mM Tris-HCl (pH 8.4), 500 mM KCl

Related products	Amount	Catalog No.
Platinum® *Taq* DNA Polymerase	100 units	10966-018
	250 units	10966-026
	500 units	10966-034
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
	500 units	11304-029
Platinum® *Pfx* DNA Polymerase	100 units	11708-013
	250 units	11708-021
	500 units	11708-039
PCR$_x$ Enhancer System	250 rxns	11495-017
Micro-to-Midi Total RNA Purification System	50 rxns	12183-018
TRIzol® Reagent	100 ml	15596-026
	200 ml	15596-018
DNase I, Amplification Grade	100 units	18068-015
Custom Primers	to order, visit www.invitrogen.com	

Quality Control

A minimum of 25 ng of a 353-bp RT-PCR product was obtained from 100 pg of total HeLa RNA and human β-actin primers. A minimum of 25 ng of a 6.8-kb RT-PCR product was obtained from 500 ng of total HeLa RNA and human Pol ε primers.

Summary of Procedure



18080051.pps Rev. date: 3 Oct 2003

Recommendations and Guidelines for First-Strand Synthesis

RNA

- High-quality, intact RNA is essential for full-length, high-quality cDNA synthesis. This kit is designed for use with 1 pg to 5 µg of total RNA or 1 pg to 500 ng of poly(A)⁺ RNA. For >5 µg total RNA, increase reaction volumes and amount of SuperScript™ III RT proportionally.
- RNaseOUT™ Recombinant RNase Inhibitor has been added to the system to safeguard against degradation of target RNA due to ribonuclease contamination of the RNA preparation.
- To isolate total RNA, we recommend the Micro-to-Midi Total RNA Purification System (Cat. no. 12183-018), TRIzol® Reagent (Cat. Nos. 15596-026/-018), or the Chomczynski and Sacchi method. Oligo (dT)-selection for poly(A)⁺ RNA is typically not necessary, although it may improve the yield of specific cDNAs.
- Small amounts of genomic DNA in the RNA preparation may be amplified along with the target cDNA. If your application requires removal of all genomic DNA from your RNA preparation, we recommend using DNase I, Amplification Grade (Catalog no. 18068-015). DNase I, Amplification Grade, has been extensively purified to remove trace ribonuclease activities commonly associated with other "RNase-free" enzyme preparations, and does not require the addition of placental RNase inhibitor.

RNase H Digestion

The sensitivity of the PCR step can be increased (especially for long templates) by removing the RNA template from the cDNA:RNA hybrid molecule by digestion with RNase H after first-strand synthesis. Presence of RNase H during first-strand synthesis will degrade the template mRNA, resulting in decreased full-length cDNA synthesis and decreased yields of first-strand cDNA. The SuperScript™ III First-Strand Synthesis System introduces RNase H activity only when it is beneficial, and thus offers a unique procedural advantage over other methods.

Primers

The first-strand cDNA synthesis reaction can be primed using random hexamers, oligo(dT), or gene-specific primers (GSPs):

- Random hexamers are the most nonspecific priming method, and are typically used when the mRNA is difficult to copy in its entirety. With this method, all RNAs in a population are templates for first-strand cDNA synthesis, and PCR primers confer specificity during PCR. To maximize the size of cDNA, you should determine the ratio of random hexamers to RNA empirically for each RNA preparation.

  Note: For most RT-PCR applications, 50 ng of random hexamers per 5 µg of total RNA is adequate. Increasing hexamers to 250 ng per 5 µg of RNA may increase yield of small PCR products (<500 bp), but may decrease the yield of longer PCR products and full-length transcripts.
- Oligo(dT), a more specific priming method, is used to hybridize to 3′ poly(A) tails, which are found in the vast majority of eukaryotic mRNAs. Since poly(A)⁺ RNA constitutes approximately 1% to 2% of total RNA, the amount and complexity of cDNA is considerably less than with random hexamers. We recommend using oligo(dT)$_{20}$ (provided in the kit).

  Note: Oligo(dT) is recommended over random hexamers or GSPs when performing RT-PCR with new mRNA targets. Oligo(dT) produces an RT-PCR product more consistently than random hexamers or GSPs.
- The most specific priming method uses a gene-specific primer for the sequence of interest. First-strand synthesis can be primed with the PCR primer that hybridizes nearest to the 3′ terminus of the mRNA. Note that some GSPs fail to prime cDNA synthesis even though they work in PCR on DNA templates. If gene-specific priming fails in RT-PCR, repeat first-strand synthesis using oligo(dT) as the primer.

First-Strand cDNA Synthesis

The following procedure is designed to convert 1 pg to 5 µg of total RNA or 1 pg to 500 ng of poly(A)⁺ RNA into first-strand cDNA:

1. Mix and briefly centrifuge each component before use.
2. Combine the following in a 0.2- or 0.5-ml tube:

Component	Amount
up to 5 µg total RNA	*n* µl
Primer*	1 µl
*50 µM oligo(dT)$_{20}$, *or* 2 µM gene-specific primer (GSP), *or* 50 ng/µl random hexamers	
10 mM dNTP mix	1 µl
DEPC-treated water	to 10 µl

3. Incubate at 65°C for 5 min, then place on ice for at least 1 min.
4. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order.

Component	1 Rxn	10 Rxns
10X RT buffer	2 µl	20 µl
25 mM $MgCl_2$	4 µl	40 µl
0.1 M DTT	2 µl	20 µl
RNaseOUT™ (40 U/µl)	1 µl	10 µl
SuperScript™ III RT (200 U/µl)	1 µl	10 µl

5. Add 10 µl of cDNA Synthesis Mix to each RNA/primer mixture, mix gently, and collect by brief centrifugation. Incubate as follows.

   Oligo(dT)$_{20}$ or GSP primed: 50 min at 50°C

   Random hexamer primed: 10 min at 25°C, followed by 50 min at 50°C
6. Terminate the reactions at 85°C for 5 min. Chill on ice.
7. Collect the reactions by brief centrifugation. Add 1 µl of RNase H to each tube and incubate for 20 min at 37°C.
8. cDNA synthesis reaction can be stored at -20°C or used for PCR immediately.

Amplification of Target cDNA

The first-strand cDNA obtained in the synthesis reaction may be amplified directly using PCR. We recommend using 10% of the first-strand reaction (2 µl) for PCR. However, for some targets, increasing the amount of first-strand reaction up to 10 µl in PCR may result in increased product yield.

We recommend the following DNA polymerases (for ordering information, see page 1):

- **Platinum® *Taq* DNA Polymerase** provides automatic hot-start conditions for increased specificity and sensitivity. It is recommended for targets up to 4 kb.
- **Platinum® *Taq* DNA Polymerase High Fidelity** provides increased fidelity and higher yields for targets up to 15 kb.
- **Platinum® *Pfx* DNA Polymerase** possesses a proofreading 3′ to 5′ exonuclease activity and provides maximum fidelity for PCR. It is recommended for targets up to 12 kb.

Consult the product documentation provided with each DNA polymerase for recommended protocols and optimization guidelines. Documentation is also available on our Web site at www.invitrogen.com.

Control Reactions

The control RNA provided with this system consists of total HeLa RNA (10 ng/µl). The sense and antisense control primers provided with this kit are designed from the human β-actin gene and produce a 353-bp RT-PCR product.

Sense primer: 5'-GCTCG TCGTC GACAA CGGCT C-3'
Antisense primer: 5'-CAAAC ATGAT CTGGG TCATC TTCTC-3'

Use the following protocol for both plus and minus RT control reactions:

1. Dilute the total HeLa RNA to 100pg/µl with DEPC-treated water.
2. Prepare the RNA/primer mixtures in sterile 0.2- or 0.5-ml tubes as follows:

Component	+ RT Control	– RT Control
Diluted total HeLa RNA (100 pg/µl)	1 µl	1 µl
$Oligo(dT)_{20}$	1 µl	1 µl
10 mM dNTP mix	1 µl	1 µl
DEPC-treated water	7 µl	7 µl

3. Incubate samples at 65°C for 5 min, then place on ice for at least 1 min. Collect by brief centrifugation and add the following:

Component	+ RT Control	– RT Control
10X RT buffer	2 µl	2 µl
25 mM $MgCl_2$	4 µl	4 µl
0.1 M DTT	2 µl	2 µl
RNaseOUT™ (40 U/µl)	1 µl	1 µl
SuperScript™ III RT (200 U/µl)	1 µl	—
DEPC-treated water	—	1 µl

4. Mix gently and collect the reactions by brief centrifugation.
5. Incubate at 50°C for 50 min.
6. Terminate the reactions at 85°C for 5 min. Chill on ice.
7. Collect the reactions by brief centrifugation. Add 1 µl of RNase H to each tube and incubate for 20 min at 37°C.
8. Prepare a PCR mixture for each control reaction. For each control reaction, add the following to a 0.2-ml tube sitting on ice:

Component	Volume
DEPC-treated water	38.1 µl
10X PCR buffer minus Mg^{++}	5 µl
50 mM $MgCl_2$	1.5 µl
10 mM dNTP mix	1 µl
Control sense primer (10 µM)	1 µl
Control antisense primer (10 µM)	1 µl
cDNA from control RNA	2 µl
Taq DNA polymerase (5 units/µl)	0.4 µl
final volume	50 µl

9. Mix the contents of the tube. Centrifuge briefly to collect the reaction components.
10. Place reaction mixture in preheated (94°C) thermal cycler. Perform an initial denaturation step: 94°C for 2 min.
11. Perform 40 cycles of PCR:

    Denature 94°C for 15 sec
    Anneal 55°C for 30 sec
    Extend 68–72°C for 1 min

    Note: For slow-ramping thermal cyclers, follow manufacturer's directions.
12. Upon completion, maintain reactions at 4°C.
13. Analyze 10 µl of each sample, using agarose gel electrophoresis and ethidium bromide staining. A 353-bp band, corresponding to at least 25 ng of product, should be visible for the + RT Control sample. No band should be visible for the – RT Control sample.

First Strand cDNA Synthesis of Transcripts with High GC Content

High-GC content mRNAs often contain stable intrinsic secondary structures that can inhibit reverse transcriptase and/or primer annealing. Problems with RT-PCR due to this secondary structure often can be overcome by increasing the volume and temperature of the RT reaction.

Note: For templates that require cDNA synthesis temperatures above 55°C, we recommend the ThermoScript™ RT-PCR System (Catalog no. 11146-024). ThermoScript™ RT supports cDNA synthesis up to 70°C.

This protocol is suitable for gene-specific or oligo(dT) primers, but not random hexamers.

1. Mix and briefly centrifuge each component before use.
2. Prepare the RNA/primer mixture in a sterile 0.5-ml tube as follows:

Component	Sample	Control RNA
1 to 5 µg total RNA	*n* µl	—
Control total HeLa RNA (10 ng/µl)	—	1 µl
$Oligo(dT)_{20}$ (50 µM) *or* 2 µM GSP	1 µl	1 µl
10 mM dNTP mix	2.5 µl	2.5 µl
DEPC-treated water	to 25 µl	to 25 µl

3. Incubate each sample at 65°C for 5 min and immediately transfer to 55°C.
4. Prepare the cDNA Synthesis Mix, adding each component in the indicated order.

Component	1 Reaction	10 Reactions
DEPC-treated water	3 µl	30 µl
10X RT buffer	5 µl	50 µl
25 mM $MgCl_2$	10 µl	100 µl
0.1 M DTT	5 µl	50 µl
RNaseOUT™ Recombinant RNase Inhibitor	1 µl	10 µl
SuperScript™ III RT	1 µl	10 µl

Note: For a minus RT control reaction, substitute 1 µl of DEPC-treated water for 1 µl of SuperScript™ III RT, and assemble reaction as described above.

5. Prewarm the cDNA Synthesis Mix to 55°C.
6. To each sample incubating at 55°C, add 25 µl of prewarmed cDNA Synthesis Mix. Mix gently, and incubate at 55°C for 50 min.
7. Terminate the reactions at 85°C for 5 min. Chill on ice.
8. Collect the reactions by brief centrifugation. Add 1 µl of RNase H to each tube and incubate for 20 min at 37°C before proceeding to PCR.

Note: Frequently, problems associated with RT-PCR of GC-rich cDNA are related to PCR as well as first-strand synthesis. We recommend using the PCR$_x$ Enhancer System (Catalog no. 11495-017) to facilitate amplification of GC-rich sequences.

Troubleshooting Guide

Problem	Possible Cause	Probable Solution
No bands after electrophoretic analysis of amplified products	Procedural error in first-strand cDNA synthesis	Use the total HeLa RNA provided as a control to verify the efficiency of the first-strand reaction (see page 3).
	RNase contamination	Add control RNA to sample to determine if RNase is present in the first-strand reaction. Maintain aseptic conditions to prevent RNase contamination. Use RNaseOUT™ Recombinant RNase Inhibitor in the first-strand reaction.
	Polysaccharide coprecipitation of RNA	Precipitate RNA with lithium chloride to remove polysaccharides, as described in Sambrook *et al.*
	Target mRNA contains strong transcriptional pauses	Use random hexamers instead of oligo(dT) in the first-strand reaction. Maintain an elevated temperature after the annealing step, as described in the protocol for cDNA synthesis from high-GC content transcripts, page 3. Increase the temperature of first-strand reaction (up to 55°C). Use PCR primers closer to the 3′ terminus of the target cDNA.
	Too little first-strand product was used in PCR	Use up to 10 µl of the first-strand reaction.
	GSP was used for first-strand synthesis	Try another GSP or switch to oligo(dT). Make sure the GSP is the antisense sequence.
	Inhibitors of RT present	Remove inhibitors by ethanol precipitation of mRNA preparation before the first-strand reaction. Include a 70% (v/v) ethanol wash of the mRNA pellet. **Note:** Inhibitors of RT include sodium dodecyl sulfate (SDS), EDTA, guanidinium salts, formamide, sodium pyrophosphate, and spermidine.
Unexpected bands after electrophoretic analysis	Contamination by genomic DNA	Pretreat RNA with DNase I, Amplification Grade (Cat. no. 18068-015), as described in the DNase I documentation. Design primers that anneal to sequence in exons on both sides of an intron or at the exon/exon boundary of the mRNA to differentiate between amplified cDNA and potential contaminating genomic DNA. To test if products were derived from DNA, perform the minus RT control.
	Nonspecific annealing of primers	Vary the annealing conditions. Use Platinum® *Taq* DNA Polymerase for automatic hot-start PCR. Optimize magnesium concentration for each template and primer combination.
	Primers formed dimers	Design primers without complementary sequences at the 3′ ends.

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Appendix 8.4 *Invitrogen protocol taken directly from the manufacturers instructions for Superscript III First-Strand synthesis system for RT-PCR.*