

# **Generation and Optimisation of Novel Recombinant Antibodies for Improved Cardiac Diagnostics**

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

**by**

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**March, 2011.**

**Declaration:**

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, 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.

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*Dedicated to*

My late father

**Mr. Gopalan Balasubramaniam Ayyar**

who died due to myocardial infarction during the  
course of this research.

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## **Abstract**

Cardiovascular diseases (CVD) are major health problems causing huge socio-economic burdens worldwide. Biomarkers play an essential role in their early and accurate diagnosis, and the objective of this research was the generation and optimisation of novel recombinant antibodies/assays to the tissue injury-associated biomarkers cardiac troponin I (cTnI) and heart fatty acid binding protein (hFABP).

Rabbits and chickens were selected as hosts for immunisations due to the advantages inherent in the genetics of their immune systems. High sensitivity antibodies were generated and their specificity, affinity and stability determined using both 'real-time' studies and 'end-point' analysis. An anti-hFABP scFv was successfully incorporated into an inhibition ELISA and a lateral flow immunoassay and its epitope specificity was examined using peptide and gene fragment libraries. The anti-cTnI scFv was engineered to overcome the expression difficulties encountered with rabbit scFvs. Eight different antibody formats (2 scFv and 6 scAb) were generated to investigate/improve expression and purification.

These studies also required the recombinant expression of both hFABP and cTnI in *E. coli*. The expressed proteins were characterised using anti-His and antigen-specific antibodies.

The work undertaken has generated a range of antibodies and antigens of significant value for assay development and potential future commercialisation.

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## ABBREVIATIONS AND ACRONYMS

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Abbreviation / Acronym	Full form
<b>Ab</b>	Antibody
<b>Abs</b>	Absorbance
<b>ACC</b>	American College of Cardiology
<b>ACS</b>	Acute coronary syndrome
<b>aFABP</b>	Adipocyte fatty acid binding protein
<b>Ag</b>	Antigen
<b>AHA</b>	American Heart Association
<b>AMI</b>	Acute myocardial infarction
<b>AST</b>	Aspartate aminotransferase
<b>ATP</b>	Adenosine triphosphate
<b>bFABP</b>	Brain fatty acid binding protein
<b>BLAST</b>	Basic Local Alignment Search Tool
<b>BNP</b>	Brain natriuretic peptide
<b>BSA</b>	Bovine serum albumin
<b>cDNA</b>	Complementary DNA
<b>CDR</b>	Complementary determining region
<b>cfu</b>	Colony-forming units
<b>C<sub>H</sub></b>	Constant heavy chain
<b>CK</b>	Creatine kinase
<b>C<sub>L</sub></b>	Constant light chain
<b>CRP</b>	C-reactive protein
<b>CSC-F</b>	Chicken scFv forward
<b>CSC-B</b>	Chicken scFv backward
<b>CT</b>	Carboxyl-terminal
<b>cTnI</b>	Cardiac troponin C
<b>cTnI</b>	Cardiac troponin I
<b>cTnT</b>	Cardiac troponin T
<b>CV</b>	Coefficient of variation
<b>CVD</b>	Cardiovascular disease
<b>C</b>	Constant kappa chain
<b>C</b>	Constant lambda chain
<b>dH<sub>2</sub>O</b>	Distilled water
<b>DMSO</b>	Dimethyl sulfoxide

<b>DNA</b>	Deoxyribonucleic acid
<b>dNTP</b>	Deoxyribonucleotide triphosphate
<b>dt</b>	Different oligo
<b>DTT</b>	Di-thiotheritol
<b>e.g.</b>	<i>exempli gratia</i> (Latin for ‘for example’)
<b>ECG</b>	Electrocardiogram
<b>ED</b>	Emergency Department
<b>EDC</b>	N-ethyl-N -(dimethyl-aminopropyl)-carbodiimide hydrochloride
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>ESC</b>	European Society of Cardiology
<b><i>et al.</i></b>	<i>et alia</i> (Latin for ‘and the rest’)
<b>etc.</b>	<i>et cetera</i> (Latin for ‘and so on’)
<b>EU</b>	European Union
<b>Fab</b>	Antibody binding fragment; of an antibody
<b>FABP</b>	Fatty acid binding protein
<b>Fc</b>	Fragment (crystallisable); of an antibody
<b>FCA</b>	Freund’s complete adjuvant
<b>FICA</b>	Freund’s incomplete adjuvant
<b>FR</b>	Framework region
<b>GALT</b>	Gut-associated lymphoid tissues
<b>HA</b>	Hemagglutinin
<b>HBS</b>	Hepes buffered saline
<b>HCS</b>	Heavy chain stuffer
<b>HEXA</b>	Hexosaminidase A
<b>hFABP</b>	Heart-fatty acid binding protein
<b>HGPRT</b>	Hypoxanthine guanine phosphoribosyl transferase
<b>His</b>	Histidine
<b>HRP</b>	Horseradish peroxidase
<b>HV</b>	Hypervariable
<b>i.e.</b>	<i>id est</i> (Latin for ‘that is’)
<b>Ig</b>	Immunoglobulin
<b>IL</b>	Interleukin
<b>IMA</b>	Ischemia modified albumin
<b>IMAC</b>	Immobilised metal affinity chromatography
<b>IMS</b>	Industrial methylated spirits

<b>IPTG</b>	Isopropyl- $\beta$ -D-1-thiogalactopyranoside
<b>K<sub>a</sub></b>	Association rate constant (units = M <sup>-1</sup> s <sup>-1</sup> )
<b>K<sub>A</sub></b>	Equilibrium association constant (units = M <sup>-1</sup> )
<b>K<sub>d</sub></b>	Dissociation rate constant (units = s <sup>-1</sup> )
<b>K<sub>D</sub></b>	Equilibrium dissociation constant (units = M)
<b>KLH</b>	Keyhole Limpet Hemocyanin
<b>LB</b>	Luria-Bertani
<b>LDH</b>	Lactate dehydrogenase
<b>IFABP</b>	Liver fatty acid binding protein
<b>LFIA</b>	Lateral flow immunoassay
<b>mAb</b>	Monoclonal antibody
<b>MI</b>	Myocardial infarction
<b>MLC</b>	Myosin light chain
<b>MOPS</b>	3-(N-Morpholino)-propane sulfonic acid
<b>MPO</b>	Myeloperoxidase
<b>MWCO</b>	Molecular weight cut-off
<b>N/A</b>	Not applicable
<b>NACB</b>	National Academy of Clinical Biochemistry
<b>NHS</b>	N-hydroxysuccinimide
<b>NSTEACS</b>	Non-ST-segment elevation myocardial infarction
<b>N-terminal</b>	Amino terminal
<b>O/N</b>	Overnight
<b>OD</b>	Optical density
<b>pAb</b>	Polyclonal antibody
<b>PaPP-A</b>	Pregnancy-associated plasma protein A
<b>PBS</b>	Phosphate buffered saline
<b>PBST</b>	Phosphate buffered saline-Tween
<b>PCR</b>	Polymerase chain reaction
<b>PEG</b>	Polyethylene glycol
<b>pfu</b>	Plaque-forming units
<b>pI</b>	Isoelectric point
<b>PIGF</b>	Placental growth factor
<b>PSA</b>	Prostate specific antigen
<b>rAb</b>	Recombinant antibody
<b>RT</b>	Room temperature
<b>RU</b>	Response unit
<b>SB</b>	Super broth

<b>scAb</b>	Single chain antibody
<b>scFv</b>	Single chain variable fragment
<b>SDS-PAGE</b>	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
<b>SOC</b>	Super optimal catabolites
<b>SOE</b>	Splice-by-overlap extension
<b>ssDNA</b>	Single-stranded DNA
<b>STEMI</b>	ST-segment elevation myocardial infarction
<b>TAT</b>	Turn-around time
<b>TMB</b>	Tetramethylbenzidine dihydrochloride
<b>TNF</b>	Tumour necrosis factor-
<b>UV</b>	Ultraviolet
<b>V<sub>H</sub></b>	Variable heavy chain
<b>V<sub>L</sub></b>	Variable light chain
<b>WHO</b>	World Health Organisation



## UNITS

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Unit	Full form
<b>μF</b>	Microfarad
<b>μg</b>	Microgram
<b>μL</b>	Microlitre
<b>μm</b>	Micrometres
<b>cm</b>	Centimetre
<b>Da</b>	Daltons
<b>G</b>	Gram
<b>kDa</b>	Kilodaltons
<b>kV</b>	Kilovolt
<b>L</b>	Litre
<b>M</b>	Molar
<b>mg</b>	Milligram
<b>mL</b>	Millilitre
<b>mm</b>	Millimeter
<b>mM</b>	Millimolar
<b>nm</b>	Nanometer
<b>nM</b>	Nanomolar
<b>°C</b>	Degrees Celcius
<b>rpm</b>	Révolutions per minute
<b>U</b>	Unit
<b>v/v</b>	Volume per unit volume
<b>w/v</b>	Weight per unit volume
<b>x g</b>	Centrifugal acceleration
	Ohm

### **Research Publications:**

**Ayyar, B.V.**, Hearty, S. and O’Kennedy, R. (2010). Highly sensitive recombinant antibodies capable of reliably differentiating heart-type fatty acid binding protein from noncardiac isoforms. *Analytical Biochemistry* **407**, 165-171.

**Ayyar, B.V.**, Hearty, S. and O’Kennedy, R. Facile domain re-formatting overcomes expression recalcitrance in a rabbit antibody. (*Manuscript in preparation*).

### **Invited Review:**

Hearty, S., Conroy, P.J., **Ayyar, B.V.**, Byrne, B. and O’Kennedy, R. (2010). Surface plasmon resonance for vaccine design and efficacy studies. *Expert Review of Vaccines* **9**, 645-664.

### **Posters and Presentations:**

**Ayyar, B.V.**, Hearty, S. and O’Kennedy, R. (2011). Development of sensitive immunoassays for reliable detection of the cardiac isoform of fatty acid binding protein. The 6<sup>th</sup> Conference on Analytical Sciences in Ireland (CASi 2011), Dublin, Ireland (February 21-22, 2011).

**Ayyar, B.V.**, Hearty, S. and O’Kennedy, R. (2011). Engineering rabbit antibody to overcome expression recalcitrance. The 3<sup>rd</sup> Annual School of Biotechnology Research Day, Dublin City University, Dublin, Ireland (January 28, 2011).

**Ayyar, B.V.**, Hearty, S. and O’Kennedy, R. (2011). Highly sensitive recombinant antibodies capable of reliably differentiating the heart fatty acid binding protein from other non-cardiac isoforms. The 3<sup>rd</sup> Annual School of Biotechnology Research Day, Dublin City University, Dublin, Ireland (January 28, 2011).

**Ayyar, B.V.**, Hearty, S. and O’Kennedy, R. (2010). Facile domain re-formatting overcomes expression recalcitrance in a rabbit antibody. IBC's 21<sup>st</sup> Annual Antibody Engineering Conference, San Diego (December 5-9, 2010).

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Hearty, S., McDonnell, B., Leonard, P., **Ayyar, B.V.**, Gilmartin, N., Conroy, P.J., Desmonts, L.B., Hill, D., MacCraith, B.D. and O’Kennedy R. (2008). Optimized isolation of recombinant antibodies for biosensor development. Europtrode IX Conference, Dublin, Ireland (March 30-April 02, 2008).

# **Chapter 1**

## **Introduction**

## **1. INTRODUCTION**

### **1.1 Cardiovascular Diseases**

#### **1.1.1 Introduction to Cardiovascular Diseases**

Cardiovascular disease (CVD) is a general term used to describe disorders affecting the heart or blood vessels and includes arteriosclerosis, coronary artery disease, heart valve disease, arrhythmia, heart failure, hypertension, orthostatic hypotension, shock, endocarditis, diseases of the aorta and its branches, disorders of the peripheral vascular system, cerebrovascular disease (stroke and transient ischemic attack) and congenital heart disease (Ha *et al.*, 2007).

Cardiovascular diseases (CVDs) are leading health problems, reaching epidemic proportions in both developed and developing countries (Jafar, 2006; Paccaud, 2006; Bartunek, 2009). CVDs are global killers and deaths resulting from CVDs surpass any other cause (Table 1.1). They are also expensive on a world-wide scale (Table 1.1). Considering the huge economic impact and premature deaths, prevention is imperative in reducing the global burden of CVDs. Timely identification can facilitate the stratification of the individuals at increased risk from CVD.

Numerous clinical and statistical studies have identified certain factors that enhance the risk of CVDs. The major risk factors include high cholesterol, tobacco and alcohol consumption, hypertension, obesity, physical inactivity, diabetes, unhealthy diets, and family history (Goyal and Yusuf, 2006; Gaziano, 2007).

**Table 1.1: Global socio-economic burden of cardiovascular diseases.**

Social burden of CVDs
<p><i>CVDs – the global picture</i></p> <ul style="list-style-type: none"> <li>• CVDs cause most deaths globally every year than any other cause</li> <li>• In 2006 the overall death rate from CVDs was 262.5 per 1,00,000</li> <li>• About 82% of CVD deaths take place in low- and middle-income countries</li> <li>• CVD occur almost equally in men and women</li> <li>• CVD risk is particularly high in women after menopause</li> <li>• CVD accounted for 17.4 million deaths (30% of all deaths) globally in 2005</li> <li>• It is estimated that almost 23.6 million people will die from CVDs, by 2030</li> </ul> <p><i>CVDs in Australia and New Zealand</i></p> <ul style="list-style-type: none"> <li>- are the leading health problem</li> <li>- affected 20% of Australian population in 2000</li> <li>- cause disability in 1.4 million Australian, preventing them from living full life</li> <li>- kill one Australian nearly every 10 minutes</li> <li>- affect more than 3.7 million people in Australia</li> <li>- accounted for death of 46,000 Australians (34% of all deaths) in 2006</li> <li>- are the leading death cause in New Zealand</li> <li>- accounted for 40% of all deaths in New Zealand in 2000</li> </ul> <p><i>CVDs in Europe and European Union</i></p> <ul style="list-style-type: none"> <li>- are leading cause of death</li> <li>- cause 48% of all deaths in Europe and 42% of all deaths in the European Union (EU)</li> <li>- are main death cause for women in all European countries and main death cause for men in all European countries except France, the Netherlands and Spain</li> <li>- account for over 4.3 million and 2 million deaths each year in Europe and EU, respectively</li> </ul> <p><i>CVDs in India and Pakistan</i></p> <ul style="list-style-type: none"> <li>- will be the largest cause of disability and death in India by 2020</li> <li>- occurs 10 to 15 years earlier in India than in the west</li> <li>- resulted in loss of 9.2 million productive years of life in India in 2000</li> <li>- accounted for 29% of all deaths in India in 2005</li> <li>- lead to 1,54,338 deaths in Pakistan in 2002</li> </ul> <p><i>CVDs in Ireland and UK</i></p> <ul style="list-style-type: none"> <li>- are the largest health problem</li> <li>- are most common cause of death in Ireland</li> <li>- cause 22% of all premature deaths (deaths before the age of 65) in Ireland</li> <li>- account for 36% of all Irish deaths</li> <li>- killed approximately 4,929 Irish men and 4,733 Irish women in 2006</li> <li>- main cause of premature deaths (deaths under the age of 75) in UK; with 30% and 22% of premature deaths in man and women, respectively in 2006</li> <li>- account for more than one in three (35%) deaths each year in UK</li> <li>- claimed around 1,98,000 lives in UK in 2006</li> </ul> <p><i>CVDs in USA and Canada</i></p> <ul style="list-style-type: none"> <li>- affect at least 80 million people in USA</li> <li>- have been the leading cause of death, in US, every year since 1900, except 1918</li> <li>- cause more deaths, in US, than the next five causes of death (cancer, chronic lower respiratory diseases, accidents, diabetes mellitus, and flu/pneumonia) combined</li> <li>- caused more than 2,300 deaths each day in US in 2006, at an average of one death every 38 seconds</li> <li>- claimed 8,31,272 lives (34.3% of all deaths) in US in 2006</li> <li>- kill a Canadian every 7 minute</li> <li>- claimed 71,338 Canadian lives (31% of all deaths) in 2005</li> </ul>
Economic burden of CVDs
<ul style="list-style-type: none"> <li>▪ In Australia, the total health care expenditure for CVDs was \$5.942 billion in 2004-2005.</li> <li>▪ CVDs cost the Canadian economy more than \$22.2 billion every year.</li> <li>▪ In China, annual direct costs were estimated at €30.76 billion or 4% of gross national income for 2007.</li> <li>▪ CVDs cost the EU economy over €192 billion every year.</li> <li>▪ India lost 9 billion dollars in national income from premature deaths due to CVDs and diabetes in 2005.</li> <li>▪ Total cost of CVDs to the Irish economy was €769 million in 2006.</li> <li>▪ In South Africa, at least 25% of the country's health care spending is devoted to CVD.</li> <li>▪ The cost of cardiovascular diseases in USA for 2009 is estimated at \$475.3 billion.</li> <li>▪ CVDs cost the health care system in UK £14.4 billion each year; with an overall cost of £30.7 billion a year.</li> </ul>

(Compiled from Beaglehole and Yach, 2003; Goenka *et al.*, 2009; Lloyd-Jones *et al.*, 2010; <http://www.ehnheart.org/content/sectionintro.asp?level0=1457>; <http://www.americanheart.org/presenter.jhtml?identifier=4475>; <http://www.americanheart.org/downloadable/heart/1236204012112INTL.pdf>; <http://www.aihw.gov.au/publications/cvd/hcecd04-05/hcecd04-05.pdf>; <http://www.healthinitiative.org/html/whd/index.htm>; <http://www.heartandstroke.com/site/c.iQILcMWJtE/b.3483991/k.34A8/Statistics.htm>; [http://www.irishheart.ie/iopen24/facts-heart-disease-stroke-t-7\\_18.html](http://www.irishheart.ie/iopen24/facts-heart-disease-stroke-t-7_18.html); [http://www.heartfoundation.org.au/HEART\\_INFORMATION/STATISTICS/Pages/default.aspx](http://www.heartfoundation.org.au/HEART_INFORMATION/STATISTICS/Pages/default.aspx); <http://www.nhf.org.nz/index.asp?PageID=2145828139>; <http://www.heartstats.org/datapage.asp?id=713>; [http://www.nhmrc.gov.au/your\\_health/facts/cvd.htm](http://www.nhmrc.gov.au/your_health/facts/cvd.htm); <http://www.who.int/mediacentre/factsheets/fs317/en/index.html>; <http://www.worldheart.org/cardiovascular-health/global-facts-map/economic-impact/>).

Lack of awareness, absence of early diagnosis of disease risk, improper proactive disease management in emergency departments, ineffective interventions, high incidences of misdiagnosis, inappropriate referrals and high treatment costs further augment the problem (Goyal and Yusuf, 2006; Gaziano, 2007).

### **1.1.2 Pathophysiology**

Atherosclerosis, which is thickening of arterial walls, is a common process initiating the pathogenesis of certain major cardiovascular diseases, such as coronary heart disease, acute myocardial infarction, and ischemic stroke (Luc and Fruchart, 1991; Ross, 1993; Kaul *et al.*, 2000).

In response to injury or to an inflammatory milieu or under certain physiological conditions (Hort, 1985; Kaul *et al.*, 2000; Carreiro-Lewandowski, 2006) the endothelial cells of coronary artery / arteries may secrete adhesion molecules and selective chemokines attracting immune cells and lipids to a weakened or damaged vessel. This leads to adherence of T-lymphocytes and monocytes to the endothelium, and subsequently penetration into the tunica intima of blood vessels, where some monocytes differentiate into macrophages (Libby *et al.*, 1996). These macrophages ingest low

density lipoproteins (LDLs), resulting in formation of lipid-laden foam cells (Libby *et al.*, 1996), and together with lymphocytes, these form fatty streaks on the inner surface of the artery.

The foam cells express cytokines attracting the inflammatory molecules (Kruth, 2001). On their death, foam cells expel their contents into the sub-intimal space attracting additional macrophages and inflammatory cytokines, such as tissue necrosis factor- (TNF ), interleukin-1 (IL-1) and macrophage colony stimulating factor (MCSF), which further increase binding of LDLs to endothelium (Libby *et al.*, 1996; Zaman *et al.*, 2000). Smooth muscle cells from the neighbouring media layer also migrate into the area of vascular injury (Libby, 2008).

The smooth muscle cells differentiate into fibroblasts and release collagen, which deposits over the foam cells. Consequently foam cells undergo either necrosis or apoptosis, resulting in the formation of a fibrous plaque, an atherosclerotic plaque, containing a pool of lipids trapped beneath a fibrous cap (Figure 1.1). The plaques may also become calcified. The point where the fibrous cap joins the normal arterial wall, i.e. the shoulder of the plaque, continues to be active, and it is here that active foam cell formation continues as the plaque advances across the inner surface of the artery. These lesions in the arterial walls narrow the arterial lumen, called stenosis, and cause the hardening of the arteries, i.e. atherosclerosis (Carreiro-Lewandowski, 2006), leading to myocardial ischemia (decreased blood flow to heart muscles).

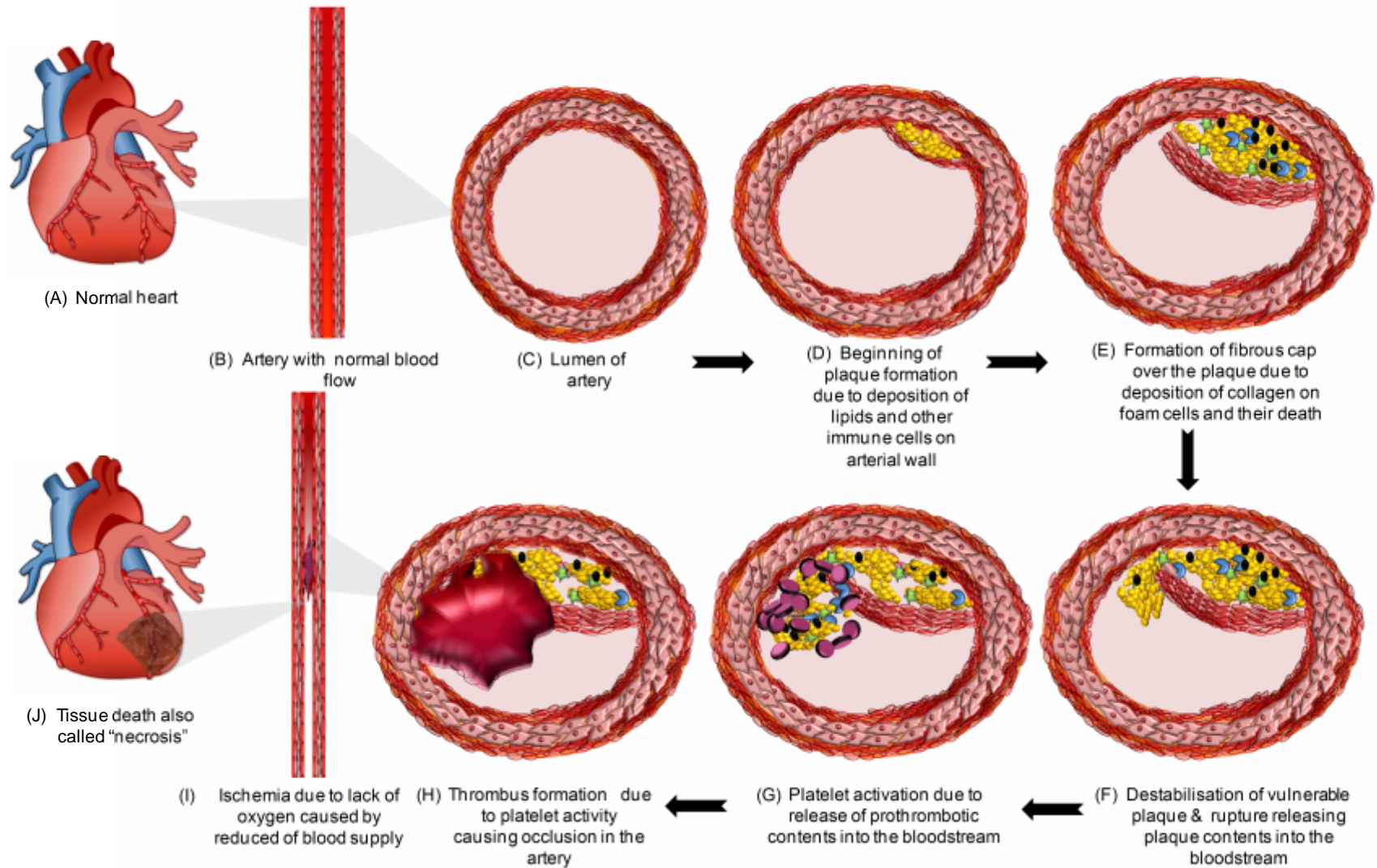
These events typically happen over a period of many years, often decades. Growth of plaques occurs discontinuously, with periods of relative quiescence interspersed by periods of rapid evolution. In certain cases, atherosclerosis may not manifest any



clinical symptoms, while in others atherosclerosis may become clinically manifested, following a silent period. The clinical manifestations may be chronic, as in the development of stable angina pectoris (chest pain or discomfort), or acute, such as myocardial infarction, stroke, or sudden cardiac death (Libby, 2008).

Another type of clinical manifestation involves rupture of plaques (Adams, 2004) (Figure 1.1). The fibrous cap of lipid-rich plaques, atheromatous plaques, is mechanically very weak, especially at the shoulder of the plaque (Penz *et al.*, 2005) as it is weakened by macrophage-derived foam cells, which release oxygen radicals and neutral proteases leading to degradation of the connective tissue matrix covering the plaque (Mitchinson and Ball, 1987; Richardson *et al.*, 1989). These plaques, also referred to as ‘vulnerable plaques’, may consequently rupture resulting in exposure of the contents of the plaque core to the circulating blood in arterial lumen (Figure 1.1). These plaques are rich in prothrombotic content causing platelet activation and leading to thrombus formation (Tipping *et al.*, 1989; Wilcox *et al.*, 1989; Semeraro *et al.*, 1990; Penz *et al.*, 2005) (Figure 1.1). The thrombus may be large enough to fully occlude the blood vessel or may be partially occluding (Figure 1.1). A fully occluding thrombus plugs the artery, stopping the supply of blood to a particular region of the heart, causing necrosis of the affected area and resulting in acute myocardial infarction (AMI); characterised as ST elevation MI (STEMI) on the basis of electrocardiogram (ECG). However, in some cases, MI, with less myocardial damage, can also result from partial occlusions, and are characterised as non-ST elevation MI (NSTEMI) (Bassand *et al.*, 2007). Cases of partial occlusion may also go undetected until a time when they clinically manifest as unstable angina pectoris (Carreiro-Lewandowski, 2006). In some cases, the thrombus may dissolve by thrombolysis or may form emboli (Brunette *et al.*, 2003). These emboli may be either cleared off, or may again lodge in smaller blood

vessels, leading to clinical symptoms, such as cardiac ischemia, unstable angina pectoris, MI, or cardiac death (Falk, 1985; Davies *et al.*, 1986).



**Figure 1.1:** *Chain of major events associated with Acute Myocardial Infarction (AMI).*

### **1.1.3 Acute Myocardial Infarction**

Acute myocardial infarction (AMI) accounts for greatest percentage of deaths among CVD. AMI strikes suddenly in the absence of previous warning, which makes timely diagnosis of AMI a major challenge. From a clinician's point of view the early recognition of cardiac ischemia and placing the patient accurately in the AMI risk spectrum are important aspects in determining the patient's outcome. Traditional diagnostic regimes fail to meet all these requirements which resulted in the World Health Organisation (WHO) defining AMI. According to the WHO criteria, AMI is diagnosed in the presence of two out of three characteristics comprising:

- Symptoms of acute ischemia (chest pain)

- Development of Q waves in ECG and

- Elevated activities of traditional serum enzymes

However, the development of more sensitive and specific serological biomarkers and precise imaging techniques over time allowed AMI to be defined accurately. Based on this AMI was redefined in 2000 by European Society of Cardiology (ESC) and American College of Cardiology (ACC) joint committee. A set of guidelines were established for characterising MI and these were added to the existing WHO criteria. The guidelines were based on the ability of biomarkers and high precision imaging techniques to measure minute amounts of necrosis. These guidelines were again revised in 2007 by ESC and other medical and scientific bodies which resulted in characterisation of AMI into different types (I, II, III, IVa, IVb and V) depending on different pathological conditions. According to the recent guidelines MI was defined as “myocardial necrosis in a clinical setting consistent

with myocardial ischemia”. Additionally, the following criteria should be met for MI to be diagnosed (Thygesen *et al.*, 2007):

- i. Detection of a rise and / or fall of cardiac troponin with at least one concentration above the 99<sup>th</sup> percentile value, together with evidence of myocardial ischemia with at least one of the following symptoms of ischemia
  - ECG changes indicative of new ischemia
  - Development of pathological Q waves in ECG;
  - Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.
- ii. Specific pathological findings.

In recent guidelines, analytical performance of the assay is taken into account which suggests optimal precision (percentage coefficient of variation (CV)) at the 99<sup>th</sup> percentile for each assay should be defined at <10% and recurrent measurement of cardiac troponins at the time of admission and 6 hours afterwards (Jaffe, 2008) (<http://www.aacc.org/publications/cln/2009/september/Pages/series0909.aspx>).

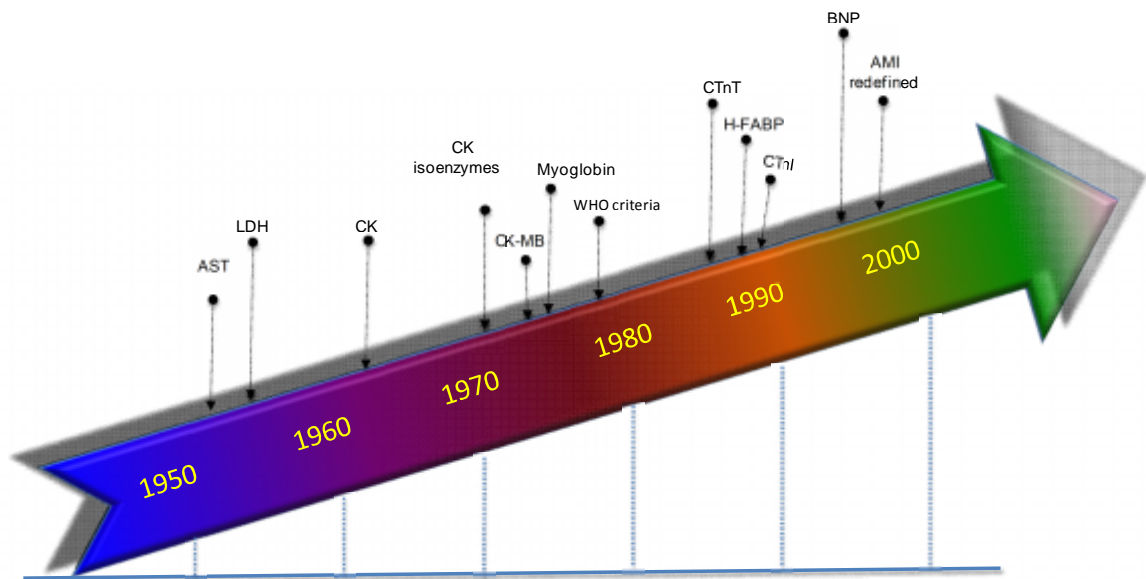
## **1.2 Biomarkers**

Biomarkers are defined as measurable biological substances in plasma and serum, apart from regular haematological and biochemical analytes (used in routine clinical studies), which serve as indicators for health and physiologically related assessments (Maas and Furie, 2009; Richards, 2009). Biomarkers are classified into three types: diagnostic aids, prognostic indicators and pathobiological markers of disease (Becker, 2005). Discovery of biomarkers lead to a revolution in medicine (Parikh and Vasan, 2007), by providing a

convenient and non-invasive insight in understanding the disease spectrum. Biomarkers play an important role in staging, diagnosis, disease classification, risk assessment, and prognosis of diseases in addition to assisting the monitoring of disease progression and the effect of therapy (Vasan, 2006; Classen *et al.*, 2008; O'Donoghue and Morrow, 2008).

### 1.2.1 Emergence of Biomarkers as Diagnostic Tools in Cardiovascular disease

Since the discovery of the first cardiovascular disease biomarker, aspartate aminotransferase, in 1954, these biochemical entities were studied intensively for understanding the disease by characterising clinical symptoms based on various pathobiological conditions in order to provide guidance in medical interventions (Lange, 2006; Bonaca and Morrow, 2008) (Figure 1.2).

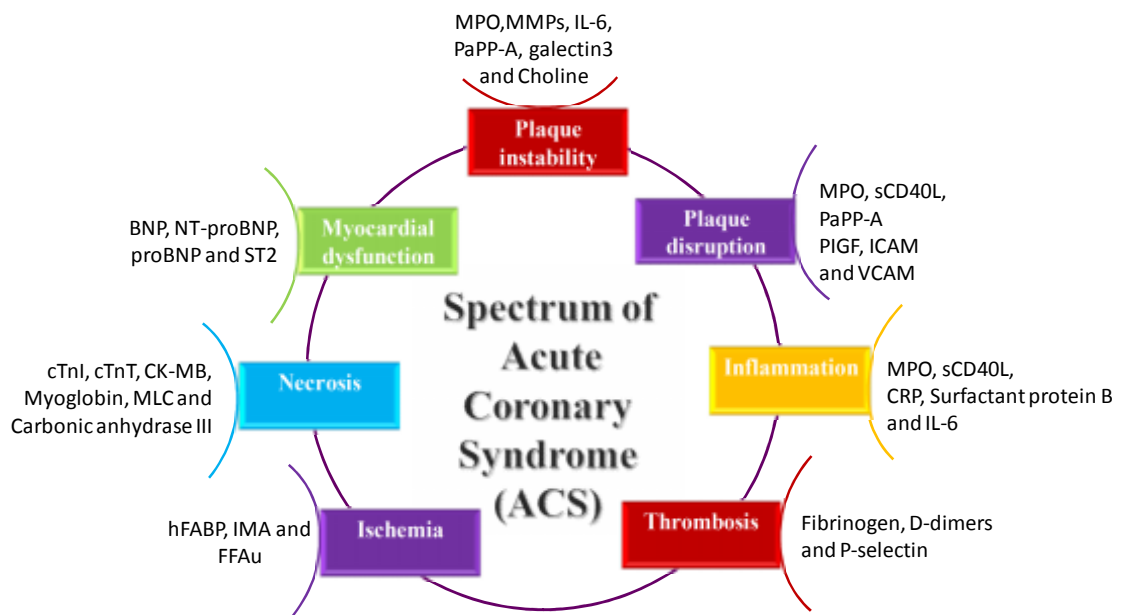


**Figure 1.2: Emergence of cardiovascular biomarkers.**

*AST: aspartate aminotransferase; LDH: lactate dehydrogenase; CK: creatinine kinase; HFABP: heart-fatty acid binding protein; IMA: ischemia modified albumin; cTnT: Cardiac troponin T; cTnI: Cardiac troponin I; and BNP: Brain natriuretic peptide.*

(Compiled from Christenson and Azzazy, 2006 and Dolci and Panteghini, 2006).

Improvement in our knowledge of pathobiology using molecular biological approaches (e.g. genomics, proteomics bioinformatics, etc.) resulted in the evolution of biomarkers from novel research aids to practical tools for clinical applications (Jaffe, 2007). Thus, they could play a pivotal role in assessment and early risk stratification of disease (Antman *et al.*, 2000; Dalzell *et al.*, 2009) (Figure 1.3). In the last half a century (Tang, 2009), over 50 biomarkers were identified, related to different pathological events in the development of disease states (Carreiro-Lewandowski, 2006; Richards, 2009).



**Figure 1.3: An overview of biomarkers associated with different stages of the disease.**

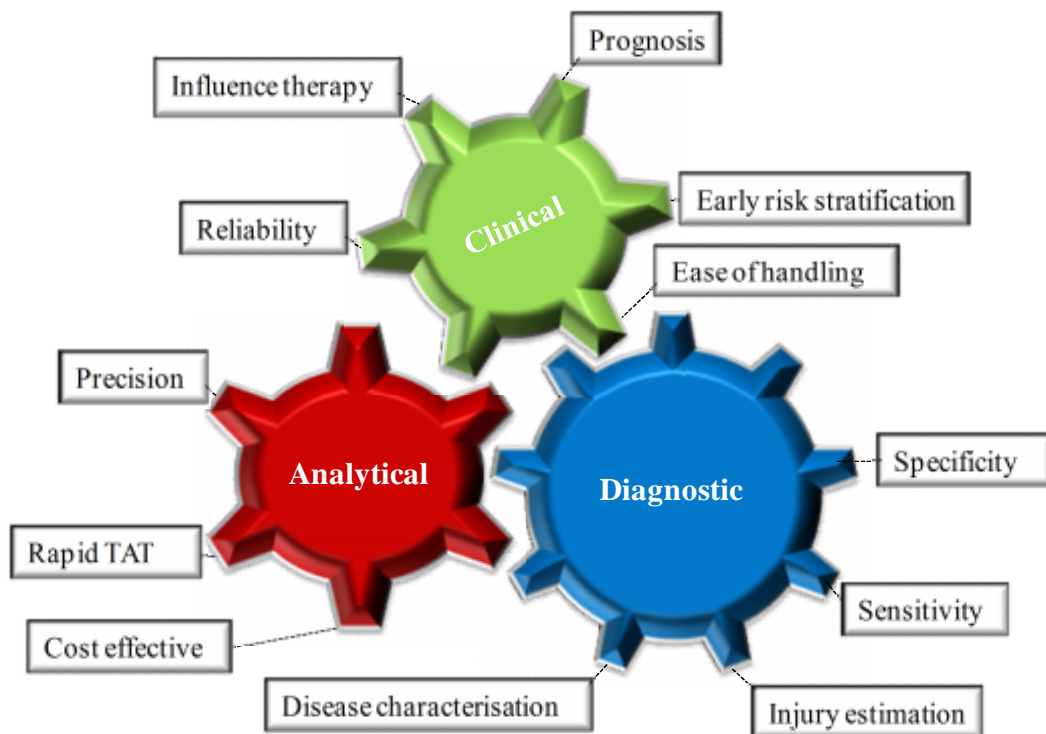
*Acute Coronary Syndrome is spectrum of different physiological events in which various clinical conditions are associated with release of different biochemical substances. These proteins, termed biomarkers, can provide a clear picture of the clinical condition. ACS-associated physiological conditions are represented in the figure while the associated specific biomarkers, indicative of the respective condition, are grouped together.*

(Compiled from Friess and Stark, 2009; McDonnell *et al.*, 2009 and Richards, 2009)

### **1.2.2 Characteristics of Cardiac Biomarkers**

Biomarkers can provide a wealth of information about a disease and can be a very useful aid in medical intervention (Howie-Esquivel and White, 2008) if they provide accurate data. A set of criteria is defined by the FDA for cardiac biomarkers to be used in assessment of disease in clinical settings (Figure 1.4). Biomarkers used in a diagnostic assay should be highly specific (Nigam, 2007). The specificity of biomarkers determines the performance of an assay both diagnostically and analytically (Dolci and Panteghini, 2006). From the perspective of the use of a cardiac biomarker, it should not be present in non-myocardial tissues and should ideally result in a high myocardium to serum ratio during injury. The release of a biomarker should be specific to the tissue injury with no or negligible presence in the individual in normal or any other disease conditions. Furthermore, it should be possible to differentiate between reversible (ischemia) and irreversible injury (necrosis). In order to obtain high diagnostic sensitivity, the marker should be small in size (Pasaolu *et al.*, 2007) and have rapid release kinetics after the myocardial injury (Friess and Stark, 2009). The marker should have a long half-life in blood allowing a large diagnostic window (Nigam, 2007). However, markers with rapid clearance rates are beneficial in detection of recurrent injury (McDonnell *et al.*, 2009). The marker should be predictive (Adams, 2004), allowing detection of the extent of injury (which should be proportional to its release). Additionally, it should be accurate, robust and cost effective with a rapid turn-around time (TAT) (Richards, 2009) along with minimal inter-operator and/or inter-institutional variability (Ludwig and Weinstein, 2005). In a nutshell, it should be able to influence the therapy, thus improving patient outcome by providing both diagnostic and prognostic information.





**Figure 1.4: Characteristics of cardiac biomarkers.**

*An assay used for diagnosis of disease affects various aspects of patients' treatment and the corresponding outcome. The interrelationship of different aspects and the characteristics possessed by the biomarkers for generating an ideal assay are presented.*

### **1.2.3 Markers of Ischemia and Necrosis**

AMI is a continuum of various processes resulting in inadequate supply of oxygen to heart muscle ultimately leading to tissue death (Jesse and Kukreja, 2001) or necrosis. Ischemia causes imbalance between the supply to and demand for oxygen in the heart muscles, impairing ion homeostasis and accumulation of waste products leading to retention of water due to osmosis. Increased osmotic load results in tissue oedema, stretching the sarcolemma, thus, increasing the permeability of membranes. Persistence of prolonged ischemia leads to irreversible tissue damage, and rupturing of the plasma membrane which results in leakage

of tissue proteins into the vascular compartment. These events contributed to attract the focus of researchers towards biomarkers of ischemia (Jesse and Kukreja, 2001) and necrosis (Peters *et al.*, 2005), which became hallmark in detection of AMI. For years there was a continuing rush to identify specific and sensitive markers that might be useful in providing diagnosis in the early onset of disease, preventing irreversible tissue injury and, thus, assisting in therapy (Table 1.2 and 1.3).

**Table 1.2: Past, present and developing markers of myocardial ischemia and necrosis.**

<b>Past</b>	<ul style="list-style-type: none"> <li>• AST (Aspartate aminotransferase)</li> <li>• LDH (Lactate dehydrogenase)</li> <li>• Myosin light chains</li> </ul>
<b>Present</b>	<ul style="list-style-type: none"> <li>• CK (Creatinine kinase) and CK isoenzymes</li> <li>• Cardiac troponins (I and T)</li> <li>• Myoglobin</li> </ul>
<b>Developing</b>	<ul style="list-style-type: none"> <li>• HFABP (heart fatty acid binding protein)</li> <li>• IMA (Ischemia modified albumin)</li> </ul>

(Compiled from Carreiro-Lewandowski, 2006 and Christenson and Azzazy, 2006).

**Table 1.3: Characteristics of present and developing biomarkers of ischemia and necrosis.**

Marker	Molecular Weight	Type of marker	Advantages	Limitations	Elevation Time	Peak time	Return to normal
Myoglobin	18 kDa	Necrotic	High sensitivity; early detection of MI; useful in re-perfusion	Low specificity in presence of skeletal injury and renal insufficiency; rapid return to normal	1–3 hours	6–12 hours	24–48 hours
HFABP	15 kDa	Ischemic	High sensitivity; early detection of MI; detects ischemia in absence of necrosis; detects reinfarction	Low specificity in skeletal injury and renal insufficiency; small diagnostic window	2–3 hours	8–10 hours	18–30 hours
CK-MB isoforms	85 kDa	Necrotic	Early marker; allows infarct size estimation and reinfarction	Lack of widespread availability and experience	4–6 hours	12–24 hours	3–4 days
Troponin T	37 kDa	Necrotic	Highly specific; tool for risk stratification; helpful to determine therapy; detection of re-perfusion.	Not an early marker, limited ability to detect reinfarction; less sensitivity in MI of less than 6 hours; serial testing needed to discriminate early reinfarction.	4–6 hours	12–24 hours	7–10 days
Troponin I	23.5 kDa	Necrotic	Highly specific; tool for risk stratification; helpful to determine therapy; detection of re-perfusion.	Not an early marker; limited ability to detect reinfarction; less sensitivity in MI of less than 6 hours; serial testing needed to discriminate early reinfarction; lack of analytical reference standards.	4–6 hours	12–24 hours	6–8 days
IMA	65 kDa	Ischemic	Early marker	Poor discriminator of ischemia caused due to AMI compared to other tissue ischemia	Not defined	Not defined	Not defined

(Compiled from Christenson and Azzazy, 2006 and McDonnell *et al.*, 2009).

#### **1.2.4 Heart-Fatty Acid Binding Protein**

Heart-fatty acid binding protein (hFABP) is a low molecular weight (15 kDa), hydrophilic, non-enzyme protein involved in the transport of long-chain fatty acids to different sites of oxidation and esterification within the cell, for maintaining myocardial lipid homeostasis (Fournier and Richard, 1990). Apart from maintaining homeostasis and satiating metabolic needs of myocytes, it is believed to play an important role in their protection from harmful effects of accumulated fatty acids during ischemia (Alhadi and Fox, 2004). The importance of hFABP can be attributed to its abundance in heart representing 10% of the whole myocyte cytosolic proteins (Pelsers *et al.*, 2005).

HFABP emerged as one of the promising early markers of AMI in the early 1990's (Kleine *et al.*, 1992; Glatz *et al.*, 1998). The rationale of hFABP being recognised as a cardiac biomarker can be attributed to its small size, high myocardium concentration and relative tissue specificity and rapid release kinetics in plasma contributing to its sensitivity. A rise above the cut-off value is achieved as quickly as 1 hour after the onset of MI (Chan *et al.*, 2005) peaking within 4-6 hours, with 30-40 times the plasma concentration compared to the baseline level (Cavus *et al.*, 2006) and returning to normal within 24-30 hours (Alhadi and Fox, 2004; Pasaolu *et al.*, 2007).

In many respects, hFABP resembles myoglobin, a recommended early biomarker in recent guidelines (NACB writing group members *et al.*, 2007). However, on comparing both the markers for their diagnostic efficacy (specificity, sensitivity and the areas under receiver operating curve (ROC)) hFABP was reported to be better than myoglobin (Okamoto *et al.*, 2000; Pelsers *et al.*, 2005; Cavus *et al.*, 2006; Zaninotto *et al.*, 2006). Several studies demonstrated the superiority of hFABP over traditionally established

markers like troponins, CK-MB, BNP, etc. in early disease characterisation (Seino *et al.*, 2003; Zaninotto *et al.*, 2007; Figiel *et al.*, 2008; Liao *et al.*, 2009).

Apart from diagnostic applications, hFABP also proved its worth as useful marker in clinical applications, e.g. in evaluating thrombolytic therapy due to accurate infarct sizing (Glatz *et al.*, 1994; Okamoto *et al.*, 2000), monitoring success of coronary reperfusion (de Lemos *et al.*, 2000; de Groot *et al.*, 2001), assessing recurrent infarction (Vannieuwenhoven *et al.*, 1995), detecting tissue loss in surgery and postoperative myocardial injury (Hayashida *et al.*, 2000; Chowdhury *et al.*, 2008). HFABP is also considered to be an independent marker able to predict adverse outcomes in patients with ACS (Iida *et al.*, 2005; Suzuki *et al.*, 2005; O'Donoghue *et al.*, 2006; Kilcullen *et al.*, 2007) along with the survival prognosis of patients (Iida *et al.*, 2005).

There are two major limitations preventing the universal acceptance hFABP as an ideal marker. These are lack of complete cardiac specificity (presence in skeletal muscles) and inappropriate measurements in patients with renal failure (hFABP clearance takes place through kidneys) (Vannieuwenhoven *et al.*, 1995; de Groot *et al.*, 1999).

However, specificity can be improved by using the hFABP / myoglobin (Vannieuwenhoven *et al.*, 1995) ratio to eliminate false positives due to skeletal injury. In the case of renal failure, it can be corrected by taking individual clearance rates into account (de Groot *et al.*, 1999). Recently hFABP / myoglobin ratio has been found useful in patients with renal dysfunction to assess cardiac damage (Furuhashi *et al.*, 2004). Another approach suggested involves two consequent measurements of hFABP, initially at admission and then 1 hour later, improves the diagnostic potential of hFABP by 100% exclusion of non-AMI patients (Chan *et al.*, 2004).

Despite limitations, hFABP still stands out to be an independent marker of various cardiovascular events owing to its rapid capacity for discrimination between AMI and non-AMI patients (Cavus *et al.*, 2006; Pasaolu *et al.*, 2007). It is a unique marker capable of diagnosing patients with atypical symptoms or silent MI, not detectable by ECG or other well established markers (Liyan *et al.*, 2009). It is also an excellent cardiac biomarker capable of detecting ischemia in the absence of necrosis (de Lemos and O'Donoghue, 2007), which still remains a challenges, as per the recent guidelines on ACS (NACB writing group members *et al.*, 2007).

### **1.2.5 Cardiac Troponin I**

Cardiac troponin I (cTnI) is a subunit of a tripartiate complex along with two other subunits TnT and TnC and is involved in the systemic process of cardiac muscle contraction and relaxation. Studies on the underlying principle of muscle contraction along with detailed knowledge of MI processes suggested that tissue injury may cause degradation of structural proteins such as troponins (Toyo-Oka and Ross, 1981). This fact paved the way for analysis of cardiac troponins as potential cardiac biomarkers. However, out of the three subunits only cTnT and cTnI was tissue-specific which favoured their further analysis as potentially useful biomarkers.

Since the 1980's cTnI and cTnT emerged as strong indicators of myocardial damage replacing early traditional cardiac enzymes such as CK and CK-MB isoforms that were referred to as "gold standard" at that time (Jaffe *et al.*, 2000; Toyo-oka and Kumagai, 2007). This was attributed to their high specificity, sensitivity and large time window (Babuín and Jaffe, 2005). Since then, both cTnI and cTnT are highly utilised specific biomarkers for diagnosis of myocardial tissue damage. Their performance as cardiac

biomarkers was primarily responsible for redefinition of AMI (Antman *et al.*, 2000) and gained them the reputation as new golden standards (Thygesen *et al.*, 2007).

Cardiac troponin I is one of the most widely used troponins in the diagnosis of infarction. It is recommended that an elevation of troponin significant to detect necrosis exceeds the 99th percentile of troponin values in a reference control group with an assay imprecision of 10% or less, within 24 hours of the clinical event (Antman *et al.*, 2000). CTnI is released in 2-3 hours after the onset of symptoms of myocardial injury (Tousoulis *et al.*, 2008), peaking at 12-24 hours and returning to normal in 6-8 days. The initial rise is due to the cytoplasmic pool which comprises 4% of the total cTnI (Adams *et al.*, 1994; Kemp *et al.*, 2004). The soluble portion (cystolic) of cTnI is rapidly released, like other cystolic proteins thus contributing to the sensitivity of the marker. Prolonged damage results in the release of the structurally bound cTnI (96%) which explains its prolonged elevation providing a large diagnostic window. Owing to the different compartmentalisation of cTnI (cytoplasmic and structural) it is expected to characterize reversible from irreversible myocardial damage, depending on its release profile (Toyo-oka and Kumagai, 2007; Vasile and Jaffe, 2007).

CTnI is a superior and a sensitive cardiac necrosis, sensitive to even slightest damage (Inbar, 2009) capable of diagnosing myocardial injury in both NSTEMI and STEMI patients (Babu and Jaffe, 2005; Lippi *et al.*, 2006; NACB writing group members *et al.*, 2007; Howie-Esquivel and White, 2008) the former being undetected by ECG. It is valuable in infarct sizing and is an independent marker in prognosis and risk stratification in a number of clinical conditions (Korff *et al.*, 2006; Anderson *et al.*, 2007; Gupta and de Lemos, 2007; Setiadi *et al.*, 2009; Thygesen *et al.*, 2007). Its role in disease management is well established earning positive recommendations from many

scientific bodies in various disease guidelines (NACB writing group members *et al.*, 2007; Thygesen *et al.*, 2007).

Four generations of cardiac troponin assays have been generated with increasing high sensitivity and specificity requirements to detect the cystolic pool of cTn, released initially, for early diagnosis of disease (Saenger and Jaffe, 2007; Tate, 2008; Wu and Jaffe, 2008). CTnI assays are produced by a variety of manufacturers (Collinson *et al.*, 2001) evolving substantially to improve the analytical performance. New generation of highly sensitive troponin assays can measure cTnI at levels down to 0.0002 µg/L (Wilson *et al.*, 2009).

Despite being the superior marker of AMI diagnosis and prognosis, cTnI suffers from limitations such as interferences in serum troponin measurements, assay standardisation and reproducibility and autoantibodies leading to false positives or negative results (Gennaro *et al.*, 2008; Lazzeri *et al.*, 2008; Tate, 2008; Agzew, 2009). Analytical variations may be due to the use of different antibodies, used by different manufacturers (Tate, 2008; Wu and Jaffe, 2008) with variable ability to bind the biomarker (Katrukha *et al.*, 1998) due to post-ischemic modification (Le Moal *et al.*, 2007).

Efforts are being made to meet the limitations posed by the troponin assays. The revised guidelines suggest using cTn values at 99<sup>th</sup> percentile at a 10% coefficient of variation to minimize inter-assay variation and false positives, to some extent (Anderson *et al.*, 2007). Serial sampling is another approach suggested for determining the rise and fall patterns of the marker (Wu and Jaffe, 2008) with time to differentiate acute injury from structural injury (Saenger and Jaffe, 2007). Usage of standard reference material for assay standardisation (Panteghini *et al.*, 2008) is also suggested to harmonize the results



from different assay platforms. A robust and well-characterised immunoassay method capable of determining the cTn levels in a serum pool containing clinically relevant concentrations of cTn was suggested to serve as a reference material for standardising upcoming new generation assays (Panteghini *et al.*, 2008). A new generation of high sensitivity assays are expected to solve the reproducibility problems associated with previous assays due to high sensitivity (Tate, 2008). Determining assay ‘cut-offs’ based on age, gender or ethnicity (Melanson *et al.*, 2007) and multimarker strategies could also be helpful in preventing false results.

With improvement in assay design strategies and careful evaluation of results cardiac troponins will probably retain their dominance as gold standards for AMI detection.

### **1.3 Antibodies**

#### **1.3.1 Antigens and Antibodies**

An antigen is any foreign substance that can induce an immune response and is capable of reacting with the products of that response, produced by the immune system, e.g. antibodies or T-lymphocytes. Antigens can be protein, carbohydrate, lipid or nucleic acid in nature. The ability of the antigen to generate an immune response depends on its immunogenicity. Such an antigen, capable of eliciting an immune response by itself, is known as an immunogen. All the immunogens are antigen but all the antigens are not immunogens. The immunogenicity of an antigen depends on the distinct molecular structural features on the surface of the molecule, recognised as foreign by the host immune system. Such features are known as “epitopes”. They act as the target for the immune system to produce an array of antibodies with different specificity, which, on binding efficiently to the immunogen, renders it ineffective.

Antibodies, also known as immunoglobulins (Igs), are Y-shaped globular protein structures produced in plasma and extracellular fluids as a first response to an invading foreign molecule by the host's immune system. Antibodies are produced from B-cell lymphocytes, which play a major role in generating the humoral response of the host immune system. Each B-cell is programmed to make a single type of antibody for a particular immunogen. When a B-cell encounters an antigen it eventually produces a plasma cell which makes antibodies with specificity and high binding affinity for the epitope of the immunogen.

### **1.3.2 Different Classes of Antibodies**

There are five different classes of antibodies (Lu *et al.*, 2005) produced by B-cells. They are characterised based on their structure, functions and localisation (Murphy *et al.*, 2007) (Table 1.4).

IgG and IgA are further subdivided into subclasses, called isotypes, due to polymorphisms in the conserved regions of the heavy chain. IgG is the most abundant Ig class, representing 80% of the total immunoglobulin population in the serum (de Voer *et al.*, 2008) with the longest half-life of approximately 21-28 days. From a biotechnology prospective they are the most important class of antibodies owing to their small size, stability and high rate synthesis by body. These properties make them ideal candidates to be used as biological reagents in various immunological assays.

**Table 1.4: Characteristics and functions of different classes of immunoglobulins.**

Type	Molecular weight	Structure	Localisation	Function	Sub-classes
<b>IgG</b>	150 kDa	Monomer	Blood and body fluids	Protection against infection	4
<b>IgA</b>	150-350 kDa	Dimer	Secreted in tears, saliva, and breast milk	Mucosal immunity, passive immunity to infants	2
<b>IgD</b>	180 kDa	Monomer	Antigen receptor on naïve B-cells	ND	1
<b>IgM</b>	900 kDa	Pentamer	Surface of B-cells, secreted in soluble form as well	Early immune response	1
<b>IgE</b>	190 kDa	Monomer	Bloodstream and mucus of the digestive system	Immune response to parasites and worms, involved in allergic reactions	1

(Compiled from Carreiro-Lewandowski, 2006 and Christenson and Azzazy, 2006).

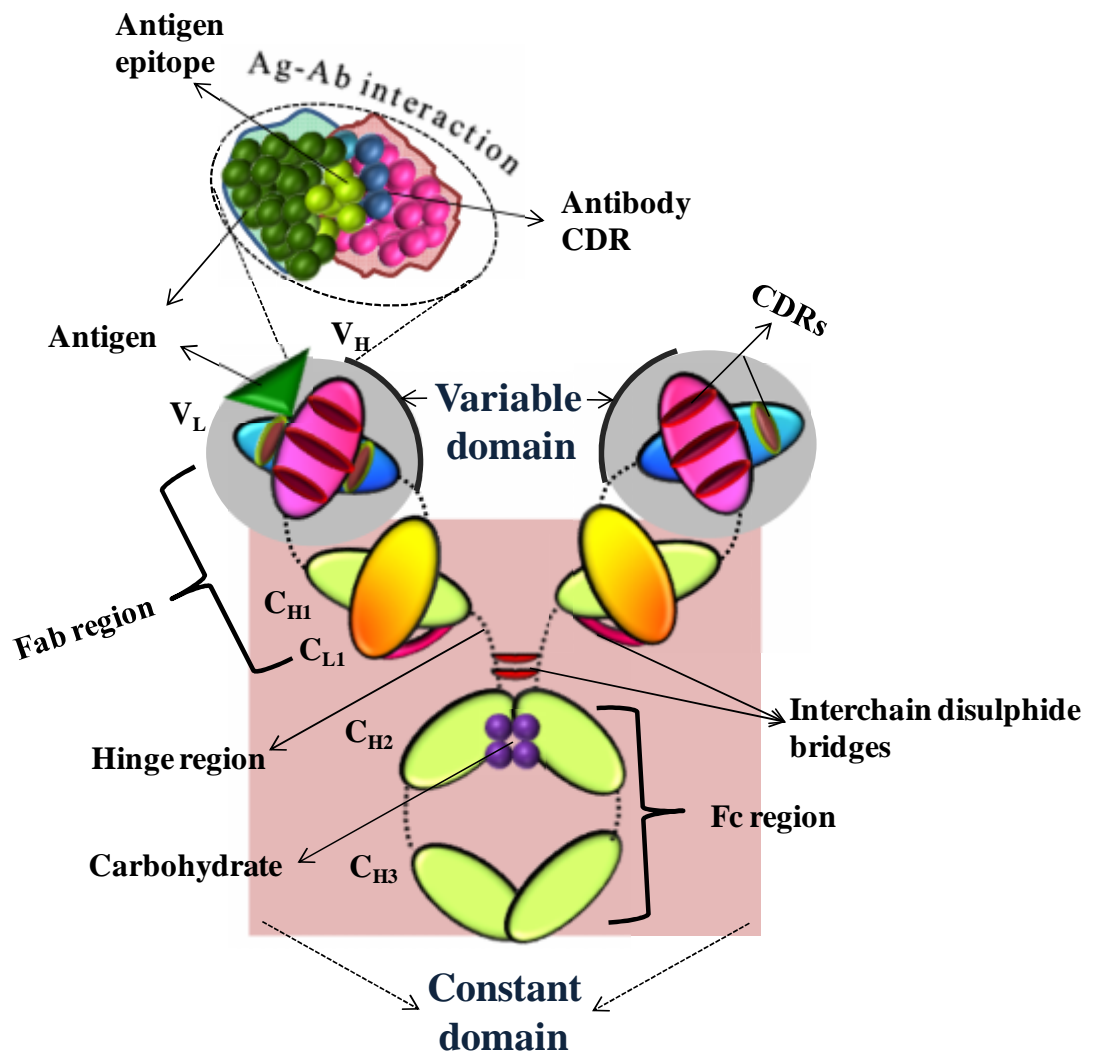
### 1.3.3 Antibody Structure

Antibody structure varies with the type of antibody. Typically an IgG antibody consists of four polypeptide chains, two heavy chains and two light chains, connected by disulfide bonds (Figure 1.5). These heavy and light chains are named as a result of the difference in their molecular weights. A light chain has a molecular weight of ~25,000 Da whereas a heavy chain has a molecular weight of ~50,000 Da. There are 5 different types of heavy chains  $\gamma$ ,  $\delta$ ,  $\mu$ ,  $\epsilon$ , and  $\alpha$ , and two types of light chains,  $\kappa$  and  $\lambda$ . Each antibody molecule contains only one type of light chain and heavy chain which determine the subclass of antibody (Copley *et al.*, 1996).

The light chain is composed of 220 amino acid residues and the heavy chain is composed of 440-550 amino acids. These chains are further divided into constant and variable regions based on the amino acid sequence. The constant region determines the

mechanism, used to destroy the antigen and segregates the antibodies into five major classes (Table 1.4) depending on its type and immune function. The variable region is further subdivided into hypervariable (HV) and framework (FR) regions. There is great variability in the sequence of the amino acids in the HV regions. They are also referred as complementary determining regions (CDR's). The stable conserved amino acid sequences are called the FR regions. In the heavy and light chains there are three HVs/CDRs which are connected to each other by four FR regions. The combination of different amino acids in the CDR regions confers on the antibody its ability to recognise a specific antigenic determinant. In addition, a few specific amino acid residues account for the predominant interaction of the antibody with the antigen. These locations are commonly termed as "hot spots" (Clackson and Wells, 1995; Copley *et al.*, 1996; Murphy *et al.*, 2007).

Each antibody is functionally divided into two regions, based on papain digestion, a F(ab)<sub>2</sub> region (fragment, antigen binding) and a Fc region (fragment, crystallisable). The F(ab)<sub>2</sub> region of an antibody is involved in antigen binding. It contains the two constant and variable domains of both light and heavy chains linked to form the paratope (antigen binding site) at the amino terminal end. The Fc region at C-terminal is glycosylated, containing 2-4 heavy chain constant domains in each polypeptide chain. Functionally, the Fc region plays no role in antigen binding, but has certain effector functions (e.g. binding complement, binding to cell receptors on macrophages and monocytes, etc.), and serves to distinguish one class of antibody from another. The F(ab)<sub>2</sub> region is connected to the Fc region by a hinge region which imparts flexibility for antigen-binding which is required by the antibody to interact efficiently with the antigen.



**Figure 1.5: Basic IgG antibody structure.**

*IgG consists of an antigen binding region, for binding the antigen and a crystallisable region, for effector functions. Disulphide bridges stabilise the interaction between heavy and light chains thus, playing a major role in the determination of antibody structure and conformation. Based on the amino acid sequences, an antibody is made up of variable (encircled in grey) and constant domains (shaded in pink).*

### 1.3.4 Characteristics of Antibodies

Over the last few decades, a revolution in antibody-based research has occurred due to its role in *in vivo* and in *in vitro* applications. For diagnostics, they are the ideal biological recognition reagents, and, thus, are useful in a range of analytical platforms e.g. Western blotting, immunohistochemistry, immunocytochemistry, enzyme-linked

immunosorbant assay (ELISA) and flow cytometric analysis (Saleem and Kamal, 2008; Pohanka, 2009).

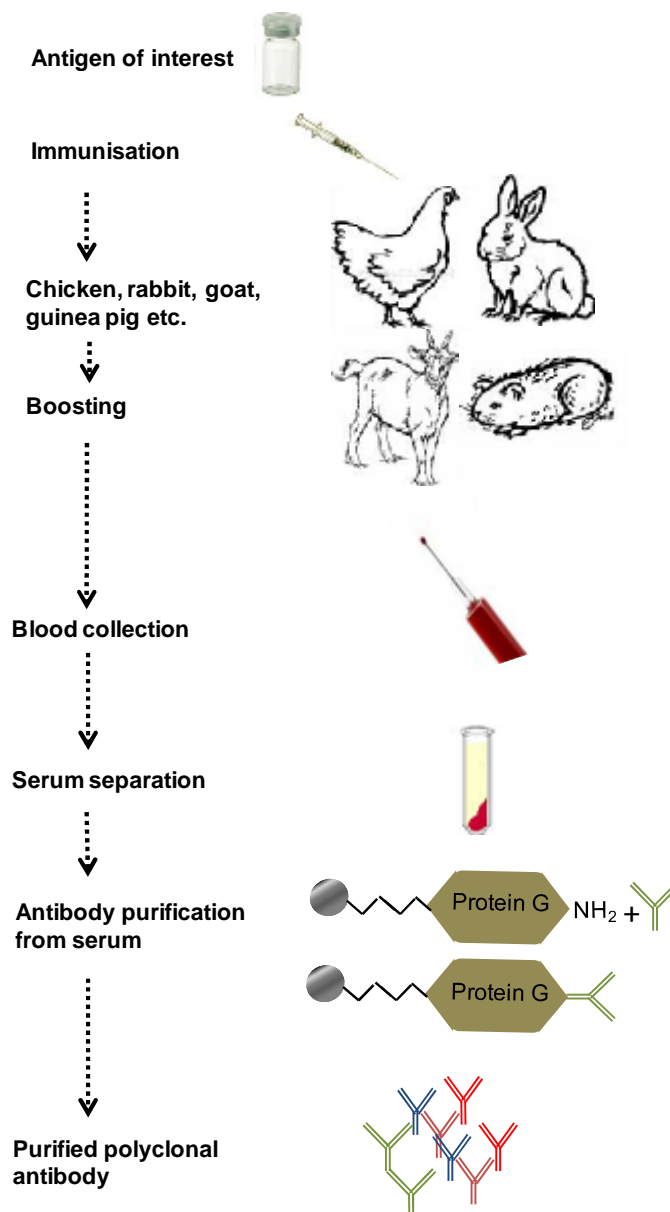
The use of antibodies in such platforms requires them to be fully characterised in terms of sensitivity, specificity, avidity and cross-reactivity. All these parameters play a significant role in designing novel antibody-based assays. Among all these characteristics, the sensitivity of an antibody is an important parameter which may determine the efficacy of an assay. Affinity is the binding strength of an antibody to a particular epitope of an antigen. A high affinity antibody is preferable in some immunoassay formats due to the need to form a stable binding complex with an antigen in a short period of time. The binding strength of antibody to antigen can also be determined by avidity. Avidity is the valency of the paratopes (antigen binding sites) on an antibody that identify and bind same or different antigenic epitopes. The avidity of an antibody can be calculated by its affinity for the epitope, the number of paratopes possessed, and the orientation of the antibody-antigen complex formed. A high avidity antibody can be very useful while dealing with complex epitopes. It can also increase the sensitivity of the assay due to its capacity for binding large amounts of antigen.

Specificity is another important characteristic of an antibody apart from sensitivity. The performance of an assay may be limited or determined by the specificity of the antibody. For an antibody to be used as a detection reagent in a diagnostic platform of assay, it should demonstrate very specific binding for the antigen and little or no cross-reactivity towards identical epitopes presented on different antigens. A less specific antibody may provide false results thus yielding a false diagnosis for a disease (Pohanka, 2009).

### 1.3.5 Types of Antibodies and their Production

Antibodies are categorised into polyclonal, monoclonal and recombinant forms. A polyclonal antibody is a mixture of antibodies to a given antigen produced by different B-cells. Such a mixture contains antibodies with different affinities and specificities to different epitopes on the antigen. Traditionally, polyclonal antibodies were the first to be recognised for their therapeutic properties, in the 1890's, when it was found that polyclonal antiserum taken from animals could treat life-threatening infections in humans (Buchwald and Pirofski, 2003; Buelow and Schooten, 2007). The diversity of the antibodies produced depends on the type and the number of species used for immunisation. Rabbits are the most common species used owing to their relative small size, ease of handling and shorter breeding times combined with the ability to produce sufficient serum volumes (Cooper and Paterson, 2009). Other animals used include mice, rats, hamsters, goats, sheep, horses, cows, guinea pigs, baboons, donkeys, frogs and fish (Euhus *et al.*, 1990; Hanly *et al.*, 1995; Reynolds *et al.*, 1995; Shariful Islam *et al.*, 1998; Nuntaprasert *et al.*, 2005). Chickens may also be a good choice for polyclonal antibody development for mammalian proteins (Hau and Hendriksen, 2005; Lipman *et al.*, 2005).

Polyclonal antibodies are produced by immunising an animal with the antigen of interest and boosting it at regular intervals with the antigen. Once a sufficiently high antibody titre is achieved the blood of the animal is harvested then serum separation is carried out. This serum contains a mixture of antibodies which is then affinity purified (Figure 1.6).

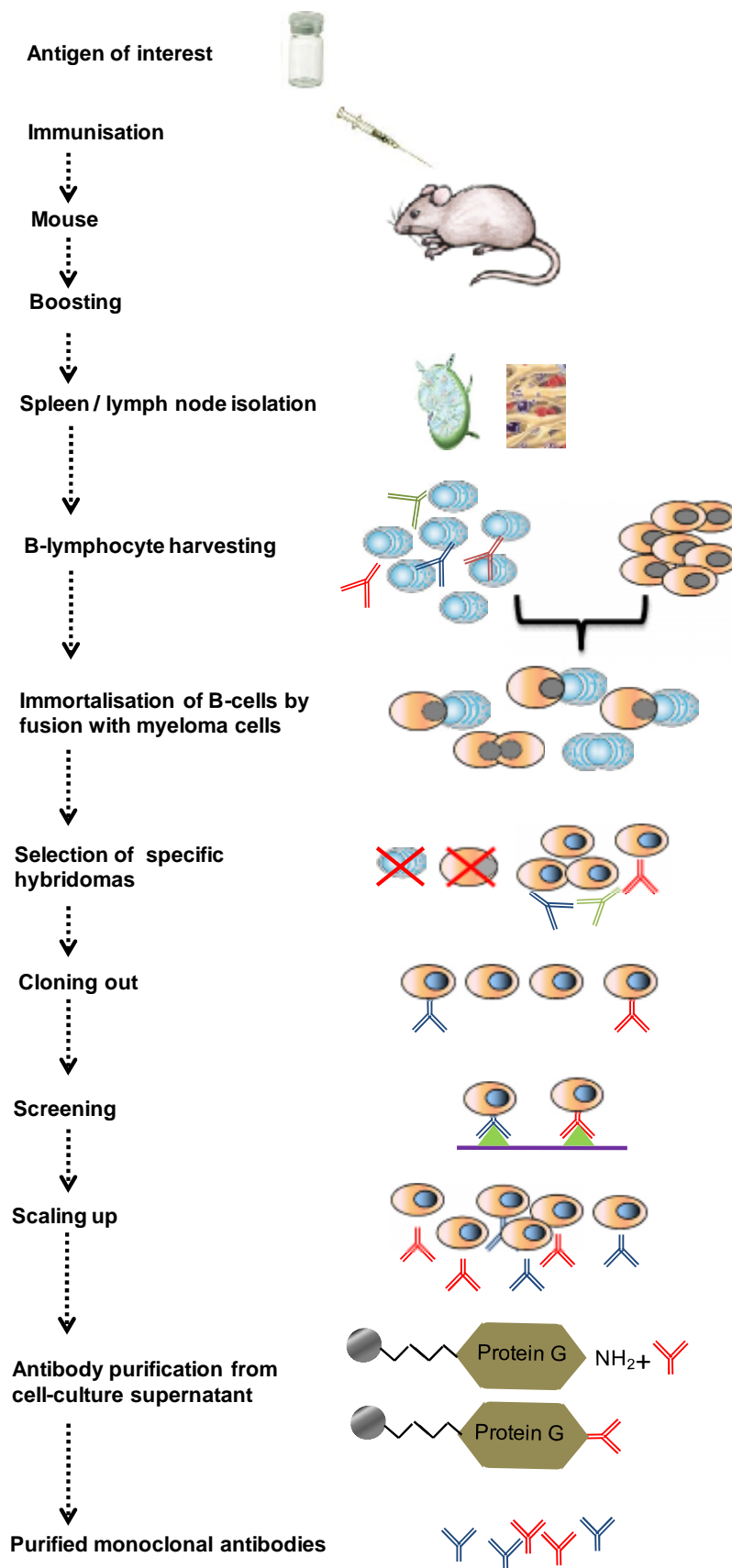


**Figure 1.6: *Production of polyclonal antibodies.***

Monoclonal antibodies are similar but are produced from a single clone of B-cells. These antibodies are identical to each other in respect to their antigen binding characteristics. The B-cells have a very short life-span so antibody production is prolonged by their immortalisation. This novel method of generating the antibodies was developed by Kohler and Milstein (Kohler and Milstein, 1975), who were awarded the Nobel Prize in 1984, for their contribution to Medicine. The underlying principle



involves immunisation of mice with the desired antigen and once a high titre in the serum antibodies is detected, the spleenocytes from the animal are fused with myeloma cells. The fusion cell product is called a “hybridoma” which possess the antibody producing ability of spleenocytes and the immortality of myeloma cells. The myeloma cells used in this procedure are deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) which serves as a means to select the hybridomas from other fusion by-products by means of a selective medium. The individual cells producing monoclonal antibodies are separated by cloning out using limiting dilution or other methods. This process helps in obtaining a single hybridoma clone producing an antibody of a single constant specificity (Figure 1.7).



**Figure 1.7: Production of monoclonal antibodies.**

Monoclonal antibodies have proved their applicability in diagnostics and therapeutics (Little *et al.*, 2000; Saleem and Kamal, 2008). However, the development of recombinantly expressed entire monoclonal antibodies in early 1980's, bypassed hybridoma technology (Neuberger *et al.*, 1984), achieving a major breakthrough in 1989, with the advent of a novel technology of cloning antibody genes in a bacterial system (Orlandi *et al.*, 1989). This technology combines the power of immune system with a group of molecular approaches to produce a large diversified antibody repertoire, offering further engineering options to modify the affinity, specificity and size of antibodies over conventional hybridoma technology. The major difference between hybridoma-derived antibodies and recombinant monoclonal antibodies is that the former immortalises antibody producing cells while the later immortalises the antibody gene. Recombinant antibody technology involves the amplification of antibody variable genes from the host cDNA by PCR and pairing them at random, creating a library of the antibody genes with different combinations of variable heavy and light chains. This library of antibody genes can be further cloned into an appropriate vector system for expression, e.g. bacteria, yeast, plants, insect cells, and mammalian cells (Kipriyanov and Little, 1999; Weisser and Hall, 2009). Each type of antibody has advantages and limitations and these are summarised in Table 1.5.

**Table 1.5: Advantages and limitations of different antibody types.**

Characteristics	Polyclonal antibody	Monoclonal antibody	Recombinant antibody
Production time	Short	Long	Short to moderately long
Ease of production	Very easy	Difficult	Easy
Production cost	Low	Moderate to high	High
Stability	High	Moderate	Depends on the format
Number of animal required for production	High	Moderate	Low to none
Batch to batch variations	Yes	No	No
Supply	Limited	Unlimited	Unlimited
Detailed analysis of antibody*	Not-possible	Possible	Possible
Epitope specificity	Polyspecific	Monospecific	Monospecific
Specificity	Moderate	High	High
Sensitivity	Low	Moderate to High	High
Generation of the antibodies of desired isotype	Not-possible	Not-possible	Possible
Ability to cross link antigens	Yes	No	No
Genetic modification (including affinity maturation)	Not-possible	Possible only when converted to rAb	Possible

\*Such as X-ray crystallography, gene sequencing, structural and chemical analysis.

### 1.3.6 Recombinant Antibody Libraries, Construction and Selection

Antibody libraries can be classified into three major categories depending on the source of antibody genes: naive, immune, and synthetic/semisynthetic (Clackson *et al.*, 1991; Hoogenboom and Chames, 2000; Weissner and Hall, 2009).

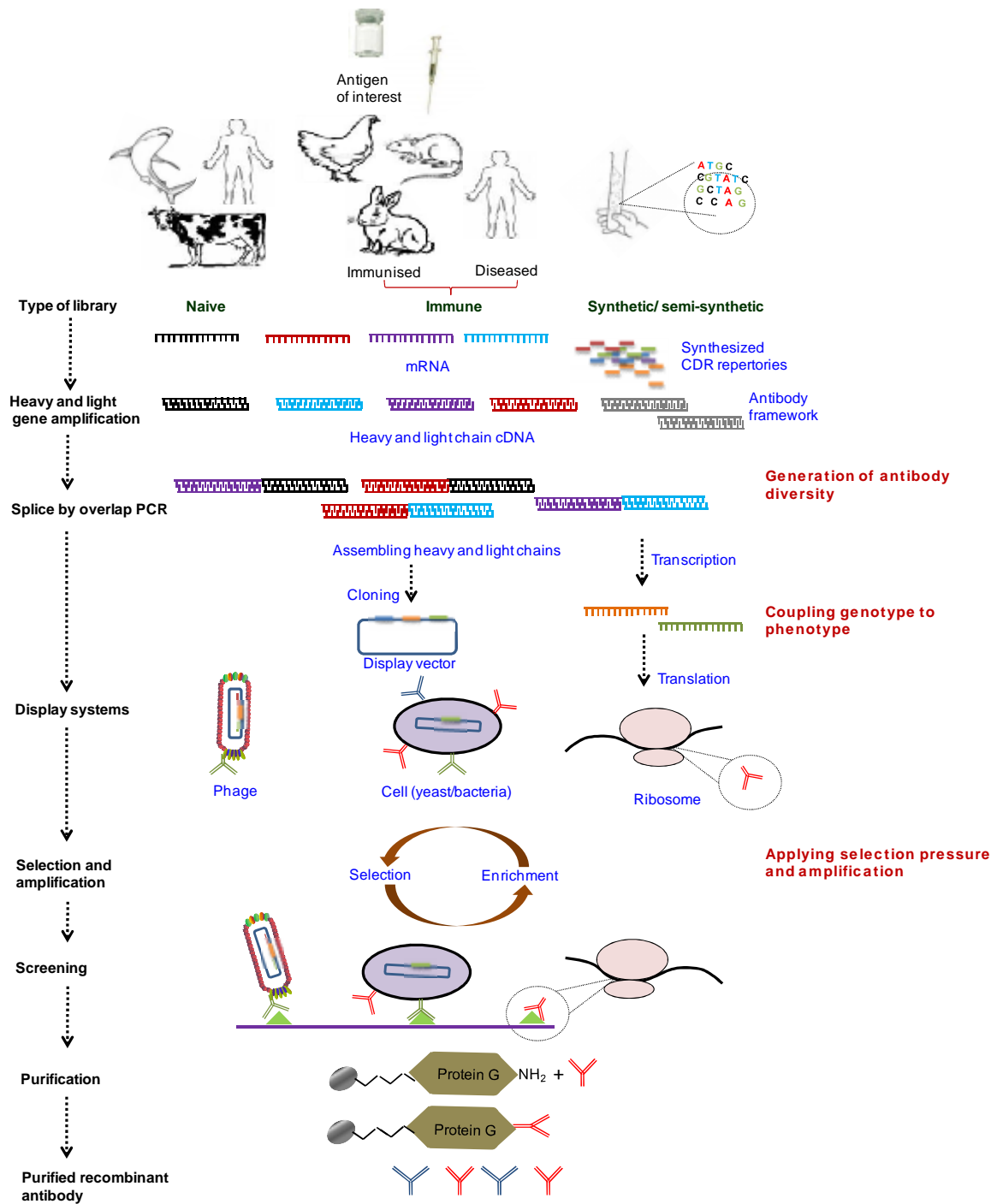
Naive and synthetic libraries are unbiased libraries not focused on any particular antigen. Such libraries are also called as “single-pot” libraries and exhibit a large

antibody diversity between  $10^7$ - $10^{11}$  (Smith *et al.*, 2004; Yin *et al.*, 2008; Lloyd *et al.*, 2009; Pansri *et al.*, 2009). Naïve antibody libraries are constructed by amplifying and rearranging germline-encoded antibody genes producing a large array of antibodies with different paratopes. This antibody pool is generated by natural variable genes present in the host immune system which may or may not have produced under any antigenic influence (Persson, 2009). Synthetic libraries are constructed *in vitro* using oligonucleotides for synthesis of antibody CDR's. Such libraries overcome the limitations of *in vivo* generated libraries based on natural bias and redundancies allowing the generation of more stable antibodies. Another version of these libraries is the semi-synthetic library, a mixture of both a naïve and a synthetic library, containing a combined synthetic and natural diversity in different CDR loops (Hoogenboom, 2005; Sidhu and Fellouse, 2006). Single pot libraries can be used for screening and selecting antibodies against a variety of antigens. Antibodies from such libraries may have low affinity towards an antigen due to lack of somatic hypermutation. However, there have been reports where such libraries yielded low nanomolar affinity antibodies (Pope *et al.*, 1996; Hanes *et al.*, 2000).

In contrast to single-pot libraries, immune libraries are biased to a specific antigen which upon exposure triggers a cascade of events in the host immune system. The immune system counteracts the exposed antigen by selecting antibodies binding to the antigenic epitopes from a wide array of germline-encoded antibodies, increasing antigen-specific antibody production, and incorporating subsequent mutations in the gene for effective antigen binding. Immune libraries have a smaller size than single pot libraries with maximum diversity of  $10^{10}$  reported. This is due to the development of selective antibodies recognised as antigen-specific by the immune system (Dubrovskaya *et al.*, 2007; Leonard *et al.*, 2007; Abi-Ghanem *et al.*, 2008).

The antibodies obtained from immune or non-immune libraries can be further improved by affinity maturation using molecular biological approaches, inducing site-directed or random mutations in CDR hot spots or chain shuffling to produce high affinity antibodies (Wu *et al.*, 1998; Boder *et al.*, 2000; Hanes *et al.*, 2000; Kurtzman *et al.*, 2001).

The selection of specific high affinity antibodies from a large diverse antibody repertoire, generated by combinatorial assembly of germline coded genes was made possible due to the development of clever screening platforms. *In vitro* selection of antibodies is a powerful approach based on a fundamental aspect of establishing a physical linkage between the encoding genotype and the experimental phenotype (Scott and Smith, 1990). Most of the display technologies use this basic principle (Leemhuis *et al.*, 2005) for selecting antibodies. All these technologies shares four major steps (I) generating genotypic diversity, (II) combining genotype to phenotype (III) applying clonal selection pressure and (IV) amplification of the selected clones. Several molecular display platforms are available among which phage display, cell surface display and ribosome display are the most commonly used (Hoogenboom, 2005).



**Figure 1.8: Production of recombinant antibodies from different libraries and their screening and selection.**

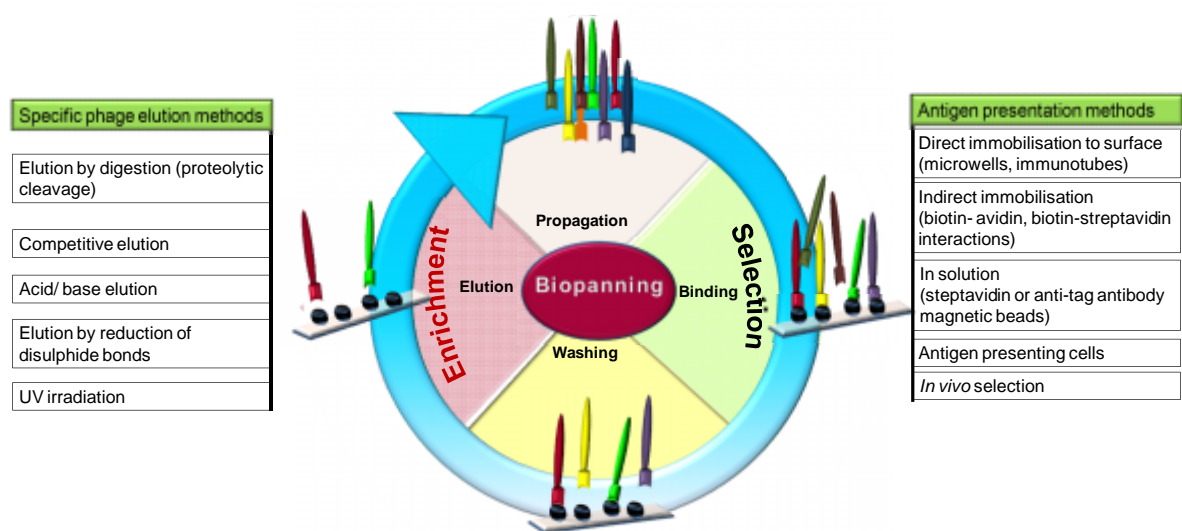
### 1.3.7 Phage Display

The frequency of desired antibody in a heterologous population is minute and selecting a specific antibody to a particular antigen in such a diverse pool of antibodies is similar

to searching for a pin in a haystack. Over many years display techniques emerged (Russell *et al.*, 1993; Georgiou *et al.*, 1997; Mössner *et al.*, 2001; Wittrup, 2001; Sepp *et al.*, 2002; Harvey *et al.*, 2004; Odegrip *et al.*, 2004; Groves and Osbourn, 2005; Hoogenboom, 2005; Leemhuis *et al.*, 2005; Reiersen *et al.*, 2005; Urban *et al.*, 2005; Taube *et al.*, 2008; Alonso-Camino *et al.*, 2009) to ease the tedious task of searching out specific antibodies in a vast library among which phage display remains the oldest and most powerful technique used (Levin and Weiss, 2006; Sidhu and Fellouse, 2006).

This technique, first reported by Smith (1985), was later used by McCafferty *et al.* (1990) for antibody display. Since then, it has developed extensively finding applicability in many fields of research for studying protein-protein interactions. This technique involves the use of filamentous bacteriophage for coupling proteins with the genetic information that encodes them and selection of genotypes whose phenotypes interact specifically with the antigen. This is achieved by cloning the antibody genes into the single stranded phage genome, coding for surface capsid or coat proteins, for displaying the antibodies as fusion product for target selection on the surface of encapsulated phage particles. The cloned library is then transformed into *E. coli* cells and grown allowing the propagation of phage particles in the presence of helper phage, which provides necessary proteins for packaging the phage particle. On assembly the phage is released from the bacterium by lysis, which is harvested and selected against the antigen. The selected phage is eluted from the bound antigen, reinfected, reamplified and reprocessed several times enriching for the specific phenotype along with the coding genotype. This cyclic process of alternating selection and enrichment of phage particles against specific target is termed “biopanning” (Hust and Dübel, 2004) (Figure 1.9).





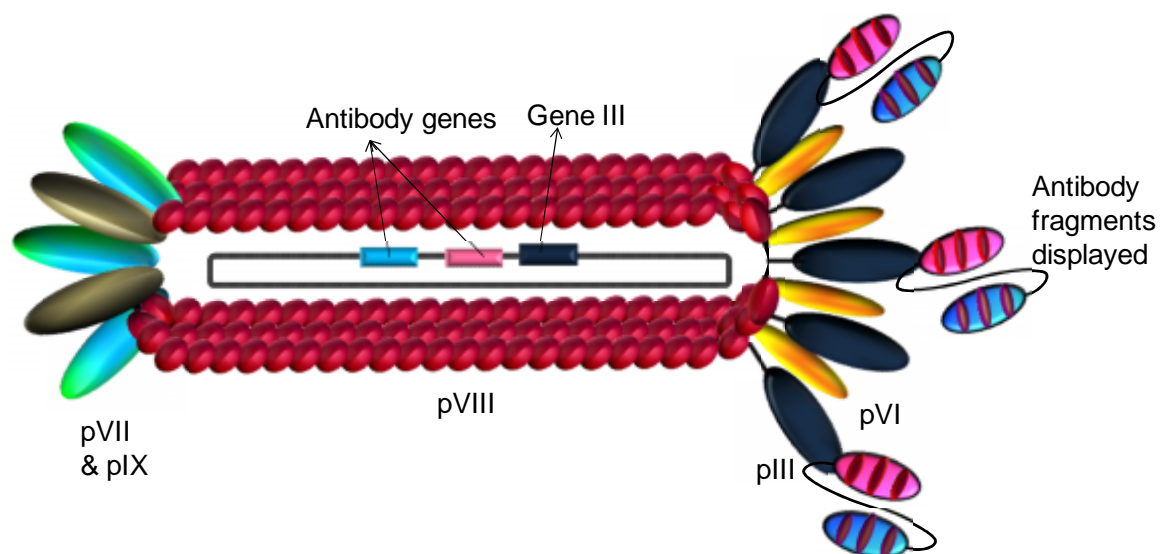
**Figure 1.9: Biopanning for selection and enrichment of phage library.**

*Biopanning is a straight forward cyclic process of selecting and enriching for the required antibodies, based on its specificity towards a particular antigen. With each round of biopanning antigen-specific phage is enriched by clonal expansion of the selected clones. By increasing the stringency of panning, high affinity clones can be selected from a diverse antibody repertoire. This process offers the advantage of flexibility for both in vitro and in vivo selection of proteins along with the choice of solid and/or solution-based panning.*

### 1.3.7.1 Biology of phage display

Phage display exploits the biology of bacteriophages and its ability to infect gram negative bacteria. Filamentous phage are the most commonly used bacteriophage in phage display systems although phage such as T4 (Ren *et al.*, 1996), T7 (O'Neil and Hoess, 1995), and phage (Gi Mikawa *et al.*, 1996) were used. Filamentous phage are a group of single stranded DNA viruses with their genome encased in long rod-shaped capsid cylinder. Among the filamentous phages, the Ff class (M13, f1 and fd) is the most extensively exploited and they use the tip of the conjugative F-pilus to facilitate infection of bacteria containing the F-plasmid. The members of this class share 98% of homology in the DNA sequences with interchangeable gene products (Russel *et al.*, 2004).

The Ff phage is approximately 6.5 nm in diameter and 930 nm in length. The single stranded phage genome is composed of 6,400 nucleotides comprising of 11 different gene proteins and an intergenic region containing an origin of replication (Figure 1.10). Five of these genes code for coat proteins, pVIII being the major coat protein producing ~2700 copies per virion and others (pIII, pVI, pVII, and pIX) being minor coat proteins producing 3-5 copies per virion (Webster, 2001; Russel *et al.*, 2004). Three genes (pI, pIV and pXI) are involved in membrane-associated assembly of phage particle and the others (pII, pV and pX) take part in DNA replication and binding (Mullen *et al.*, 2006).



**Figure 1.10: Structure of a filamentous bacteriophage.**

*Filamentous phage consists of five coat proteins (pIII, pVI, pVII, pVIII and pIX). The majority of the phage body is covered with coat protein pVIII, which is produced in high copies by the phage. Minor coat proteins, which are produced in low copies, are present on either side of the phage. Proteins pVII and pIX are displayed on one end, whereas, pIII and pVI are at the other end of the phage. The phage shown here is displaying antibody fragments on the minor coat protein, pIII.*

NMR analysis of phage infection *via* pIII revealed that the pIII consists of three domains: N1, N2 and CT. The first two domains (N1 and N2) are involved in the bacterial infection process while CT is essential for forming a stable phage particle. N1



The properties of the phage display platform are affected by the choice of coat proteins, vector system, *E. coli* strain and the helper phage used. Most commonly the libraries are generated by cloning into mature or truncated phage coat proteins III or VIII and expressing as N-terminal or C-terminal fusion products (Levin and Weiss, 2006). pVIII is a major coat protein of filamentous phage, produced in about 2700 copies per virion, whereas, pIII is a minor coat protein with 3-5 copies per virion present. Despite the low number of copies, this is widely used due to its advantage for accommodating larger polypeptides and monovalent display allowing selection of high affinity clones due to less avidity effects, which occurs in multivalent display (Goodyear and Silverman, 2008). Other coat proteins like pVI (Jespers *et al.*, 1995; Fransen *et al.*, 1999) or pVII and pIX (Gao *et al.*, 1999) were also examined for the ability to display proteins.

The cloning of the foreign DNA into phage genome is achieved by using phage display vectors. Basically, there are two types of vectors used for constructing a phage display library; phage vector and phagemid vector. Phage vectors are derived from the phage genome and contain a phage origin of replication and genes for assembly of phage particles. Such vectors are capable of polyvalent display, are faster to select and are easier to use. In contrast, the phagemid vector is a phage-plasmid vector containing both phage and plasmid origin of replication. Such vectors are capable of recombinant fusion protein production but lack the necessary proteins for producing phage. Phagemid vectors need the assistance of helper phage (e.g. M13K07 and VCSM13) with mutated or deleted pIII and a defective packaging signal (provides the genes essential for phage replication and assembly). Phagemid vectors are preferred over phage due to their monovalent display, selection of high affinity clones, due to less avidity effects, high stability, ease of handling, the presence of the antibiotic resistance gene, the *lac* promoter, affinity tags and affinity maturation options (Bradbury and Marks, 2004). The

phagemid system with improved expression was designed to facilitate phage display (Pavoni *et al.*, 2007).

The bacterial strains used during different phases determine the type of expression of antibody in phage display. Generally during the biopanning the phagemid containing the cloned library is expressed in a suppressor *E. coli* strain like XL1-Blue, TG1, in which an amber stop codon is incorporated between the displayed protein and the phage coat protein allowing the transcription of the pIII fusion protein. Non-suppressor strains, such as Top10F and HB2151, will not incorporate a glutamine at the amber codon, thereby solubly expressing the antibody moiety in *E. coli* periplasmic space without the pIII gene (Carmen and Jermutus, 2002).

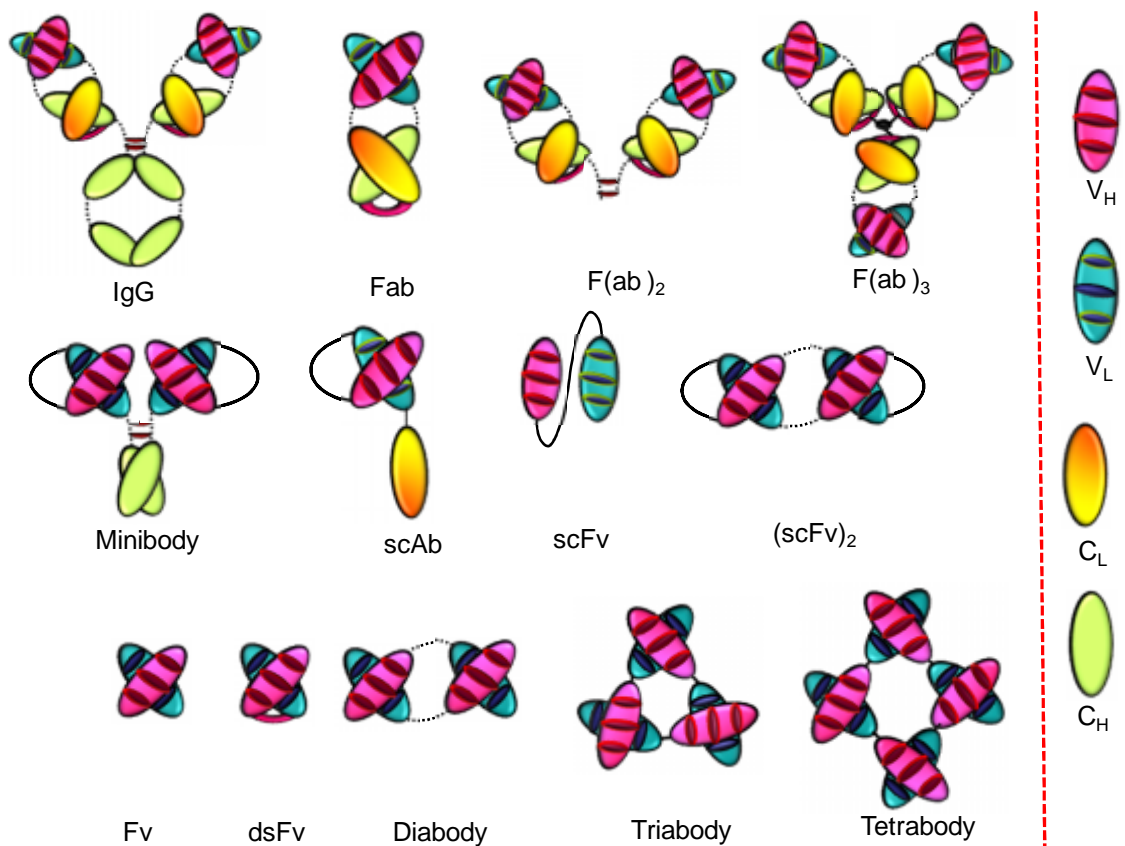
In phage display, helper phage plays an important role in display and selection of antibody. Several attempts were made to optimise the helper phage genome either to increase the display level or to reduce the background from non-displaying phage particles. The former was achieved by deleting the helper phage pIII gene (g3p) and introducing g3p by packaging the helper phage in bacterial cell lines expressing g3p (hyperphage) (Rondot *et al.*, 2001). However, this method suffered from low helper phage titre and helper phage backgrounds. Other methods involved partial deletion of g3p in the helper phage by introducing amber codons and producing the helper phage in suppressor strains and packaging of phagemids in non suppressor strains (EX-phage, phaberge) (Baek *et al.*, 2002; Soltes *et al.*, 2003). Other methods involved deletion of the infective g3p portion responsible for infecting bacteria (CT helper phage) (Kramer *et al.*, 2003) or introduction of a trypsin cleavage site on the pIII gene (KM13) (Jestin *et al.*, 2001). In a few studies bacterial packaging cell lines were used that were produced with a helper phage genome construct, eliminating the need for separate helper phage

addition, thus, simplifying the process. Such a system can be used for both monovalent and multivalent display using phagemid vectors (Chasteen *et al.*, 2006).

### **1.3.8 Recombinant Antibody Formats**

With the aid of molecular biology tools, the antibodies obtained from different libraries can be improved for their specificity, sensitivity, effector functions, pharmacokinetic and biophysical properties and to reduce immunogenicity (Carter, 2006). Antibody engineering is the most promising feature of this recombinant DNA technology overcoming the natural constraints of the immune system.

For more than a decade the molecular domain architecture of antibodies has undergone recombinant engineering to produce a range of antibody formats (Figure 1.12), that have been used as biological reagents in a variety of biosensor and other platforms (Ghatnekar-Nilsson *et al.*, 2007; Saerens *et al.*, 2008). Antibody formats basically fall into three main divisions, i.e. full length, small, and conjugated (Hudson and Souriau, 2003).



**Figure 1.12: Various antibody formats.**

Antibody heavy and light chains are joined with linker and disulphide bonds to form various monovalent, divalent and multivalent antigen binding formats. These recombinant antibody fragments may be monospecific or multispecific. Different types of long and short linkers can also be used to stabilise the fragments.

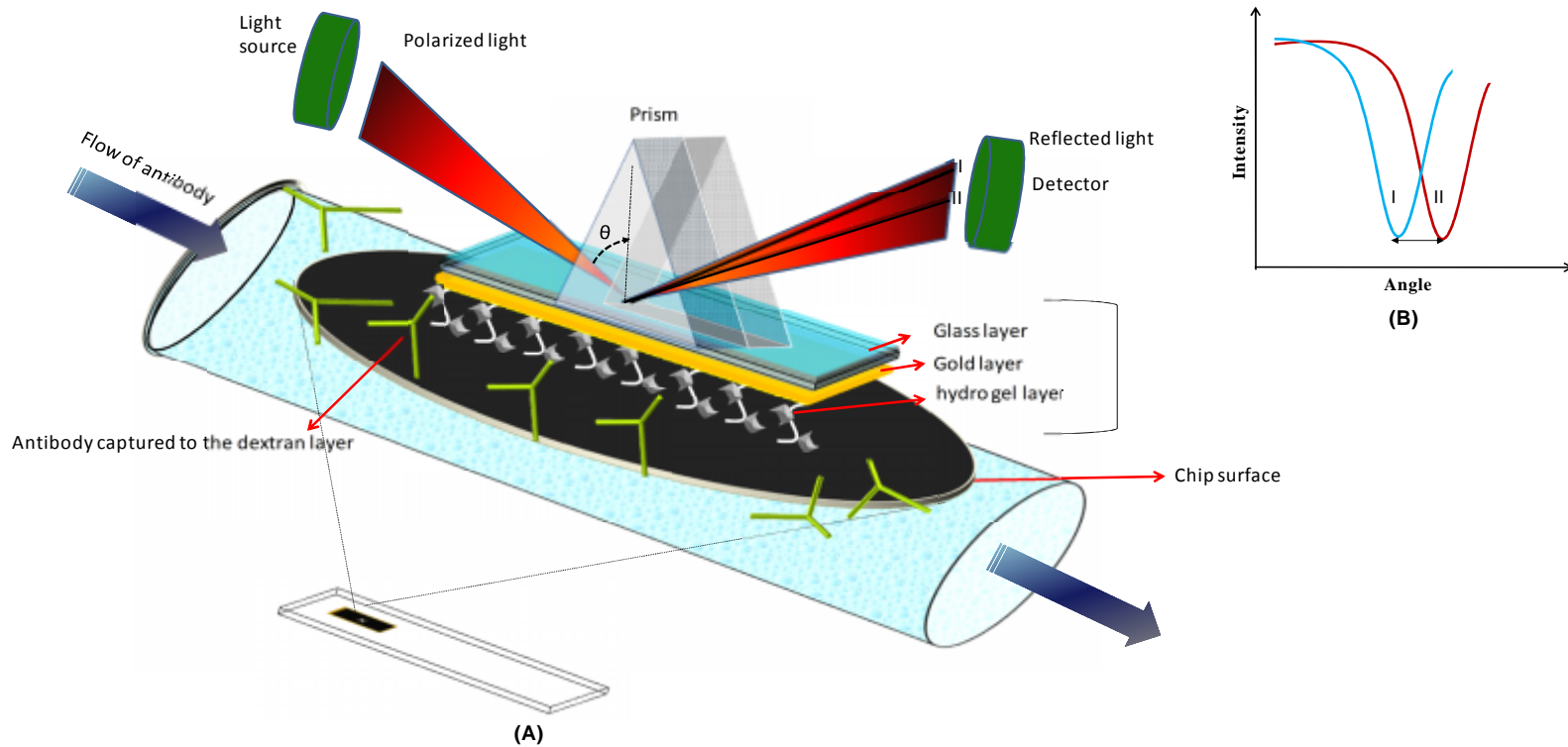
#### **1.4 Surface Plasmon Resonance in Antibody Characterisation**

Surface plasmon resonance (SPR) has emerged as the foremost and favoured technology for monitoring molecular interactions, due ostensibly to the fact that binding events can be monitored in ‘real-time’ and without the requirement for ancillary labels (Proske *et al.*, 2005). These key attributes facilitate accurate kinetic measurements, rapid analysis times and reduce the additional costs and possible heterogeneities or other complications associated with labelling of interactants. Since its discovery in 1968 (Kretschmann and Raether, 1968; Otto, 1968), SPR has witnessed numerous advances in terms of technology and its evolution for numerous applications such as immunoassays (for hormones, drugs, steroids, immunoglobulins, toxins, biomarker detection, fungi, viruses, and bacterial antigens) (Daniels *et al.*, 1988; Hsieh *et al.*, 1998; Gustafson, 2003; Leonard *et al.*, 2004; Masson *et al.*, 2004; Rella *et al.*, 2004; Leonard *et al.*, 2005; Berre and Kane, 2006; Mitchell *et al.*, 2006; Townsend *et al.*, 2006; Zezza *et al.*, 2006; Dutra *et al.*, 2007; Habauzit *et al.*, 2007; Mao and Brody, 2007; Meslet-Cladière *et al.*, 2007; Skottrup *et al.*, 2007; Bright *et al.*, 2008; Jiang *et al.*, 2008; Roche *et al.*, 2009), gas detection and biosensing (Liedberg *et al.*, 1983; Nylander *et al.*, 1983; Chabot *et al.*, 2009), and DNA binding assays (Straume *et al.*, 2009).

SPR biosensor represents an interdisciplinary field of research involving photonics, electronics, chemistry, and molecular biology. SPR is an optical transduction technique based on evanescent wave phenomenon to measure the changes in refractive index of the sensor surface. The term ‘surface plasmon resonance’ arises from the name of highly charged quasi-free electron density waves called surface plasmon, propagating at the interface between metal and dielectric. SPR is a resultant of the total internal reflection of

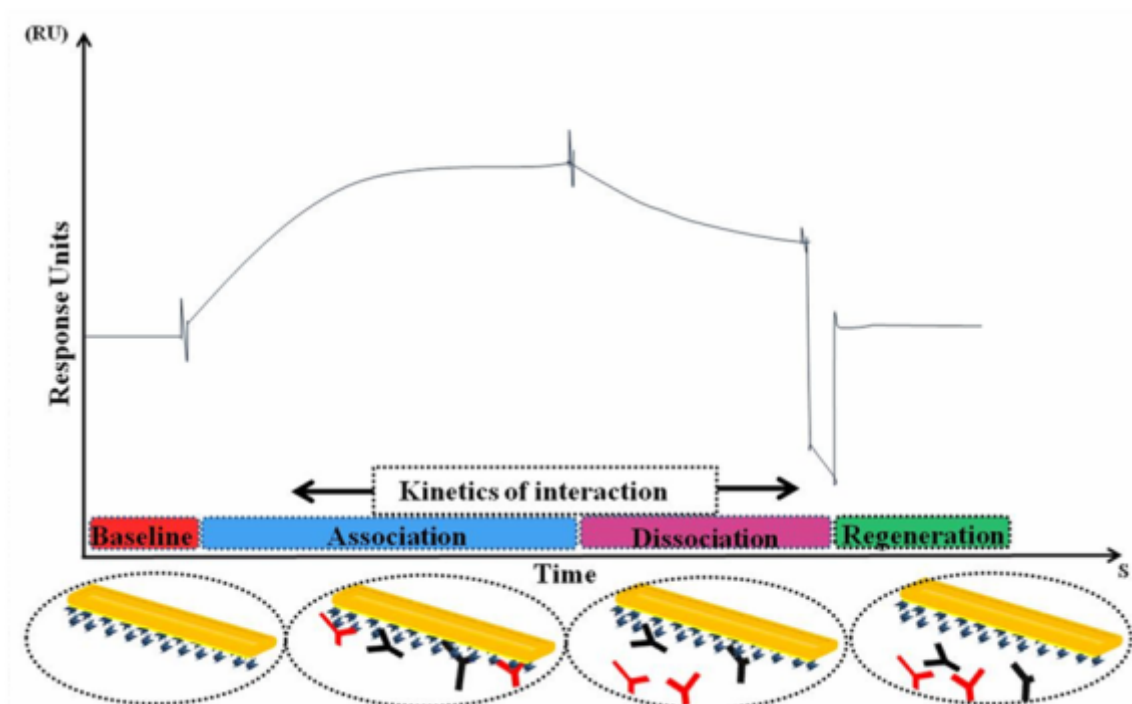


the incident polarized light when light travels from a denser medium to a rarer medium at an angle greater than the critical angle ( ) (Schasfoort and Tudos, 2007). This phenomenon occurs under certain conditions when photons of incident light are coupled with the resonance of surface electrons of a metal film (generally gold). A part of the energy is transferred to the electrons resulting in the formation of a surface plasmon. These surface plasmons create an evanescent field which penetrates a short distance into the lower refractive index medium. Photon-plasmon energy transfer depends on the dielectric properties of the material which makes this field sensitive to the refractive index at the metal surface. For plasmon excitation to take place the wave vector for the photon and plasmon should be equal in magnitude and direction. Any change in the refractive index of media is compensated by either changing the angle of incidence or the wavelength of light to equate the wave vector of plasmon. This resonance match between the photon and the plasmon results in absorption of a part of incident light, exciting the plasmon, thus diminishing the intensity of reflected light which can be observed as sharp attenuation of reflectivity (Figure 1.13) (Campbell and Kim, 2007). Thus the progress of reaction can be monitored by detecting the SPR angle shift and plotting it with respect to time ([http://www.rci.rutgers.edu/~longhu/Biacore/pdf\\_files/SPR\\_TechNote.pdf](http://www.rci.rutgers.edu/~longhu/Biacore/pdf_files/SPR_TechNote.pdf)).



**Figure 1.13. Schematic of the typical set-up for a surface plasmon resonance (SPR) biosensor.**

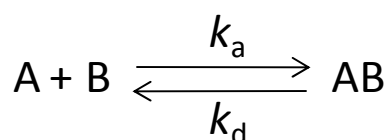
SPR refers to the excitation of surface plasmons propagating in the metal/dielectric interface. These plasmons are sensitive to the changes in the refractive index in the immediate vicinity to the interface. Depending on the mass on a sensor chip surface, at a critical angle of incidence ( ), total internal reflection of the light occurs resulting in absorption of a part of the light causing a shadow in the reflected light. (A) A typical Kretschmann prism set-up integrated with different components of biosensor. (B) Change in the mass at the surface layer resulting in shift in the resonance angle (from I to II in the diagram) at the dip in the intensity of reflected light.



**Figure 1.14. Sensorgram obtained as a plot of resonance signal (proportional to mass change) versus time obtained as a change in resonance angle.**

A typical sensorgram consists of various regions indicating different phases of a biomolecular interaction enabling 'real-time' monitoring. The baseline represents the beginning of the sensorgram with buffer flowing over the chip surface which has immobilised analyte attached. Association is the phase of the sensorgram where the interactant is passed over the surface resulting in the formation of complex between the immobilised analyte and the interactant, thus, increasing the mass on the surface. The continuous flow of buffer results in the dissociation of interactant from the analyte resulting in the gradual decrease in the mass over the surface. The surface is regenerated using acid or alkaline solutions removing any bound analyte returning the response to the baseline level.

SPR is widely employed in studying antigen-antibody reactions (Ayela *et al.*, 2007; Tang *et al.*, 2007a) and the antibodies are characterised based on their affinity, epitope binding and kinetic characteristics (Cachia *et al.*, 2004; Mahmood *et al.*, 2008; Doern *et al.*, 2009). The Biacore family of SPR biosensors are widely used in the antibody characterisation (Mytych *et al.*, 2009) and may be further used for immunoassay development. The key application of Biacore in antibody characterisation is determination of affinity and kinetic characteristics of biomolecular interactions which in turn is determined by a number of biophysical parameters. The simplest manifestation is the 1:1 binding interaction, which can be described for immobilised ligand (A) binding to its cognate analyte (B). This is presented below with different biophysical determinants described in Table 1.6.



**Table 1.6: Definition of biophysical determinants of biomolecular interactions.**

Constant	Definition	Description	Proportionality	Units
$k_a$	Association rate constant (Also referred to as 'on-rate' or ' $k_{on}$ ')	No. of AB complexes formed per unit time at unit concentration of A and B.	Affinity $\propto k_a$	$M^{-1}s^{-1}$
$k_d$	Dissociation rate constant (Also referred to as 'off-rate' or ' $k_{off}$ ')	No. of AB complexes dissociating per unit time.	Affinity $\propto 1/k_d$	$s^{-1}$
$K_A$	Equilibrium association constant	Affinity propensity to association of A + B	$K_A = k_a/k_d = [AB]/[A][B]$	$M^{-1}$
$K_D$	Equilibrium dissociation constant	Affinity contribution to stability of AB complex	$K_D = k_d/k_a = \{[A][B]\}/[AB]$	M

## **1.5 Aims**

The key aims of the research described in this thesis were:

- Generation of chicken anti-hFABP recombinant antibodies.
- Generation of rabbit anti-cTnI recombinant antibodies.
- Cloning and expression of recombinant hFABP and cTnI.
- Characterisation of the generated anti-hFABP and anti-cTnI recombinant antibodies based on specificity, sensitivity and affinity measurements.
- Optimisation of recombinant antibody format for expression of rabbit anti-cTnI antibody.

# **Chapter 2**

## **Materials and Methods**

## 2. MATERIALS AND METHODS

### 2.1 Materials

Materials used in the study were divided into equipment, culture media, bacterial strains, buffers, commercial kits, commercial antigens and antibodies.

#### 2.1.1 Equipment

This list consists of the standard equipment used in and their supplier information.

Equipment item	Supplier
Biometra T <sub>GRADIENT</sub> PCR machine	LABREPCO, 101 Witmer Road, Suite 700, Horsham, PA19044, USA.
Nanodrop™ ND-1000	NanoDrop Technologies Inc., 3411 Silverside Rd 100BC, Wilmington, DE19810-4803, USA.
Gene Pulser Xcell™ electroporation system	Bio-Rad Laboratories Inc., 2000 Alfred Nobel Drive, Hercules, California 94547, USA.
Roller mixer SRT1	Sciencelab Inc., 14025 Smith Rd, Houston, Texas 77396, USA.
Safire 2 plate reader	Tecan Group Ltd., Seestrasse 103, CH-8708 Männedorf, Switzerland.
PX2 thermal cycler	Thermo Electron Corporation, 81 Wyman Street, Waltham, Massachusetts (MA), 02454, USA.
Biacore 3000 and A100	GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden.

Vibra Cell™ sonicator	Sonics and Materials Inc., 53 Church Hill Road, Newtown, CT 06470-1614, USA.
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### 2.1.2 Culture Compositions

This section describes the various media compositions used for culturing bacterial cells. All chemicals were purchased from Sigma-Aldrich Co., Poole, Dorset, England and Cruinn Diagnostics Ltd., Hume Centre, Parkwest Business Park, Nanogor Road, Dublin 12, Ireland.

2x Tryptone Yeast extract (2X TY) Media	Tryptone	16 g/L
	Yeast extract	10 g/L
	NaCl	5 g/L

Super Broth (SB) Media	MOPS	10 g/L
	Tryptone	30 g/L
	Yeast extract	20 g/L

Super Optimal Catabolite (SOC) Media	Tryptone	20 g/L
	Yeast extract	5 g/L
	NaCl	0.5 g/L
	KCl	2.5 mM
	MgCl <sub>2</sub>	20 mM
	Glucose	20 mM



100x 505 media	Glycerol	50% (v/v)
	Glucose	5% (w/v)

### 2.1.3 Bacterial strains

The *E. coli* strains used for various purposes of expression are listed below:

*E. coli* TOP10 F' strain: {*lacI<sup>q</sup>*, Tn10(Tet<sup>R</sup>)} *mcrA*  $\Delta$ (*mrr-hsdRMS-mcrBC*) 80*lacZ*  $\Delta$ M15  $\Delta$ *lacX74* *recA1* *araD139* (*ara-leu*)7697 *galU* *galK* *rpsL* (Str<sup>R</sup>) *endA1* *nupG*

*E. coli* XL1-Blue strain: *recA1* *endA1* *gyrA96* *thi-1* *hsdR17* *supE44* *relA1* *lac* [F *proAB* *lacI<sup>q</sup>*Z M15 Tn10 (Tet<sup>R</sup>)].

*E. coli* BL21-CodonPlus(DE3)-RIPL strain: B F<sup>-</sup> *ompT* *hsdS*(*r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>*) *dcm*+ Tetr *gal* (DE3) *endA* *Hte* [*argU* *proL* Camr] [*argU* *ileY* *leuW* Strep/Specr]

*E. coli* K12 ER2738 strain: F'*proA<sup>+</sup>B<sup>+</sup>* *lacI<sup>q</sup>* (*lacZ*)M15 *zzf::Tn10*(Tet<sup>R</sup>)/*fhuA2* *glnV* (*lac-proAB*) *thi-1* (*hsdS-mcrB*)5

### 2.1.4 Buffer Compositions

Compositions of various commonly used buffers are enlisted below:

Phosphate buffered saline (PBS) (150 mM, pH 7.4)	0.15 M NaCl
	2.5 mM KCl
	10 mM Na <sub>2</sub> HPO <sub>4</sub>
	18 mM KH <sub>2</sub> PO <sub>4</sub>
d in 800 mL of ultra pure H <sub>2</sub> O, and the pH was adjusted to 7.4. The volume ra pure H <sub>2</sub> O.	

BSA-Milk-PBST (MPBST)	PBST
	Specified % (w/v) milk marvel powder
	Specified % (w/v) BSA

Phosphate buffered saline Tween (PBST) (150 mM, pH 7.4)	PBS
	0.05% (v/v) Tween 20 detergent

Hepes buffered saline (HBS)	HEPES	2.38 g/L
	NaCl	8.77 g/L
	EDTA	1.27 g/L
	Tween	0.05% (v/v)
<i>HEPES, NaCl and EDTA were dissolved in 800 mL of ultra-pure water. The pH was adjusted to pH 7.4 by adding 1M NaOH. The solution was filtered through a 0.2 µm filter and degassed for 1 hour followed by addition of Tween 20.</i>		

1 M TrisHCl	Trizma base	121.14 g/L
	<i>Trizma base was dissolved in 800 mL of ultra-pure water. The pH was adjusted to pH 8.8 or 6.8, for separation and stacking gels, by adding 1M HCl. The solution was filtered through a 0.2 µm filter and used for SDS-PAGE preparations.</i>	

## **SDS-PAGE**

<b>12.5% Separation gel</b>	<b>1 gel/6 mL</b>
1 M TrisHCl, pH 8.8	1.5 mL
30% (w/v) acrylamide	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) APS	30 µL
TEMED	6 µL

<b>12.5% Stacking gel</b>	<b>1 gel/ 2.5 mL</b>
1 M TrisHCl, pH 6.8	300 µL
30% (w/v) acrylamide	375 µL
2% (w/v) methylamine bisacrylamide (Bis-Acrylagel)	150 µL
Water	1.74 mL
10% (w/v) sodium dodecyl sulfate (SDS)	24 µL
10% (w/v) APS	24 µL
TEMED	2.5 µL

<b>Sample treatment buffer (4x)</b>	<b>10 mL</b>
0.5M Tris, pH 6.8	2.5 mL
Glycerol	2.0 mL
2 mercaptoethanol	0.5 mL
20% SDS (w/v)	2.5 mL
Bromophenol blue	20 ppm
Water	2.5 mL

<b>10x electrophoresis buffer</b>	<b>1 L</b>
Tris pH, 8.3	30 g
Glycine	144 g
SDS	10 g
Water	to 1

<b>Coomassie blue stain dye</b>	<b>1 L</b>
Coomassie blue R-250	2 g
Methanol	450 mL
Acetic acid	100 mL
Water	450 mL

<b>Coomassie destain solution</b>	<b>1 L</b>
Acetic acid	100 mL
Methanol	250 mL
Water	650 mL

### 2.1.5 Commercial Kits

<b>Kit</b>	<b>Supplier</b>
Superscript III reverse transcriptase kit	Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008, USA.
Perfectprep gel cleanup kit	Eppendorf AG, Barkhausenweg 1, Hamburg 22339, Germany.
Wizard Plus SV Miniprep™ kit	Promega, 2800 Woods Hollow Road, Madison, WI 53711, USA.
QIAquick™ gel extraction kit	Qiagen, 28159 Avenue Stanford, Valencia, CA 91355, USA.

### 2.1.6 Commercial Antigens and Antibodies

Antigen	Supplier
HFABP (heart-fatty acid binding protein) CTnI antigen	Life Diagnostics Inc., PO Box 5205, West Chester, PA 19380.
CTnI peptide 1 CTnI peptide 2 CTnI peptide 3 CTnI peptide 4	HyTest Ltd., Intelligate 6 <sup>th</sup> Floor, Joukahaisenkatu 6 20520 Turku, Finland.
mFABP(Myelin fatty acid binding protein) bFABP (brain fatty acid binding protein) lFABP (liver fatty acid binding protein)	Randox Laboratories Ltd., 55 Diamond Road, Crumlin, Co. Antrim, Northern Ireland.
aFABP (adipocyte fatty acid binding protein)	BioVendor GmbH, Im Neuenheimer Feld 583, D-69120 Heidelberg, Germany.

Antibody	Supplier
Anti-bFABP Anti-mFABP Anti-bFABP Anti-aFABP Anti-lFABP	Hycult Biotech, Frontstraat 2a, 5405 PB Uden, The Netherlands.
Mouse anti-HA mAb	Cambridge Bioscience, 24-25 Signet Court, Newmarket Road, Cambridge CB5 8LA, UK.
Anti-M13 antibody (HRP-labelled)	GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden.
Anti-His (HRP-labelled) mAb Anti-chicken (HRP-labelled) pAb Anti-rabbit (HRP-labelled) pAb Anti-mouse (HRP-labelled) pAb	Sigma Aldrich, 3050 Spruce Street, St. Louis, MO 63103, USA.

## **2.2 Methods**

### **2.2.1 Generation of Chicken Anti-hFABP scFv**

#### **2.2.1.1 Immunisation of chicken with hFABP**

The work on the hFABP library, from immunisations to biopanning, was carried out by Dr. Stephen Hearty. All the procedures involving animals were first approved by the ethics committees of School of Biotechnology and Dublin City University and they then underwent approval and licensing by the Department of Health and Children under licence number B100/3816. The Laboratory Animal Science and Training (LAST) Ireland course was undertaken to ensure that legal aspects of animal usage in research are well understood and experimental procedures are performed with high standards of care. All the necessary precautions were taken to ensure minimal distress to the animals during the entire study.

Two white male leghorn chickens were injected with 200  $\mu$ L of a homogeneous mixture of 200  $\mu$ g/mL of the hFABP, myoglobin and CRP mixed thoroughly with an equal volume of Freund's complete adjuvant (FCA). Adjuvants are substances used to enhance, accelerate, or prolong, antigen-specific immune responses when used in combination with a specific antigen. These substances lack any specific antigenicity in an animal when used on their own. Freund's adjuvant is an emulsified mineral oil-antigen solution that is used as an immune-potentiator.

Subsequent boosting injections were given using 1:1 ratio of antigen (50  $\mu$ g/mL), along with Freund's incomplete adjuvant at 4 weekly intervals. A bleed was taken after each boost in 15 mL tubes and the serum was separated by incubating the blood in a tilted position at 4°C overnight (O/N). The next day the blood was centrifuged at 805 g for 10 minutes resulting in the separation of the straw coloured serum from the clotted

remainder. The serum was removed carefully and 0.2% (w/v) sodium azide was added. The serum was then aliquoted into 1.5 mL tubes and stored in -20°C.

The serum was assayed by ELISA to determine the antibody titre against hFABP. A Nunc ELISA plate, coated with 100 µL of 2 µg/mL hFABP, was incubated O/N at 4°C. It was then blocked with 200 µL of 2.5% (w/v) BSA-milk marvel in PBS (pH 7.4, 150 mM) for 1 hour at 37°C. Doubling dilutions of immune serum along with the pre-immune serum (starting from 1:1,600 to 1:1,638,400) in 0.5% (w/v) BSA-milk marvel-PBST were prepared and 100 µL of 5 dilutions (in duplicate) were added to the plate. Following incubation for 1 hour at 37°C, 100 µL of 1:2,000 dilution of the detection antibody (HRP-conjugated anti-chicken antibody from Sigma) in 150 mM PBS (pH 7.4) was added. The plates were again incubated for 1 hour at 37°C. The plates were washed 3 times with 0.05% (v/v) PBST and 3 times with PBS after each step. The plate was incubated for 1 hour at 37°C, washed, and developed with 100 µL of Tetramethylbenzidine dihydrochloride (TMB). After 20 minutes, the reaction was quenched using 50 µL of 10% (v/v) HCl. The absorbance values were read at 450 nm on a Safire 2 plate reader (Tecan). Once the titre had reached a satisfactory level (refer to section 3.2), the chickens were again boosted 5 days prior to sacrifice.

#### **2.2.1.2 RNA extraction and cDNA preparation**

A Gelaire BSB 4 laminar unit was prepared by spraying with both IMS and RNase<sup>TM</sup> ZAP, thus ensuring an uncontaminated environment (free from contaminant RNA). The chicken was sacrificed by cervical dislocation and both the spleen and bone marrow were harvested immediately using sterile surgical implements.

The bone marrow from chicken femurs was washed out with 10 mL of chilled TRIzol<sup>®</sup> reagent (Invitrogen, USA) using a 25 gauge needle and 5 mL syringe. Ten mL of chilled TRIzol<sup>®</sup> reagent was added to the avian spleen and all samples were fully homogenised using a sterile (autoclaved and baked O/N at 180°C) homogeniser. The tubes were incubated at room temperature for 5 minutes, to allow for the total dissociation of nucleoprotein complexes while maintaining the integrity of the RNA, and centrifuged (Eppendorf centrifuge 5810R) at 2,465 g for 10 minutes at 4°C. The supernatants were carefully removed and transferred to fresh 'RNase-free' 50 mL Oakridge tubes (Thermo Fisher Scientific, USA). For each sample, 3 mL of 'RNase-free' chloroform were added and tubes were shaken vigorously for 15 seconds and incubated for 15 minutes at room temperature. Addition of chloroform leads to the separation of the homogenised spleen into an upper aqueous phase (containing RNA) and a lower organic phase (containing DNA and protein). Tubes were centrifuged at 12,000 x g at 4°C for 15 minutes producing 3 layers, a lower red phenol/chloroform phase, a protein interphase and a colourless liquid upper phase with the RNA. The upper aqueous phase, containing the RNA, was carefully removed and transferred to a fresh 'RNase-free' 50 mL Oakridge tube. To each tube, 15 mL of propan-2-ol was added and contents were shaken vigorously for 15 seconds, stored at room temperature for 10 minutes and centrifuged at 12,000 x g at 4°C for 30 minutes. RNA was precipitated as a white pellet on the bottom and side of the tube. The supernatant was decanted carefully and the pellet was washed with 30 mL of 75% (v/v) ethanol and centrifuged at 12,000 x g at 4°C for 10 minutes. This step was repeated and after removal of the supernatant, the RNA pellet was allowed to air dry for 5 minutes. The pellet was then resuspended in 250 µL of 'RNase-free' water. The RNA concentrations were determined by spectrophotometric measurement at 260 nm with a NanoDrop<sup>™</sup> spectrophotometer ND-1000 (Thermo Fisher Scientific, USA).



An aliquot of freshly isolated RNA was used for cDNA synthesis. The remaining RNA solution was precipitated at -20°C with 1/10 the volume of 'RNase-free' sodium acetate, pH 5.2, (Sigma-Aldrich), and twice the total sample volume of 100% (v/v) ethanol. To enhance RNA precipitation, 'nuclease-free' glycogen (Fermentas) was added at a final concentration of 1µg/µL.

### **2.2.1.3 Reverse transcription of total RNA to cDNA**

A PCR reaction was set up for synthesizing first strand-cDNA from mRNA using oligo dT20 priming. All the components, excluding the enzymes, were placed on ice and slowly thawed.

Enzymes were removed from the freezer just before the use, to prevent loss of activity. A 20X reaction was set up for both the mixtures. 8 x 25 µL aliquots were made for mixture 1(RNA, Oligo (dT) primer and dNTP mix), in sterile 'RNase-free' PCR tubes and left to incubate at 65°C for 5 minutes and then placed on ice. This allows initial denaturation of the sample. To this sample 25 µL of mixture 2 (RT Buffer, MgCl<sub>2</sub>, DTT, RNase Out, Superscript enzyme) was added. This step allows the annealing of oligo (dT) to the RNA template containing poly (A) tail at the 3' end. The reaction mixture was incubated at 50°C for 50 minutes, during which cDNA strand was synthesized. The reaction was terminated by heating the mixture at 85°C for 5 minutes. Finally, 5 µL of RNase<sup>TM</sup> H was added to each of the 8 x 50 µL reactions and incubated at 37°C for 20 minutes. This step ensures the removal of residual RNA template which might decrease the sensitivity of PCR and other analysis carried out with the final cDNA product.

**Table 2.1: Composition of cDNA synthesis reaction mixtures.**

Mixture 1 Components	Conc. in 10 $\mu$ L volume	Mixture 2 Components	Conc. in 10 $\mu$ L volume
Total RNA	5 $\mu$ g	10x RT Buffer	2x
Oligo (dt) primer	0.5 $\mu$ g	MgCl <sub>2</sub>	2.5 mM
dNTP mix	1 mM	DTT	20 mM
Water	N/A	'RNase-Out'	40 U
		Superscript enzyme	200 U

#### 2.2.1.4 PCR primers for amplification of chicken scFv (pComb series)

The primers, listed below, were obtained from Eurofins-MWG-Operon (Eurofins MWG Operon, Anzingerstr. 7a, 85560 Ebersberg, Germany). The oligonucleotides obtained were used to generate a chicken scFv library from both the bone marrow and spleen, as described in Barbas *et al.* (2001). The amplified heavy and light chains will contain a long linker sequence along with *Sfi*I sites compatible for cloning into pComb vector series.

##### V<sub>H</sub> primers

CHICK-VH-FOR 5 sense primer

5 GGT GGT TCC TCT AGA TCT TCC TCC GGT GGC GGT GGC TCC GGC GGT  
GGT GGC TCT TCC GCC CTG ACG TTG GAC GAG 3

CHICK-VH-REV 3 antisense primer

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

##### V<sub>L</sub> primers

CHICK-VL-FOR 5 sense primer

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

CHICK-VL-REV 3 antisense primer

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

### Overlap extension Primers

CHICK-SOE-FOR 5 sense primer

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

CHICK-SOE-REV 3 antisense primer

5 GAG GAG GAG GAG GAG GAG GAG CTG GCC GGC CTG GCC ACT AGT

GGA GG 3

### **2.2.1.5 Amplification of antibody variable domain genes using pComb series primers**

For amplification of variable heavy and light genes the components for a 1x reaction were as follows:

**Table 2.2: Composition of PCR mix for chicken heavy and light antibody chain amplification.**

Components	Conc. in 50 $\mu$ L volume
GoTaq Buffer (5x)	1x
Sense Primer	60 pM
Anti-sense Primer	60 pM
cDNA	0.5 $\mu$ g
dNTP	0.2 mM
MgCl <sub>2</sub>	1mM
H <sub>2</sub> O	N/A
GoTaq™ Polymerase	2.5 U

The PCR for the amplification of the antibody variable domain genes was performed in the Biometra T<sub>GRADIENT</sub> PCR machine under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	94	300	1
2	94	30	30
	56	30	
	72	60	
3	72	600	1

#### **2.2.1.6 Purification of V<sub>H</sub> and V<sub>L</sub> variable gene-fragments using Qiagen gel extraction kit**

Gel extraction was carried out for purification of the amplified gene (distinctively amplified using gene specific primers) from the whole cDNA template. The V<sub>H</sub> and V<sub>L</sub> chain gene amplicons were resolved on 1% (w/v) agarose gel. This allows separation of the heavy and light chain genes from the rest of the PCR amplified products, which is identified based on molecular weight. The bands for V<sub>H</sub> chain will be between 386-440 bp, for V<sub>L</sub> chain will be between 375-402 bp. Bands of correct size were excised carefully from the gel by using sterile scalpels to avoid any cross-over of the DNA and transferred to sterile clean 1.5 mL micro-centrifuge tubes. DNA binding buffer, provided by the manufacturer, was added to the gel fragment in 1:3 ratio (w/v). The gel-buffer mixture was dissolved by placing it in a water bath for 10 minutes at 50°C. One volume of isopropanol, equal to the original weight of the gel slice, was then added and mixed to allow for precipitation of the DNA. The resulting mixture was then added to the silica column placed on a collection tube. The column was centrifuged at 22,000 x g for 1 minute to remove any residual buffer. The flow-through was discarded and the column was washed with 750 µl wash buffer. The column was centrifuged as before and washed with 250 µl wash buffer. The column was centrifuged at 22,000 x g for 2 minutes and the DNA was eluted from the column using 30 µL molecular grade H<sub>2</sub>O. The purified DNA absorbance was measured @ 260 nm using Nanodrop™ ND-1000 and the concentration calculated using the Beer-Lambert law.

#### **2.2.1.7 Splice-by-Overlap extension (SOE) PCR**

The V<sub>H</sub> and V<sub>L</sub> purified fragments were joined using an SOE-PCR *via* a glycine-serine linker (G<sub>4</sub>S)<sub>3</sub>, producing a fragment of approximately 750 bp.

The following composition of PCR mix was used for the SOE-PCR:

**Table 2.3: Reaction mix for overlapping chicken heavy and light chains.**

Components	Conc. in 50 $\mu$ L volume
HiFi buffer (10x)	1x
CSC-F Primer	60 pM
CSC-B Primer	60 pM
V <sub>H</sub> chain	100 ng/rxn
V <sub>L</sub> chain	100 ng/rxn
dNTP	0.2 mM
MgSO <sub>4</sub>	1 mM
H <sub>2</sub> O	N/A
Platinum HiFi™ <i>Taq</i>	2.5 U

The SOE-PCR was performed in the Biometra T<sub>GRADIENT</sub> PCR machine under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	94	300	1
2	94	30	30
	56	30	
	72	60	
3	72	600	1

#### 2.2.1.8 *Sfi* restriction digest of purified SOE-PCR fragment and ligation into pComb3XSS vector

The scFv fragment (SOE product) and pComb3XSS vector (phagemid vector) were digested using *Sfi* restriction enzyme. The *Sfi* sites on both forward and reverse SOE primers are different and similar to that of the *Sfi* sites on the vector thus allowing unidirectional cloning of the scFv gene into the phagemid vector. The enzyme recognises 8 bases which are interrupted by 5 non-recognised nucleotides (5'-GGCCNNNNNGGCC-3'), thus virtually eliminating internal digestion in antibody sequences. Both the digestions (outlined below) were carried out for 5 hours at 50°C. The reaction mix was further treated with *Xho*I and *Xba*I, to further digest the stuffer released, thus eliminating the possibility of stuffer contamination in the resulting library. Finally the vector was treated with calf alkaline phosphatase to prevent vector re-ligation.

**Table 2.4: Reaction mix for digestion of vector and chicken scFv SOE-product.**

Components	Conc. in 70 $\mu$ L volume	Components	Conc. in 70 $\mu$ L volume
10x buffer II	1x	10x buffer II	1x
pComb3XSS vector	50 $\mu$ g	SOE product	10 $\mu$ g
<i>Sfi</i> enzyme	300 U	<i>Sfi</i> enzyme	160 U
100x BSA	1x	100x BSA	1x
Water	N/A	Water	N/A

Both digests were resolved *via* electrophoresis on a 1% agarose gel and then gel-purified as described in section 2.2.1.6. The restricted scFv gene was then ligated into the pComb3XSS vector in a 3:1 (insert: vector) ratio as outlined below and incubated at room temperature O/N.

**Table 2.5: Ligation mixture for cloning chicken scFv SOE-product into pComb3XSS vector.**

Components	Conc. in 70 $\mu$ L volume
10x Ligase buffer	1x
Digested pComb3XSS vector	5 $\mu$ g
Digested scFv gene	100 U
T <sub>4</sub> DNA Ligase enzyme	400 U
Water	N/A

The ligation mixture was then subjected to ethanol precipitation by adding 1/10th the volume of 'RNase-free' sodium acetate (pH 5.2), 2 times the volume of 100% (v/v) ethanol and 1  $\mu$ L of glycogen. After O/N precipitation at -20°C, the sample was centrifuged at 22,000 x g for 20 minutes at 4°C and the pellet was washed with 70% (v/v) ice-cold ethanol. The mixture was centrifuged at 22,000 x g for 10 minutes at 4°C and the pellet was allowed to air-dry briefly and resuspended in 5  $\mu$ L of molecular grade water.

### **2.2.1.9 Transformation of cloned gene into *E. coli* by electroporation**

Commercially available electrocompetent XL1-Blue *E. coli* cells (Stratagene, Agilent technologies, 11011 N. Torrey Pines Road, La Jolla, CA 92037, USA) were transformed with the ligated scFv vector construct, by electroporation, using the Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, USA). The program was set at 25  $\mu$ F, 1.25 kV and the pulse controller at 200 . The *E. coli* cells (100  $\mu$ L) were thawed on ice just before transformation. The ligated product (10  $\mu$ L) was added to the cells, mixed, left to incubate for 1 minute and immediately transferred it to an ice-cold 0.2 cm electroporation cuvette (Bio-Rad Laboratories, USA). The cuvette was tapped gently to move the cell suspension between the electrodes and was placed in the ShockPod and pulsed once. The cuvette was quickly removed from the chamber and 1 mL of SOC medium was added immediately to the cuvette, thus, resuspending the cells in medium. The period between applying the pulse and transferring the cells to the growth media is crucial for recovering *E. coli* transformants. A delay in transfer, even by 1 minute, causes a 3-fold drop in transformation. The resuspended cells were transferred to a 20 mL sterile universal container containing 2 mL of SOC media, which was later incubated for 1 hour at 250 rpm at 37°C shaking, to facilitate recovery of the cells. The transformants from both bone marrow and spleen libraries were pooled and plated on LB plates, supplemented with 100  $\mu$ g/mL carbenicillin and 1% (v/v) glucose. Untransformed XL1-Blue *E. coli* cells (negative control) were plated out in parallel on agar plates with 100  $\mu$ g/mL carbenicillin and 1% (v/v) glucose. The plates were incubated O/N at 37°C. The pComb3XSS transformant colonies were scraped off the plates and were suspended in 20% (v/v) glycerol, snap frozen in liquid nitrogen and stored at -80°C as stocks.

#### **2.2.1.10 Rescue and subsequent precipitation of scFv-displaying phage**

The anti-hFABP library was propagated by inoculating 400  $\mu$ L of the transformed library glycerol stocks into 400 mL sterile SB medium supplemented 100  $\mu$ g/mL carbenicillin. The culture was grown at 37°C at 200 rpm until mid-exponential phase of growth (O.D.  $\sim$  0.6 @ 600 nm) was achieved. Helper phage ( $2 \times 10^{11}$  pfu/mL) was added and the culture was left static at 37°C for 30 minutes for subsequent F-pilus conjugation to occur (an essential part of infection process) and then transferred into a 37°C shaking incubator for 2 hours at 250 rpm. Subsequently, kanamycin (70  $\mu$ g/mL) was added and the culture was incubated O/N at 37°C and 250 rpm. Kanamycin addition ensures the propagation of bacteria co-infected with helper phage.

The O/N culture was centrifuged at 3,220 x g for 15 minutes at 4°C and the supernatant was transferred to clean sterile centrifuge tubes (Thermo Fisher Scientific, USA), leaving the bacterial pellet.

Phage particles in the supernatant were precipitated by addition of 8 g of PEG 8000 and 6 g of sodium chloride. The mixture was agitated at 250 rpm in a 37°C shaking incubator until both additives were dissolved and then it was incubated on ice at 4°C for 1 hour for phage precipitation. The precipitated phage was harvested by centrifugation at 18,500 x g for 25 minutes at 4°C with brakes off. After centrifugation, the supernatant was carefully decanted and the phage pellet was resuspended in 2 mL of 0.5% (w/v) BSA-PBS buffer solution. The resuspended phage pellet was then added to a clean 1.5 mL micro-centrifuge tube and centrifuged at 22,000 x g for 10 minutes at 4°C to remove any bacterial debris. Lastly, the phage supernatant was transferred to a fresh micro-centrifuge tube and stored on ice at 4°C.



#### **2.2.1.11 Selection and enrichment of hFABP clones *via* biopanning**

An immunotube (Maxisorp™, Nunc) was coated O/N at 4°C with 500 µL of 50 µg/mL hFABP in PBS solution, and blocked for 2 hours at room temperature with 3% (w/v) BSA in PBS (150 mM, pH 7.4). Following one wash with both PBST and PBS, 500 µL of rescued phage was added to the immunotube and incubated on a roller mixer (SRT1) for 2 hours at room temperature. The immunotube was then washed 5 times with PBST and PBS, to remove non-binding phage. Specific bound phages were then eluted by incubation with 500 µL of 10 mg/mL trypsin-PBS.

Eluted phages were added to 4 mL of mid-exponential phase XL1-Blue cells, allowing infection for 30 minutes (static) at 37°C. Four mL of SB media supplemented with 2.4 µL of 100 mg/mL carbenicillin and 12 µL of 5 mg/mL tetracycline were added. Two µL of infected culture was removed and diluted 1:100 in SB media. Ten and 100 µL of the diluted culture was plated on LB agar plates containing 100 µg/mL carbenicillin. An input titre was performed by making serial dilutions ( $10^{-1}$ - $10^{-12}$ ) of the PEG-precipitated phage in SB media and infecting the XL1-Blue cells in exponential growth phase, with phage dilutions ( $10^{-8}$ -  $10^{-12}$ ). Following a 15 minutes infection at 37°C, the cells were plated on LB agar plates containing 100 µg/mL carbenicillin and incubated overnight at 37°C.

All of the subsequent rounds of panning were performed as described above with the following amendments:

**Table 2.6: Parameters varied in subsequent rounds of panning of avian anti-hFABP scFv library.**

	PAN 1	PAN 2	PAN 3	PAN 4
Culture vol.	200 mL	200 mL	200 mL	200 mL
hFABP coated	25 µg	25 µg	5 µg	1 µg
Washes	3 x PBST	5 x PBST	10 x PBST	13 x PBST
	3 x PBS	5 x PBS	10 x PBS	13 x PBS

#### 2.2.1.12 Polyclonal phage ELISA and colony pick PCR

A Nunc ELISA plate, coated with 100 µl of 0.5 µg/mL hFABP, was incubated O/N at 4°C. It was then blocked with 200 µL of 2.5% (w/v) BSA-milk marvel in PBS (pH 7.4, 150mM) for 1 hour at 37°C.

Hundred µL of phage inputs from each round of biopanning (diluted 1:10 in 1% (w/v) BSA 1x PBST, pH 7.4, 150mM) were added in duplicate to the ELISA plate and incubated for 1 hour at 37°C. Following incubation of the phage, the plate was washed 3 times with both PBST and PBS. Next, 100 µL of 1:5,000 dilution of a HRP-conjugated anti-M13 antibody (GE Healthcare Life Sciences) in 0.5% (w/v) BSA-milk marvel-PBST was added to each well and incubated for 1 hour at 37°C. Detection of the antigen-antibody complex was achieved as described in section 2.2.1.1.

Twenty single colonies were randomly picked from the fourth round of panning and incorporated into a ‘colony pick’ PCR to ensure the vector was harbouring 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<sub>GRADIENT</sub> PCR machine. The amplified scFv fragments were then analysed *via* gel electrophoresis on a 1% (w/v) agarose gel.

**Table 2.7: Composition of PCR mix for colony pick PCR.**

Components	Conc. in 50 $\mu$ L volume
Buffer (5x)	1x
CSC-F Primer	50 pM
CSC-B Primer	50 pM
Colony	N/A
dNTP	0.2 mM
H <sub>2</sub> O	N/A
REDTaq® Polymerase	5 U/ $\mu$ L

The SOE-PCR was performed in the Biometra T<sub>GRADIENT</sub> PCR machine under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	94	300	1
2	94	30	30
	56	30	
	72	60	
3	72	600	1

#### 2.2.1.13 Infecting pan 4 output phage to TOP10F cells for soluble expression

Soluble antibody fragments, without the pIII protein, were produced by infecting phagemid DNA from round 4 of biopanning into *E. coli* TOP10F cells (Stratagene, USA) at mid-logarithmic growth phase. After incubation for 30 minutes at 37°C, serial dilutions were prepared in SB media ( $10^{-2}$  to  $10^{-10}$ ), and plated on LB plates containing 1% (w/v) glucose and 100  $\mu$ g/mL carbenicillin. Single colonies were inoculated into individual wells (inner 60) of a 96-well ELISA plate containing 200  $\mu$ L of SB in the presence of carbenicillin (100 $\mu$ g/mL) and glucose (1.0% (w/v)). After an O/N incubation at 37°C, master plates of the original clones were prepared by adding glycerol (20% (v/v)) and storing at -80°C. These plates were used as a backup stock for each putative clone of interest. Thirty  $\mu$ L from the O/N subculture plates were inoculated into fresh SB media (150  $\mu$ L) containing 1x 5O5 medium and 100  $\mu$ g/mL carbenicillin. The sterile 96 well plates were propagated at 37°C at 180 rpm until an O.D. of ~0.8 (Abs @ 600 nm) was achieved. A final concentration of 1 mM isopropyl-

-D-thiogalactoside (IPTG) was added to each individual well and the plates were induced O/N at 180 rpm at 30°C. The O/N cultures were subjected to three cycles of freeze-thaw for periplasmic scFv extraction. Cell extracts were cleared by centrifugation (3,220 x g, 10 minutes) and the lysates were diluted 1:3 in 0.5% (w/v) BSA-milk-PBS (pH 7.4, 150 mM). Indirect ELISA was carried out as described in 2.2.1.12, with the exception that a 1:2,000 dilution 0.5% (w/v) BSA-milk 1x PBST of rat HRP (horse radish peroxidase)-conjugated-anti-HA (hemagglutinin) was used to detect scFvs that were specific for the hFABP.

#### **2.2.1.14 Titration of scFvs and inhibition ELISA**

Clones were checked for their antibody titre and competitiveness by inhibition ELISA. From a total of 240 clones screened, 8 positive clones were selected and grown O/N into 5 mL cultures in SB media supplemented with 100 µg/mL carbenicillin. The clones were later subcultured in 10 mL SB media, containing 1x 5O5 medium and 100 µg/mL carbenicillin, and expressed O/N (section 2.2.1.13). The cultures were pelleted by centrifugation (3,220 x g for 30 minutes) and the pellets were dissolved in 500 µL of PBS. Periplasmic scFv extraction was carried out by three cycles of freeze-thaw and cell extracts were cleared by centrifugation (22,000 x g, 10 minutes). Serial double dilutions of scFv lysates were made starting from 1:2 to 1:1024.

ELISA was carried out by coating 100 µl of 0.5 µg/mL hFABP and incubating O/N at 4°C. It was blocked by adding 200 µL of 2.5% (w/v) BSA-milk marvel in PBS (pH 7.4, 150mM) for 1 hour at 37°C. The scFv titre was determined by assaying 100 µL of each scFv dilution in duplicate by indirect ELISA (section 2.2.1.13). Graphs were plotted by using antibody dilution on the X-axis and absorbance on the Y-axis. Midpoints were

determined for each scFv curve, as they represent the dilution at which scFv is most sensitive to the antigen. These dilutions were later on used for inhibition ELISA.

An inhibition ELISA was carried out on hFABP-coated plate (100  $\mu$ L of 0.5  $\mu$ g/mL), incubated O/N at 4°C, and blocked with 200  $\mu$ L of 2.5% (w/v) BSA-milk marvel in PBS (pH 7.4, 150mM) for 1 hour at 37°C. Doubling dilutions of the hFABP antigen were made from 2  $\mu$ g/mL to 3.9  $\mu$ g/mL. The antigen dilutions were mixed with appropriate scFv dilutions (as determined by the titre) in a 1:1 ratio and incubated for 30 minutes at 37°C. 100  $\mu$ L of antigen-antibody mixture was then added to the plate and incubated for 1 hour at 37°C. Following incubation, the plates were washed 3 times with PBST and PBS. The scFv-bound hFABP complex was detected using a 100  $\mu$ L per well of a 1:2,000 dilution of a HRP-labelled anti-HA secondary antibody. After a 1 hour 37°C incubation with the labelled secondary mAb, 100  $\mu$ L per well of TMB was added, followed by incubation for 20 minutes at 37°C. The reaction was quenched by the addition of 50  $\mu$ L per well of 10% (v/v) HCl, after which the absorbance was read at 450 nm on a Safire 2 plate reader (Tecan).

#### **2.2.1.15 Preconcentration study of mouse anti-HA monoclonal antibody onto a CM5 sensor chip**

Preconcentration studies were carried out to select the buffer pH at which maximum binding of anti-HA to the CM-dextran sensor chip surface will take place. Commercial mouse anti-HA monoclonal antibody (Cambridge Bioscience, 24-25 Signet Court, Newmarket Road, Cambridge CB5 8LA, U.K.) was diluted in sterile-filtered 10 mM sodium acetate buffers with different pH (4.0, 4.2, 4.4, 4.6, 4.8 and 5.0), that had been adjusted with 10% (v/v) acetic acid, to a final concentration of 5  $\mu$ g/mL. The diluted antibody was sequentially passed over an un-activated carboxymethylated dextran

sensor chip surface at a flow-rate of 5  $\mu\text{L}/\text{minutes}$ . The pH giving greatest theoretical binding (reflected by the highest response unit (RU) value and the sharpest slope) was chosen as the carrier buffer for the immobilisation of the anti-HA antibody. The optimal condition for EDC/NHS linking to take place is at a pH of approximately 5.0.

#### **2.2.1.16 Preconditioning and immobilisation of mouse anti-HA monoclonal antibody on a CM5 sensor chip surface**

The carboxymethylated dextran matrix on the sensor chip was preconditioned by passing over twice 10  $\mu\text{L}$  of 0.05% (w/v) SDS and 5  $\mu\text{L}$  of 50 mM NaOH, at a flow-rate of 10  $\mu\text{L}/\text{minutes}$ . Preconditioning hydrates and cleans the dextran layer before immobilisation.

The anti-HA mAb was immobilised on the carboxymethyl dextran surface using amine-coupling chemistry. The carboxyl groups on the chip surface were activated by injecting 35  $\mu\text{L}$  of 0.1 M NHS (N-hydroxysuccinimide) and 0.4 M EDC (N-ethyl-N -(dimethyl-aminopropyl) carbodiimide hydrochloride), mixed at 1:1 (v/v) ratio, at a flow-rate of 5  $\mu\text{L}/\text{minute}$ .

The anti-HA mAb was diluted in 10 mM sodium acetate, pH 5.0, to a final concentration of 25  $\mu\text{g}/\text{mL}$ . This solution was then injected over the activated chip surface for 30 minutes, at flow-rate 5  $\mu\text{L}/\text{minute}$ . Any free reactive carboxylic groups on the surface were capped by passing over 1 M ethanolamine hydrochloride solution (pH 8.5) at flow-rate 5  $\mu\text{L}/\text{minute}$  for 20 minutes. Finally, loosely bound material was further removed using two 30 second pulses of 10 mM NaOH at a flow-rate of 30  $\mu\text{L}/\text{minute}$ .

### **2.2.1.17 Ranking of clones by ‘off-rate’ analysis using Biacore 3000**

Clones selected, from screening the hFABP library (section 2.2.1.13), were subjected to a Biacore-based ‘ranking’ approach, using an anti-HA immobilised chip described in section 2.2.1.16. This ‘ranking’ approach ensured a reliable and high-throughput analysis of positive clones giving a basic idea of the ‘off-rates’ and antibody/antigen complex stability.

Overnight cultures of the anti-FABP scFv clones were grown in 5 mL of SB media with 100 µg/mL carbenicillin. The clones were subcultured in 10 mL SB carbenicillin media supplemented with 1x 5O5 medium, and incubated shaking at 37°C. The cultures were induced and expressed O/N (section 2.2.1.13). The cultures were pelleted by centrifugation (3,220 x g for 10 minutes) and dissolved in 500 µL of HBS buffer. ScFv extraction was carried out by 3 rounds of alternating freeze-thaw cycles. The lysates were centrifuged at 22,000 x g for 10 minutes and the supernatant was collected. The supernatants containing the antibodies were supplemented with 12 mg/mL BSA and 12 mg/mL CM-dextran to reduce non-specific binding to the sensor chip surface. The scFvs were subsequently injected at a flow-rate of 10 µL/minute for 1 minute, over the HA-coated flow channel along with a reference channel. HFABP antigen (30 nM) was passed over the captured scFvs at a flow-rate of 30 µL/minute for 3 minutes, with a dissociation time of 10 minutes. Regeneration of the surface was carried out by injecting 25 mM NaOH at a flow-rate of 30 µL/minutes for 30 seconds. The data obtained from ranking analysis was reference subtracted using BIAevaluation 4.1 software to remove any systemic artefacts before calculating percentage stability. A total of 80 avian scFv antibody fragments were analysed.

#### **2.2.1.18 DNA fingerprinting of the selected clones to check the diversity**

Ranking of clones using Biacore 3000 enabled selection of a few high affinity clones out of the total of 80 clones selected initially after screening. All the clones were grown in 10 mL cultures O/N at 37°C, while shaking at 200 rpm. The cultures were then subjected to plasmid purification using the Promega SV Miniprep™ kit.

The cells were harvested by centrifugation at 3,220 x g for 20 minutes. The cell pellets were resuspended in 250 µL cell resuspension solution, which was then transferred to a 1.5 mL sterile micro-centrifuge tube. Cell lysis solution (250 µL) was added to each tube along with 10 µL of alkaline protease, to lyse the cell and digest periplasmic protein released, and the contents were mixed by inverting the tubes 4 times. The tubes were incubated at room temperature for 5 minutes followed by addition 350 µL of neutralisation solution, containing guanidine hydrochloride, potassium acetate and glacial acetic acid. The contents were mixed by inverting the tube several times. This neutralisation solution causes the genomic DNA and proteins present in the solution to precipitate. The lysed cells were then pelleted by centrifugation at 22,000 x g for 10 minutes and the supernatant containing the plasmid DNA transferred to a spin column supplied with the kit. The spin column was placed in a clean collection tube and centrifuged at 22,000 x g at room temperature for 1 minute causing the plasmid DNA to bind to the spin column membrane. The membrane-bound DNA was then washed once with 750 µL followed by one more wash with 250 µL of wash solution, containing ethanol, at 22,000 x g. The wash solution was removed by centrifuging the column at 22,000 x g for 1 minute followed by an additional centrifugation step to remove any residual wash solution on the membrane before eluting the DNA. The washed spin column was then transferred to a new sterile 1.5 mL micro-centrifuge tube and the



plasmid DNA eluted with 100 µL of molecular grade H<sub>2</sub>O. The scFv fragments were then PCR-amplified from the plasmid DNA.

**Table 2.8: Composition for PCR mix for amplification of anti-hFABP SOE product.**

Components	Conc. in 50 µL volume
Buffer (5x)	1x
CSC-F Primer	50 pM
CSC-B Primer	50 pM
Plasmid DNA	1 µg
dNTP	0.2 mM
H <sub>2</sub> O	N/A
REDTaq® Polymerase	5U/µL

The PCR was performed in the Biometra T<sub>GRADIENT</sub> PCR machine under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
<b>1</b>	<b>94</b>	<b>300</b>	<b>1</b>
<b>2</b>	<b>94</b>	<b>30</b>	<b>30</b>
	<b>56</b>	<b>30</b>	
	<b>72</b>	<b>60</b>	
<b>3</b>	<b>72</b>	<b>600</b>	<b>1</b>

Fingerprinting was carried out by digesting the amplified SOE product with two different restriction enzymes. The PCR amplified SOE product was subsequently digested with two frequently cutting enzymes, *Bst*N1 and *Alu*1 (refer section 3.4.3) for 8 hours at 60°C and 37°C, respectively, using the following conditions:

**Table 2.9: Composition of reaction mix for anti-hFABP fingerprinting.**

Components	Conc. in 30 µL volume
10x buffer II	1x
PCR product	200 ng
<i>Bst</i> N1/ <i>Alu</i> 1 enzyme	10 U
100x BSA	1x
Water	N/A

#### **2.2.1.19 Kinetic analysis of anti-hFABP clones to analyse their binding affinity to hFABP**

Biacore was used to determine the association and dissociation rate constants of the anti-hFABP scFvs. ScFv lysates (section 2.2.1.17) and different hFABP antigen dilutions were prepared (100, 50, 25, 2 x 12.5, 6.25, 3.12, 1.56 and 0 nM). A wizard was set up for passing different samples of scFv over the immobilised anti-HA antibody, followed by dilutions of antigen and regeneration solutions at various flow-rates for defined periods of time. Before starting the wizard scFv capture was optimised to 100-200 RU by manually passing different scFv dilutions over the anti-HA surface. ScFv samples were injected for 2 minutes at a flow-rate of 10  $\mu$ L/minutes with a stabilisation time of 3 minutes. The hFABP dilutions (12.5 nM dilution in duplicate, programmed *via* wizard) were injected, randomly, over the captured scFv surfaces for 3 minutes and the dissociation phase was monitored for 12 minutes. Regeneration of the surface was carried out by injection of 25 mM NaOH at a flow-rate of 30  $\mu$ L/minute for 30 seconds. The dataset was evaluated using the kinetics evaluation module, BIAevaluation 4.1 software. Data from the reference flow cell channel was subtracted to remove any systematic artefacts and each antigen response was then double referenced by subtracting the buffer response (zero analyte concentration) for each antibody tested during each cycle. The rate constants were fitted with a pre-defined fitting algorithm using the BIAevaluation 4.1 software for kinetic analysis.

#### **2.2.1.20 Large-scale production of anti-hFABP scFvs and purification using IMAC**

Overnight cultures of the 3 selected clones (after kinetic analysis) were grown in SB media, containing 100  $\mu$ g/mL carbenicillin, by inoculating with 10  $\mu$ L of the stock culture and grown overnight at 37°C. A 5 mL volume of this culture was then

inoculated into 500 mL SB media containing 100 µg/mL carbenicillin and 1x 5O5 media. The subcultured clone was incubated at 37°C at 250 rpm until an OD ~0.8 (OD 800 nm) was achieved. The cultures were then induced by adding IPTG to a final concentration of 1mM and incubating at 30°C (250 rpm) O/N.

The O/N expressed culture was then transferred to two 250 mL Sorval tubes and centrifuged at 18,500 x g in a GSA rotor for 20 minutes ('brake on') to pellet the bacterial cells. The supernatant was discarded and the excess media removed by inversion of the Sorval tube onto a paper towel. The cell pellet was thoroughly resuspended in 30 mL of sonication buffer (1 x 150 mM PBS, pH 7.4, 0.5 M NaCl and 20 mM imidazole) and aliquoted into 1 mL volumes in 2 mL micro-centrifuge tubes. Each aliquot was sonicated on ice for 45 seconds (with 3 second interval between each pulse) at an amplitude of 40, using a microtip Vibra Cell<sup>TM</sup> sonicator and the cell debris removed by centrifuging at 22,000 x g for 10 minutes at 4°C. The lysate supernatant was then passed through a 0.2 µm filter to remove any residual cell debris that might clog the resin column later.

Purification of scFv fragments using immobilised metal affinity chromatography (IMAC) was carried out using Ni<sup>+</sup>-NTA agarose resin (QIAGEN). A 4 mL aliquot of Ni<sup>+</sup>-NTA agarose resin (QIAGEN) was added to a 20 mL column and allowed to settle for 10 minutes. The column was equilibrated using 30 mL of running buffer (1 x 150 mM PBS, 0.5 M NaCl, 20 mM imidazole and 1% (v/v) Tween-20). The filtered lysate was then applied to the equilibrated column and the flow-through was collected in a 50 mL tube. The column was then washed with 30 mL of running buffer to remove any loosely bound non-specific proteins, and the wash collected in a 50 mL tube. The scFv fragment was eluted using 100 mM sodium acetate, pH 4.4, and collected in 24 x 400 µL

aliquots in 1.5 mL micro-centrifuge tubes containing 100  $\mu$ L of filtered neutralisation buffer (50  $\mu$ L 100 mM NaOH and 50  $\mu$ L of 10 x PBS). The neutralised scFv (12 mL) was then thoroughly buffer-exchanged against 20 mL PBS (filtered) using a 5 kDa cut-off Vivaspin™ 6 column (AGB, VS0611). The buffer-exchanged scFv was quantified using the Nanodrop™ ND-1000, transferred into clean PCR tubes and stored at -20°C.

#### **2.2.1.21 Protein purification analysis by SDS-PAGE**

Proteins were analysed for their purity and apparent molecular weight by separation on 12.5% (w/v) SDS-PAGE with a separation and stacking gel. The separation gel was cast between two clean glass plates (provided with the apparatus) and left to polymerise. After the gel polymerised, stacking gel was poured and wells were prepared by inserting 1mM comb between the plates, for loading protein samples. The samples were prepared by adding appropriate volumes of 4x sample treatment buffer and deionised water. Each protein sample was added into each well in a total volume of 20  $\mu$ L. The gels were placed in an electrophoresis apparatus and submerged in electrophoresis buffer. The gel was run at 100 V until the tracker dye had reached the bottom of the gel, taking approximately 1 hour. The gels were taken out and stained using Coomassie blue for 1 hour and the destained for 3-4 hours until the protein band is clearly visible and the background was removed.

#### **2.2.1.22 Cross-reactivity analysis of anti-hFABP scFv with closely related FABP isoforms**

The selected anti-hFABP scFvs were checked for cross-reactivity against three closely related FABP isoforms and one unrelated isoform as a negative control. Both ELISA and Western blotting techniques were used for this analysis.

Brain (bFABP), myelin (mFABP), adipocyte (aFABP) and liver FABP (lFABP) along with hFABP were coated on an ELISA plate (Nunc maxisorb) at a concentration of 0.5 µg/mL in 1x PBS (150 mM, pH 7.4) and incubated O/N at 4°C. An indirect ELISA was carried out as described in section 2.2.1.13, in triplicate, using a 1:500 dilution of anti-hFABP scFvs in 0.5% (w/v) BSA-milk-PBST. Commercial antibodies against bFABP, mFABP, aFABP and lFABP were also used at a similar dilution, i.e. as positive controls. Two different secondary antibodies were used in 1:2,000 dilution. These were HRP conjugated anti-HA antibody for the anti-hFABP scFvs and HRP conjugated anti-L protein for the commercially available antibodies. The plate was incubated for 1 hour at 37°C and then washed 3 times with PBST and PBS. The plate was developed for 20 minutes using 100 µL of TMB and the reaction was quenched by adding 50 µL of 10% (v/v) HCl, per well, after which the absorbance was read at 450 nm on a Safire 2 plate reader (Tecan).

Western blot analysis was also carried out to confirm the results obtained by ELISA. SDS-PAGE gels were run, as outlined in section 2.2.1.21, loading with 2.5 µg of bFABP, mFABP, aFABP, lFABP and hFABP. One gel was stained with Coomassie blue and other gels were prepared for transfer. Six sheets of Whatman® Gel Blot Papers (Sigma) and one sheet of 3 mm Protran BA 85 Nitrocellulose membrane (Carl Stuart Ltd.) were cut to the same dimensions as the SDS-PAGE gel. Each of the layers of filter paper, and the nitrocellulose membrane was soaked in transfer buffer for 15-30 minutes along with the gels. Three layers of the soaked blotting paper were placed between the electrodes of Trans-Blot® Semi-Dry Transfer cell (BioRad) apparatus. Buffer-soaked nitrocellulose membrane was placed over the blotting paper followed by the SDS-PAGE gel containing the resolved proteins to be transferred. The gel was sandwiched by placing three buffer-soaked blotting papers to complete the set up. All air bubbles

were removed by carefully rolling each of the layers with a disposable 10 mL serological pipette. Proteins were transferred from the gel to the nitrocellulose by applying 20 V electric current for 20 minutes. The nitrocellulose membrane was then carefully transferred into a large weighing boat containing 20 mL of 2.5% (w/v) BSA-milk-PBS solution and blocked O/N at 4°C. The blocked membrane was washed 3 times with PBST and PBS followed by incubating it with 20 mL of 0.5% (w/v) BSA-milk-PBST solution containing of anti-hFABP scFvs with a HA tag (1:10,000 dilution), for 1 hour at room temperature with gentle agitation. The membrane was then washed 3 times with PBST and PBS and again incubated for an hour with 1:2,000 dilution of HRP-conjugated anti-HA antibody in 20 mL of 0.5% (w/v) BSA-milk-PBST. After 1 hour incubation, the membrane was washed as before and developed by the addition of TMB substrate. Once sufficient development had occurred the reaction is stopped by multiple washes with distilled water.

#### **2.2.1.23 Inhibition assays using anti-hFABP scFv**

The ELISA procedure was same as previously described (section 2.2.1.14). Interday and intraday inhibition ELISA assays were carried out using a 1:250,000 dilution of the purified scFv, 3.G9. The scFv was incubated with different concentrations of the free hFABP antigen (2 µg to 0.059 ng/mL), along with one without any antigen ( $A_0$ ). The antigen-antibody mixture was incubated at 37°C for 30 minutes. The absorbance obtained for each antigen dilution was divided from the  $A_0$  absorbance and all the values were plotted to determine the  $IC_{50}$  and limit of quantification (LOQ) of 3.G9 scFv. Accuracy of each value was calculated using BIAevaluation 4.1 software.

#### **2.2.1.24 Lateral flow immunoassay using purified chicken anti-hFABP scFv**

A 1:50,000 dilution of anti-hFABP scFv in 50 mM sodium phosphate buffer pH 7.5 containing 0.05% Tween 20 was prepared. This dilution was selected from the mid-point of the titration curve obtained against hFABP (Figure 3.14). Equal volumes (200  $\mu$ L) of the scFv dilution and 4 different concentrations of commercial hFABP (2  $\mu$ g/mL, 200 ng/mL, 20 ng/mL and 2 ng/mL) were mixed. One dilution of scFv mixed with equal volume of buffer, containing no hFABP, was used as a control. The reaction mix was incubated for 30 minutes at 37°C.

Meanwhile, a strip of HiFlow Plus HF07504 nitrocellulose membrane (Millipore) was cut to specific dimensions 7 cm x 5 cm. The antigen, hFABP, was diluted in 50 mM sodium phosphate buffer pH 7.5 (spotting buffer), containing 1% (w/v) trehalose to a final concentration of 4  $\mu$ g/mL. Seventeen  $\mu$ L of the diluted hFABP was sprayed across the width of the strip (4 cm up the strip - test line) at a flow-rate of 400 nL/second using the Linomat 5 system (CAMAG). Anti-CRP scFv, previously isolated in the laboratory, was also diluted in spotting buffer to a concentration of 2  $\mu$ g/mL and was sprayed across the width of the strip approximately 1 cm above the test line (control line). The membrane was then dried at 37°C for 20 minutes. Once drying was complete, the membrane was cut in strips 1 cm in width and clamped with blotting paper. The nitrocellulose strips were set up to stand vertically, with the bottom of the strips touching a strip of parafilm.

Two hundred  $\mu$ L of each of the antigen-antibody dilutions prepared earlier were then applied separately to the individual strips. The solutions travelled up the length of the strip *via* capillary action, assisted by wicking with the absorbent paper. A wash buffer solution consisting of 50 mM sodium phosphate pH 7.5 (0.05% Tween 20) was then

applied (200  $\mu$ L volume) to the parafilm at the bottom of the strip. The Tween-20, a mild detergent, breaks up any non-specific binding between the components of the solution and the nitrocellulose membrane, allowing movement of the solution components up the strip towards the test and control lines.

The hFABP in the sample inhibits the binding to the chicken anti-hFABP scFv (3.G9) to the hFABP coated on the membrane as test line. The degree of inhibition of the scFv to bind the hFABP on test line is dependent upon the concentration of free hFABP in the inhibition mix. The bound scFv was then probed by the addition of anti-HA-HRP conjugated antibody, which binds to the HA-tag in the scFv. The secondary antibody travelled up the strip binding to any anti-hFABP scFv (containing HA-tag) on the test line and the anti-CRP scFv at the control line. The bound anti-HA antibody was then detected by the addition of TMB liquid substrate to the bottom of the strip, which travels up *via* capillary action and reacts with any anti-HA captured antibody producing a blue colour at the test and control lines. The assay was initially standardised with phosphate buffer, which was later on validated on hFABP spiked serum samples.

#### **2.2.1.25 Rabbit anti-hFABP polyclonal antibody**

A New Zealand white rabbit was immunised with 200  $\mu$ g of hFABP in PBS, mixed in 1:1 ratio with Freund's complete adjuvant (FCA), used as immunopotentiator. Two subsequent boosts were given with 200  $\mu$ g of hFABP (in PBS, mixed in 1:1 ratio with Freund's incomplete adjuvant (FICA)) at three week intervals, followed by three boosts with 50  $\mu$ g of hFABP at 1, 2 and 6 months intervals. After 10 days following each boost, blood was collected from the rabbit and the serum was separated (section 2.2.1.1). The serum was then aliquoted into 1.5 mL tubes and stored at -20°C.



## 2.2.2 Expression of Human hFABP

### 2.2.2.1 PCR primers for amplification of hFABP

Primers were designed from the gene sequence (Pubmed accession NP\_004093). Both the primers were checked for ORFs (open reading frames) and ordered from Integrated DNA Technologies with standard desalting option.

#### FABPFOR 5 sense primer

5 CAT GCC ATG GTG GAC GCT TTC CTG GGC AC 3

#### FABPREV 3 Anti-sense primers

5 CCTTTAGCGGCCGCTGCCTCTTTCTCATAAGTGC 3

### 2.2.2.2 Amplification of hFABP from human heart cDNA

HFABP was amplified from human heart cDNA (First choice R, Ambion # AM3326) by PCR using specific primers (section 2.2.2.1).

**Table 2.10: Composition of reaction mix for hFABP gene amplification for expression.**

Components	Conc. in 50 $\mu$ L volume
HiFi buffer (10x)	1x
FABPFOR primer	50 pM
FABPREV primer	50 pM
cDNA	1.0 ng
dNTP	0.2 mM
MgSO <sub>4</sub>	1 mM
H <sub>2</sub> O	N/A
Platinum HiFi <sup>TM</sup> Taq	2.5 U

The PCR was performed in the Biometra T<sub>GRADIENT</sub> PCR machine under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	94	180	1
2	94	15	30
	61	30	
	72	60	
3	72	600	1

**Table 2.11: Composition of reaction mix for digestion of hFABP gene and pET-26b(+) vector.**

Components	Conc. in 50 µL volume	Components	Conc. in 50 µL volume
10x FAST digest buffer	1x	10x FAST digest buffer	1x
pET-26b(+) vector	10 µg	Insert (hFABP)	5 µg
<i>NotI</i> enzyme	10 µL	<i>NotI</i> enzyme	5 µL
<i>NcoI</i> enzyme	10 µL	<i>NcoI</i> enzyme	5 µL
Water	N/A	Water	N/A

### 2.2.2.3 Digestion and ligation hFABP gene into pET-26b(+) vector

The PCR amplified product was resolved on 1% (w/v) agarose gel and the band of appropriate size (399 bp) was purified, as described in section 2.2.1.6. Purified product was digested along with the pET-26b(+) vector using *NotI* and *NcoI* restriction enzymes. The digestion was carried out for 2 hours at 37°C using the following reaction mixture:

The digested vector was treated with 2 µL of alkaline phosphatase for 15 minutes at 37°C which was further inactivated by incubating the mix at 65°C for 10 minutes. Alkaline phosphatase prevents self-ligation of the vector by removing the phosphatase group from the 5' end, while keeping the vector linearised until ligation. The digested products were gel-purified and ligated overnight at RT.

**Table 2.12: Ligation mix for *hFABP* gene into *pET-26b(+)* vector**

Components	Conc. in 10 $\mu$ L volume
10x ligation buffer	1x
Digested pET-26b(+) vector	100 ng
Digested insert (hFABP)	45 ng
T <sub>4</sub> DNA Ligase enzyme	400 U
Water	N/A

#### **2.2.2.4 Transformation of cloned gene into *E. coli* cells**

Cloned hFABP gene was transformed into commercial BL21-CodonPlus<sup>®</sup>(DE3)-RIPL competent cells. These are specialised cells, producing an increased supply of rare *E. coli* tRNAs thus improving the expression of many proteins that are difficult to express in conventional *E. coli* due to codon bias.

An aliquot (100  $\mu$ L) of cells were thawed on ice. Two  $\mu$ L of  $\beta$ -mercaptoethanol mix (1:10 dilution) provided with the kit, was added to the cells followed by a 10 minutes incubation on ice. Addition of  $\beta$ -mercaptoethanol increases the efficiency of transformation. Five  $\mu$ L of ligation mix was added to the cells and the cells were further incubated on ice for 30 minutes. A heat pulse was given by transferring the cells to a water bath at 42°C, for 20 seconds, followed by incubating the cells on ice for 5 minutes. This step is critical as it allows entry of cloned DNA into the cells. SOC media (1 mL) was added to the cells and they were grown at 37°C for 1 hour. Finally, 200  $\mu$ L of cells were plated on LB agar plates supplemented with 50  $\mu$ g/mL kanamycin and incubated O/N at 37°C.

#### **2.2.2.5 Growing transformants and protein expression**

Single colonies were inoculated into 8 individual wells of a 12-well plate containing 1 mL of SB media with 50  $\mu$ g/mL kanamycin. Following O/N incubation at 37°C, 100  $\mu$ L from these plates were subcultured into 10 mL of fresh SB media containing 1x 505

medium and 50 µg/mL kanamycin. Stock plates were prepared by adding glycerol (20% (w/v)) to the O/N grown plates and storing the clones at -80°C. The subcultured cells were grown until an O.D. of ~0.8 (@ 600 nm) was reached followed by addition of IPTG to 1 mM final concentration and inducing them O/N at 200 rpm at 30°C. The O/N cultures were centrifuged (Eppendorf 5810R) at 3,220 x g for 20 minutes and the pellets were resuspended in 500 µL PBS. The resuspended culture was transferred to 1.5 mL tubes and sonicated to enable periplasmic protein extraction (section 2.2.1.20). The cell debris was removed by centrifuging at 22,000 x g for 10 minutes at 4°C, followed by transferring the supernatant, containing the protein, to fresh tubes and storing them on ice at 4°C.

#### **2.2.2.6 SDS-PAGE and Western blot analysis of the expressed proteins**

HFABP expression was checked by performing SDS-PAGE and Western blot analysis. The samples were prepared by adding a 4x sample treatment buffer to the lysates followed by denaturing the protein at 95°C for 10 minutes. The SDS-PAGE gel was run at 100 V for 1 hour with 20 µL of each sample added to each well (section 2.2.1.21). Nine samples were analysed which included 8 induced clones and one uninduced cell control. One gel was stained by Coomassie blue while the other was processed further for Western blotting.

The procedure performed for blotting was similar to that of section 2.2.1.22. The membrane containing the transferred protein from the SDS-PAGE gel was blocked O/N with 2.5% (w/v) BSA-milk-PBS, at 4°C. Following 3 washes with PBST and PBS the membrane was incubated with highly specific anti-hFABP monoclonal antibody (1:10,000 dilution), with gentle agitation, for an hour at RT. The membrane was washed, as before, and incubated with anti-HA-HRP-labelled antibody (1:2,000) for a

further hour at RT. After washing, the membrane was developed by addition of TMB substrate. The reaction was stopped by washing the membrane three times with water.

### **2.2.3 Epitope mapping of chicken anti-hFABP antibody**

#### **2.2.3.1 Epitope mapping using commercial peptide library**

Epitope mapping of anti-hFABP scFv 3.G9 was carried out using Ph.D.<sup>TM</sup>-12 Phage Display Peptide Library (#E8110S; New England Biolabs Ltd., UK). It is a pre-made, dodecapeptide, random peptide library exceeding complexities of 2 billion independent clones. Phage display technology was used for the selection and enrichment of anti-hFABP scFv binding peptides by biopanning. Three rounds of panning were carried out, alternating between anti-HA and anti-His antibody-coated magnetic beads as scFv capture carriers.

##### **2.2.3.1.1 Depletion of library against capture antibody-coated magnetic beads**

The Ph.D.<sup>TM</sup>-12 Phage Display Peptide Library was depleted against the antibody-coated magnetic beads before panning. A total of 120  $\mu$ L of anti-His antibody-coated magnetic beads (Novagen #71002) were mixed with 100  $\mu$ L of 3% (w/v) BSA-PBS in 1.5 mL micro-centrifuge tube, and incubated for 1 hour at RT. The blocked beads were recovered from the suspension by keeping the tube in a magnetic separation rack. The solution was aspirated and the beads were washed once with 500  $\mu$ L wash buffer (4 M NaCl, 160 mM Tris.HCl, 480 mM Imidazole, pH 7.9). Wash buffer was removed by placing the tube in magnetic separation rack and 120  $\mu$ L of binding buffer was added (4 M NaCl, 160 mM Tris.HCl, 40 mM Imidazole, pH 7.9). To 60  $\mu$ L anti-His beads, 15  $\mu$ L of Ph.D.<sup>TM</sup>-12 phage library was added and the mixture was incubated on a roller for an hour at RT. This step was carried out to deplete the phage specific to anti-HA antibody in the library. The depleted library was recovered from the mixture by keeping

the tube in a magnetic separation rack, this retained the magnetic beads in the suspension allowing a clear solution of depleted library to be transferred to a fresh 1.5 mL micro-centrifuge tube by pipetting.

#### **2.2.3.1.2 Selection and enrichment of anti-hFABP scFv binding peptides**

The depleted library (section 2.2.3.1.1) was diluted to 200  $\mu$ L with PBST and anti-hFABP was added to a final concentration of 1  $\mu$ M. The mixture was incubated for 1 hour on a roller at RT. To recover the specific phage, displaying peptides binding specifically to the anti-hFABP scFv, 60  $\mu$ L of anti-His antibody-coated magnetic beads were added to the above mixture and incubated at RT for 1 hour. The beads capturing the scFv together with the bound phage were retained from the solution by placing the tube in a magnetic separation rack (section 2.2.3.1.1). Non-specific bound phage were removed by washing the beads 5 times with 200  $\mu$ L of wash buffer. Specifically bound phage were eluted by adding 425  $\mu$ L of glycine elution buffer (0.2 M glycine, pH 2.2) to the beads. The supernatant was recovered and neutralised by the addition of 75  $\mu$ L neutralisation buffer (1 M Tris-HCl, pH 9.1). The eluate was titered by making serial dilutions, starting from  $10^1$ - $10^4$ , and 2  $\mu$ L of each dilution was infected to 98  $\mu$ L of mid-logarithmic phase K12 ER2738 cells (O.D.  $\sim$  0.6 @ 600 nm). The cells were incubated at 37°C for 15 minute and subsequently, added to 3 mL of molten top agar. The molten agar was mixed with cells by gentle shaking and spread evenly on to LB-IPTG-X-gal plates. The plates were allowed to dry and incubated O/N at 37°C. Titre was calculated the following day by counting blue plaques on the plates.

#### **2.2.3.1.3 Rescue and subsequent precipitation of peptide-displaying phage**

The eluate from section 3.3.1.2 was amplified by infecting 200  $\mu$ L of O/N cells, diluted in 20 mL SB media in 250 mL Erlenmeyer flask and propagating the cells by shaking at

200 rpm at 37°C. After 5 hours of incubation the culture was transferred to 50 mL sterile sorbals and cells were pelleted by centrifugation at 18,500 x g in a GSA rotor ('brakes-on') for 20 minutes at 4°C. The supernatant was transferred carefully into a fresh sorval and supplemented with 4 mL of 20% PEG 2.5 M NaCl solution. The contents in the tube were mixed thoroughly and the tube was incubated on ice at 4°C for 1 hour. The precipitated phage was pelleted by centrifugation at 18,500 x g ('brakes-off') for 30 minutes. The supernatant was discarded carefully and the residual liquid from the pellet was removed by inverting the tubes on dry tissue towels. The phage pellet was resuspended in PBS solution. The phage was reprecipitated by adding 200 µL of 20% PEG 2.5 M NaCl solution and incubating the suspension on ice for 1 hour at 4°C. The precipitated phage was pelleted as before and resuspended in 200 µL of PBS with 1% BSA. Phage was titered as before and  $2 \times 10^{11}$  pfu/mL was used for each subsequent round of panning.

**Table 2.13: Variable parameters of panning of Ph.D.<sup>TM</sup>-12 Phage Display Peptide Library.**

Components	PAN 1	PAN 2	PAN 3
Capture antibody	Anti-His	Anti-HA	Anti-His
ScFv concentration	1 µM	100 nM	50 nM
Number of washes	5	10	15

Panning was repeated 3 times (Table 2.13) and the phage outputs were checked by polyclonal phage ELISA. Polyclonal phage ELISA was carried out in 2 different ways:

- The scFv was captured on the plate and the phage outputs were added as primary reagent. The reaction was probed with anti-M13-HRP conjugated antibody.
- The plate was coated with phage outputs from different outputs and the scFv was added to bind specific phage which was later probed with HRP-conjugated-anti-

HA antibody. A slight increase in the signal was obtained in the former which was further used for infecting the K12 ER2738 for screening monoclonal phage.

#### **2.2.3.1.4 DNA isolation from plaques and sequencing**

Overnight culture of K12 ER2738 cells were diluted 1:100 with SB media and 1 mL of diluted cells were aliquoted into 15 mL tubes. Blue plaques from round 3 titre plates were infected into the cells by pricking the plaque with sterile tips and dipping the tip into cells. To ensure that the phage is completely transferred to the cells from the tip, the media was pipetted up and down few times. The infected cells were grown for 4-5 hours shaking at 200 rpm at 37°C. The cultures were pelleted by centrifuging the tubes at 3,220 x g for 20 minutes. The supernatant was carefully transferred to 1.5 mL micro-centrifuge tubes and DNA was extracted using QIAprep spin M13 kit (#27704). Briefly, 1 mL of supernatant was mixed with 10 µL of MP buffer followed by mixing. The mixture was incubated at RT and applied to a spin column provided with the kit and placed in a 2 mL tube. The tube was briefly centrifuged at 11,000 x g for 15 seconds and the flow through was discarded. The column was washed twice with 700 µL of MLB buffer for M13 lysis, creating appropriate conditions for binding of M13 DNA to the QIAprep silica-gel membrane. The column was further washed with 700 µL of PE buffer to remove the residual salt. The empty column was centrifuged at 19,500 x g for 1 minute to remove the residual buffer. DNA elution was carried out by transferring the column to a fresh tube, adding 100 µL of EB buffer to the center of spin column membrane, incubating it for 10 minutes at RT followed by centrifuging the tube at 19,500 x g for 1 minute.



### 2.2.3.2 Epitope mapping using gene-fragment library

A specific gene-fragment library was constructed by digesting the hFABP gene and cloning it into a modified pComb3HSS phagemid vector. Gene-fragment libraries are highly specific for epitope mapping, thus, excluding the possibility of non-specific binding of the phage to the carrier antibody.

#### 2.2.3.2.1 Modification of vector

Modification of the vector was carried out by adding an *Xho*I restriction site at the end of the heavy chain stuffer (HCS) so as to allow the gene-fragments containing *Xho*I adapters on both the sides to be cloned. The HCS along with the PelB site was amplified from the pComb3HSS plasmid by PCR, using the following primer combination:

pComb3HSS\_For\_Xba 5 Sense Primer

5 GCCTGAGCTTGCCCGTCAC 3

pCombXhoIRev13 Anti-sense Primer

5 GTAGTCCGGAACGTCGTACG 3

Following composition of the reaction mixture was used:

**Table 2.14: Composition of PCR mix for pComb3H vector modification.**

Components	Conc. in 50 $\mu$ L volume
GoTaq Buffer (5x)	1x
pComb3HSS_For_Xba	50 pM
pCombXhoIRev1	50 pM
cDNA	0.5 $\mu$ g
dNTP	0.2 mM
MgCl <sub>2</sub>	1 mM
H <sub>2</sub> O	N/A
GoTaq <sup>TM</sup> Polymerase	2.5 U/ $\mu$ L

The PCR was performed in the Biometra T<sub>GRADIENT</sub> PCR machine under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	94	300	1
2	94	30	30
	61	30	
	72	60	
3	72	600	1
	4	Pause	

The amplified product was checked for appropriate size (~ 400 bp) and gel purified (section 2.2.1.6). This purified DNA was used as a template for the next PCR reaction for the addition of the *Xho*I site followed by a *Spe*I site in the product. The composition of the reaction mix and conditions were as outlined before (Table 2.14). Following primer combination used for this purpose was:

pComb3HSS For Xba 5 Sense Primer

5 GCCTGAGCTTGCCCGTCAC 3

pCombXhoIRev2 3 Anti-sense Primer

5 GAGGGCCACTAGTAGAACCCTCGAGGTAGTCCGGAACGTCGTACG 3

The pComb3HSS vector and the PCR amplified product were sequentially digested with *Xba*I and *Spe*I enzymes. The reaction mixture consisted of:

**Table 2.15: Composition of reaction mix 1 for sequential digestion of pComb3HSS vector and HCS insert.**

Components	Conc. in 50 µL volume
10x buffer II	1x
Insert (HCS)	7 µg
<i>Xba</i> I enzyme	160 U
100x BSA	1x
Water	N/A

Components	Conc. in 50 µL volume
10x buffer II	1x
pComb3HSS vector	7 µg
<i>Xba</i> I enzyme	160 U
100x BSA	1x
Water	N/A

The reaction was incubated at 37°C for 5 hours followed by the purification of the digested product by gel extraction (section 2.2.1.6). *Xba*I digested vector and insert were further digested with *Spe*I enzyme O/N at 37°C using the following composition of reaction mixture.

**Table 2.16: Composition of reaction mix 2 for sequential digestion of pComb3HSS vector and HCS insert.**

Components	Conc. in 50 $\mu$ L volume	Components	Conc. in 60 $\mu$ L volume
10x buffer II	1x	10x buffer II	1x
pComb3HSS vector	5 $\mu$ g	insert (HCS)	2.1 $\mu$ g
<i>Spe</i> I enzyme	50 U	<i>Spe</i> I enzyme	50 U
100x BSA	1x	100x BSA	1x
		Water	N/A

Both the digested vector and product were gel purified and ligated O/N at room temperature. Ligation mix consisted of:

**Table 2.17: Ligation mix for cloning HCS into pComb3HSS vector.**

Components	Conc. in 10 $\mu$ L volume
10x ligation buffer	1x
Digested pComb3HSS vector (modified)	112 ng
Digested insert (HCS along with pelB)	100 ng
T <sub>4</sub> DNA Ligase enzyme	400 U
Water	N/A

#### 2.2.3.2.2 Preparation of chemically competent cells

An overnight culture of cells were prepared by inoculating 5 mL of SB media with a single colony of Top10F' cells from a fresh plate and growing it at 37°C shaking at 200 rpm. Following incubation, the cells were subcultured to 250 mL of sterile SB media in 1 L flask and propagated till the O.D. @ 600 nm ~ 0.6. The bacterial cells were cooled by placing the flask on ice and incubating for 20 minutes. From this stage onwards it

was critical to maintain the cells in cold to get maximum efficiency of cells. The cells were transferred to 50 mL centrifuge tubes and centrifuging at 3,220 x g for 10 minutes at 4°C. The media was carefully decanted, and the tubes containing pelleted cells were placed on ice. The cells were resuspended in 80 mL of 0.1 M ice-cold MgCl<sub>2</sub> solution whilst keeping the tubes on ice. The cells were pooled into two tubes and incubated for 10 minutes on ice. The cells were resuspended in 80 mL of 0.1 M ice-cold CaCl<sub>2</sub> solution and incubated on ice for 30 minutes. The longer the cells are incubated the better the efficiency of the cells. The cell suspension was spun down at 3,220 x g for 10 minutes at 4°C and the pellet was resuspended in 10 mL of 0.1 M ice-cold CaCl<sub>2</sub> solution containing 20% (w/v) glycerol. The cells were aliquoted into 1.5 mL micro-centrifuge tubes and stored in -80°C after snap freezing in liquid nitrogen. The efficiency of the cells was verified by transforming one aliquot with 1 ng of plasmid DNA.

#### **2.2.3.2.3 Transformation of cloned HCS into the *E. coli* and screening of transformed clones**

Chemically competent cells (XL1-Blue), prepared in-house (section 2.2.3.2.2), were used for transforming the cloned HCS. Transformation was performed in the similar way as in section (section 2.2.2.4) with few exceptions. -mercaptoethanol was not added to the cells prior to transformation and a heat pulse was given for 2 minutes. After growing the transformation culture in SOC media for an hour, 200 µL of cells were plated on LB agar plates containing 100 µg/mL carbenicillin and incubated O/N at 37°C.

From the plates, containing transformed clones, 10 clones were checked by colony pick PCR using the same forward and reverse primers used for amplification of HCS from

the vector. Colonies were picked from the plate with a sterile tip and streaked on a plate (divided into 10 sections and numbered) followed by dipping the tip into the PCR mix containing the following components:

**Table 2.18: Composition of PCR mix for checking transformants containing modified vector.**

Components	Conc. in 50 $\mu$ L volume
GoTaq Buffer (5x)	1x
pComb3HSS_For_Xba	60 pM
pCombXhoIRev1	60 pM
Colony	N/A
dNTP	0.2 mM
MgCl <sub>2</sub>	1 mM
H <sub>2</sub> O	N/A
GoTaq™ Polymerase	2.5 U

The plate was incubated O/N at 37°C as a backup which was used later to scale up the PCR positive clones for plasmid extraction.

The PCR was performed in the Biometra T<sub>GRADIENT</sub> PCR machine under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
<b>1</b>	<b>94</b>	<b>300</b>	<b>1</b>
<b>2</b>	<b>94</b>	<b>30</b>	<b>30</b>
	<b>61</b>	<b>30</b>	
	<b>72</b>	<b>60</b>	
<b>3</b>	<b>72</b>	<b>600</b>	<b>1</b>
	<b>4</b>	<b>Pause</b>	

The clones were checked by resolving the PCR product on 1% agarose gel and checking for the amplicon of appropriate size (~ 400 bp). Positive clones were scaled up by growing them into 10 mL SB media containing 100  $\mu$ g/mL carbenicillin and incubating them shaking at 200 rpm O/N at 37°C. Plasmid extraction was carried out (section

2.2.1.18) followed by digesting the plasmid with *XhoI* restriction enzyme to check the release of new modified HCS. The reaction mix consisted of following components:

**Table 2.19: Digestion mix for checking HCS release using *XhoI* enzyme.**

Components	Conc. in 10 $\mu$ L volume
10x buffer III	1x
Modified pComb3HSS vector	200 $\mu$ g
<i>XhoI</i> enzyme	50 U
100x BSA	1x
Water	N/A

#### 2.2.3.2.4 Optimising *DNaseI* digestion of hFABP gene

Overnight culture was prepared by inoculating 10 mL SB media containing 100  $\mu$ g/mL of carbenicillin with 5  $\mu$ L of hFABP expressing clone. Plasmid was extracted (section 2.2.1.18) and hFABP gene was amplified from the plasmid using specific primers (section 2.2.2.1). A 20x reaction was set up using conditions similar to that of section 2.2.2.2. The amplified product was gel purified and subjected to digestion by different concentrations of *DNaseI* to get 50-125 bp DNA fragments. The enzyme was diluted in fresh *DNaseI* buffer (50 mM Tris.HCl, pH 7.6, containing 1 mg/mL BSA) containing 10 mM  $\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$ , in order to generate double stranded cuts in the *DNaseI* digested product. The buffer needs to be prepared fresh to avoid degradation of  $\text{MnCl}_2$ . One  $\mu$ g of purified hFABP gene was digested with 0.0312, 0.0156, 0.0078, 0.0039, 0.0019 and 0.0009 U of enzyme. The reaction was made up to 10  $\mu$ L with *DNaseI* buffer. All the reactions were incubated at 37°C for 10 minutes and inactivated later on at 70°C for 10 minutes.

The digested products were resolved on a 2.5% agarose gel and fragments were checked for appropriate size distribution (50-125 bp). The fragments appear as smear concentrated in particular region of the gel. Due to the low density of fragments (smear)

in the desired size range, time of digestion was optimised using 0.010 U of enzyme per  $\mu\text{g}$  of DNA. The samples were incubated at  $37^{\circ}\text{C}$  for 2, 4, 6, 8, 10 and 12 minutes respectively followed by inactivating the sample at  $70^{\circ}\text{C}$  for 10 minutes. The samples were checked on an agarose gel as before and the appropriate incubation time, for the enzyme to digest the gene, was determined.

The enzyme concentration was scaled up for digesting 25  $\mu\text{g}$  of hFABP gene and the digestion was performed in  $37^{\circ}\text{C}$  water bath for 6 minutes. The reaction was stopped by inactivating the *DNaseI* enzyme as before. The sample was resolved on the gel as before and the DNA band was cut using a clean scalpel, starting from 50 bp to 125 bp. The DNA was gel purified and ethanol precipitated (section 2.2.1.6).

#### **2.2.3.2.5 Modifying the gene-fragments for cloning into the vector**

The fragments generated by digesting the hFABP gene (section 2.2.3.2.4) were prepared for ligating *XhoI* adapters, by treating them with T4 polymerase and Klenow fragment of *E. coli* DNA polymerase I, to generate blunt ends.

The ethanol precipitated fragments were centrifuged at  $19,500 \times g$  for 20 minutes at  $4^{\circ}\text{C}$ . The supernatant was carefully decanted and the pellet was washed with 200  $\mu\text{L}$  of 70% chilled ethanol. The resulting suspension was re-centrifuged and the supernatant was decanted as before. The DNA pellet was air dried and resuspended in 100  $\mu\text{L}$  of polishing buffer (40 mM tris-HCl, pH 8.0, 10 mM ammonium sulphate, 10 mM - mercaptoethanol, 5 mM  $\text{MgCl}_2$ , 0.5 mM EDTA).

This suspension was further supplemented with 100  $\mu\text{M}$  dNTPs and mixed gently. Thirty units of T4 DNA polymerase were added to the suspension and incubated for an

hour at 16°C. The process was completed by the addition of 10 U of Klenow fragment of *E. coli* DNA polymerase I and incubating the final mix for 30 minutes at 37°C. The reaction was stopped by the addition of 5 µL of 250 mM EDTA. The polished DNA fragments generated in this process were purified by using QIAEX II (# 28304), as per manufacturer's instructions. Briefly, the DNA was mixed with 10 volumes of binding buffer (PN1) and the reaction mix was added to a spin column placed in a 2 mL collection tube. The column was centrifuged at 16,500 x g for 1 minute and the solution in the collection tube was discarded. The column was washed with 750 µL of wash buffer (PE) and was centrifuged as before. The residual buffer in the spin column was removed by centrifuging the empty column for 1 minute additional time. The DNA was recovered from the column by placing the column in a clean 1.5 mL micro-centrifuge tube and adding 50 µL of molecular grade water to the membrane and centrifuging at 19,500 x g allowing the DNA to be eluted.

The *Xho*I adapters were ligated to the polished DNA fragments by adding 2 µg of 5 phosphorylated linker (5' pdCCCTCGAGGG) to the DNA eluted (20 µL) using 1200 U of T4 DNA ligase. The reaction mix was made up to 9 µL with water. The mix was denatured for 5 minutes at 94°C followed by annealing at 25°C for 5 minutes. Another 400 U of T4 DNA ligase was added to the reaction to facilitate ligation. The reaction was incubated at RT O/N. Ligase was heat inactivated at 65°C for 10 minutes. The mix was cooled on ice. The DNA fragments ligated to the adapters along with the modified pComb3HSS vector were further digested with *Xho*I enzyme to generate cohesive ends. The composition of digestion mix comprised of:



**Table 2.20: Composition of reaction mix for digesting modified pComb3HSS and gene-fragments with adapters for cloning.**

Components	Conc. in 50 $\mu$ L volume	Components	Conc. in 100 $\mu$ L volume
10x buffer III	1x	10x buffer III	1x
pComb3HSS vector	5 $\mu$ g	DNA fragments	10 $\mu$ g
<i>Xho</i> I enzyme	100 U	<i>Xho</i> I enzyme	200U
100x BSA	1x	100 x BSA	1x
Water	N/A	Water	N/A

The digestion was carried out at 37°C for 4 hours. Additional 60 U of *Xho*I enzyme was added to the DNA fragments after 4 hours and the mix was incubated for another 1 hour at 37°C. The digested vector and the DNA fragments were gel purified.

#### 2.2.3.2.6 Cloning the gene-fragments into the vector and transforming it into *E. coli* cells

The gene-fragments were cloned into the vector by incubating the ligation mix overnight at RT. Ligation mix was composed of:

**Table 2.21: Ligation mix for cloning gene-fragments into modified pComb3HSS vector.**

Components	Conc. in 80 $\mu$ L Volume
10x Ligase buffer	1x
Digested pComb3HSS vector (modified)	4000 ng
Digested insert (gene-fragments)	400 ng
T <sub>4</sub> DNA Ligase enzyme	4000U
Water	N/A

Ligase was heat inactivated at 65°C for 10 minutes the following day and the ligation mix was ethanol precipitated for 1 hour at -80°C. The ethanol was removed and the pellet was finally resuspended in 40  $\mu$ L of water.

The cloned gene-fragments were transformed into commercial XL1-Blue cells as indicated in section 3.1.7. The transformants were grown shaking at 200 rpm for an hour at 37°C. The culture was centrifuged at 3,220 x g for 15 minutes and the pellet was resuspended in 1 mL of SOC media and plated on LB plates, supplemented with 100 µg/mL carbenicillin and 1% (v/v) glucose. The library was titred to determine its size and plated in parallel along with untransformed XL-1 blue *E. coli* cells as negative control. The plates were incubated O/N at 37°C. The following day, the colonies were scraped-off the plates and were suspended in SB media containing 20% (v/v) glycerol, snap frozen in liquid nitrogen and stored at -80°C as stocks.

The titre was calculated and the colonies were checked by using the following primer combinations using conditions similar to that of section 2.2.1.12.

pComb3HSS\_For\_Xba 5 Sense Primer

5 GCCTGAGCTTGCCCGTCAC 3

HSS\_pick\_rev 3 Sense Primer

5 CCACCACCCTCCTGGCCGGCCTGGC 3

### **2.2.3.2.7 Changing anti-hFABP (3.G9) scFv into scAb**

A 20 mL culture of 3.G9 scFv was carried out and plasmid was extracted. The scFv gene was amplified from the plasmid using primers containing *Sfi*I sites compatible to the pMoPac vector *Sfi*I sites enabling the gene to be cloned for scAb generation. The primers used for scFv gene amplification were:

i. Chi VL-VH-PAK\_for = GGAATTCGCGGGCCCAGCCGGCCATGGCG CTG ACT CAG

ii. Chi VL-VH-PAK\_rev =TTACTCGCGGGCCCCGAGGCCGCACTAGTGGA

The SOE product was amplified from the scFv 3.G9 using the above primers and cloned in pMoPac vector construct containing chicken constant domain (section 2.2.5.1). The transformed clones were screened by ELISA against hFABP. Positive 3.G9 chicken scAb with highest signal was selected, expressed in large volume (250 mL) and purified using IMAC column (section 2.2.1.20).

#### **2.2.3.2.8 Biopanning of gene-fragment library**

A sandwich ELISA was carried out to decide the format for panning the gene-fragment library. ELISA plate was coated with two concentrations (20 µg/mL and 5µg/mL) of goat anti-chicken IgY Fab antibody by adding 100 µL of the antibody to each well and incubating it overnight at 4°C. The plate was blocked for an hour at 37°C with 200 µL of 2.5% (w/v) BSA-milk marvel in PBS. The plate was washed 3 times with PBST and PBS. Anti-hFABP scAb was diluted in 0.5% (w/v) BSA-milk marvel PBS to the final concentration of 20 µg/mL. Diluted scAb (100 µL) was added to each well, followed by incubation at 37°C for 1 hour. The plates were washed as before and again incubated with 100 µL of hFABP antigen (1 µg/mL). After washing 100 µL of 1:1,000 dilution of rabbit anti-hFABP polyclonal sera was added to the plate. The plate was incubated and washed as before followed by addition of 1:1,000 dilution of HRP-conjugated-anti-rabbit (whole molecule)-antibody. After an hour incubation and washing with PBST and PBS the plate was developed by adding 100 µL per well of TMB followed by an incubation of 20 minutes. The reaction was stopped with 100 µL per well of 10% (v/v) HCl, after which the plate was read at 450 nm on a Safire 2 plate reader (Tecan).

Peptide displaying phage were rescued by inoculating 200 mL sterile SB medium supplemented 100 µg/mL carbenicillin with 200 µL of the transformed library (section 2.2.1.10). The culture was propagated at 37°C shaking at 200 rpm until mid-exponential

phase of growth (O.D. ~ 0.600 @ 600 nm) and then  $1 \times 10^{12}$  pfu/mL Hyperphage<sup>TM</sup> was added. The culture was left static for 30 minutes at 37°C and then transferred into a 37°C shaking incubator. After 2 hours, kanamycin (70 µg/mL) was added and the culture was incubated O/N at 37°C and 250 rpm.

The following day the phage was precipitated (section 2.2.1.10) and stored in ice at 4°C. An Immuntube (Maxisorp<sup>TM</sup>, Nunc) was coated O/N at 4°C with 500 µL of 50 µg/mL goat anti-chicken IgY Fab antibody. The tube was blocked with 5 mL of 3% (w/v) BSA in PBS for 1.5 hours at RT on a roller mixer. After blocking, the tube was washed with PBST and PBS once, followed by addition of 100 µg/mL of chicken anti-hFABP 3.G9 scAb (D2). The tube was again incubated for 1.5 hours at RT and washed 3 times with PBST and PBS. Five hundred µL of the rescued phage was added to the immuntube and incubated on a roller mixer for 2 hours at RT. The immuntube was then washed 5 times with PBST and PBS and specifically bound phage was eluted by adding 500 µL of 10 µg/mL trypsin solution in PBS followed by incubating the tube at 37°C for 30 minutes.

Eluted phage were infected to 4 mL of mid-exponential phase XL-1 Blue cells for 30 minutes static at 37°C. Additional 4 mL of SB media supplemented with 2.4 µL of 100 mg/mL carbenicillin and 12 µL of 5 mg/mL tetracycline were added to the infected cells and phage titre was carried out (3.1.11). After titering, the phage-infected culture was incubated at 37°C for 1 hour shaking at 200 rpm followed by addition of 1.6 µL carbenicillin. The culture was incubated at 37°C for an additional hour after which the phage was rescued and precipitated. Three rounds of panning were performed with similar conditions just to enrich the 3.G9 epitope specific phage.

#### **2.2.3.2.9 Polyclonal phage ELISA of gene library panned outputs**

Polyclonal ELISA was carried out in similar format as section 2.2.1.12. ELISA plate was coated with 50 µg/mL of goat anti-chicken IgY Fab O/N at 4°C. The plate was blocked with 2.5% (w/v) BSA-milk marvel in PBS for an hour at 37°C followed by washing 3 times with PBST and PBS. 100 µL of the diluted scAb (100 µg/mL) was added to each well and incubated at 37°C for 1 hour. The plates were washed as before and again incubated with 100 µL of the panned output phage diluted 1:3 in 0.5% (w/v) BSA-milk marvel in PBS. The plate was washed as before and 100 µL of 1:1,000 dilution of HRP-conjugated-anti-HA antibody was added to each well. After 1 hour incubation at 37°C the plates were washed with PBST and PBS and 100 µL per well of TMB was added followed by an incubation of 20 minutes. The reaction was stopped as before with 10% HCl and the plate was read.

#### **2.2.3.3 Gene walking**

Gene walking was another approach carried out for the epitope mapping of 3.G9 scFv. It was performed by expressing small overlapping peptides all over the length of the hFABP gene which was then checked against the scFv by Western blotting analysis for binding. Overlapping regions from the gene were amplified using various primer combinations enlisted below:

##### hFABP 5 Sense Primers

FABPFOR

5 CAT GCC ATG GTG GAC GCT TTC CTG GGC AC 3

FABP 4 FOR

5 CAT GCC ATG GGG CAC CTG GAA GCT AGT GG 3

FABP 5 FOR

5 CAT GCC ATG GTT CGA TGA CTA CAT G 3

FABP 6 FOR

5 CAT GCC ATG GAT GAA GTC ACT CGG TGT GGG 3

hFABP 3 Reverse Primers

FABPREV

5 CCT TTA GCG GCC GCT GCC TCT TTC TCA TAA GTG C 3

FABP Rev 1

5 CCT TTA GCG GCC GCG GCC ACC TGC CTG GTA GC 3

FABP Rev 2

5 CCT TTA GCG GCC GCC ACC CCC AAC TTA AAG CTG 3

FABP Rev 3

5 CCT TTA GCG GCC GCT TGC CCG TCC CAT TTC TGC 3

Primer combinations for peptide expression

Various primer combinations were used for amplifying the fragments from the gene. Each combination was labelled specifically to avoid any confusion with the peptides expressed.

Primer combinations:

<u>Label:</u>	<u>Forward:</u>	<u>Reverse:</u>
Peptide 1	FABPFOR -	FABP Rev 1
Peptide 2	FABPFOR -	FABP Rev 2
Peptide 3	FABPFOR -	FABP Rev 3
Peptide 4	FABP 4 FOR -	FABPREV
Peptide 5	FABP 5 FOR -	FABPREV
Peptide 6	FABP 6 FOR -	FABPREV
Peptide 7	FABP 7 FOR -	FABPREV

### 2.2.3.3.1 Cloning and expression of hFABP gene-fragments

PCR was carried using clone 3 expressing hFABP (section 2.2.2.6), using different sense and reverse primer combinations.

**Table 2.22: PCR mix for amplification overlapping gene-fragments from hFABP gene.**

Components	Conc. in 50 $\mu$ L volume
HiFi buffer (10x)	1x
Sense Primer	60 pM
Anti-sense Primer	60 pM
Clone 3 plasmid	100 ng/rxn
dNTP	0.2 mM
MgSO <sub>4</sub>	2 mM
H <sub>2</sub> O	N/A
Platinum HiFi <sup>TM</sup> Taq	2.5 U

The PCR for the amplification of the rabbit antibody variable domain genes was performed in the PX2 thermal cycler (Thermo Electron Corporation) under the following conditions:

Stage	Temperature ( $^{\circ}$ C)	Time (seconds)	No. of cycles
1	94	300	1
2	94	15	30
	56	30	
	72	60	
3	72	600	1

The amplified products were gel purified and cloned in pET-32b(+) vector using *NotI* and *NcoI* enzymes (section hFABP expression). Conditions used for both vector and insert are given below:

**Table 2.23: Reaction mix for digesting *hFABP* gene-fragments along with digestion of *pET-32b(+)* vector.**

Components	Conc. in 50 $\mu$ L volume	Components	Conc. in 50 $\mu$ L volume
10x buffer II	1x	10x buffer II	1x
pET-32b(+) vector	5 $\mu$ g	Gene-fragment	5 $\mu$ g
<i>Nco</i> I enzyme	100 U	<i>Nco</i> I enzyme	100 U
<i>Not</i> I enzyme	100U	<i>Not</i> I enzyme	100U
100x BSA	1x	100x BSA	1x
Water	N/A	Water	N/A

The digested products were gel purified and ligated O/N.

**Table 2.24: Ligation mix for cloning gene-fragments into *pET-32b(+)* vector.**

Components	Conc. in 10 $\mu$ L volume
10x Ligase buffer	1x
Digested pET-32b(+) vector	30-45 ng (based on size)
Digested cTnI gene	60 ng
T <sub>4</sub> DNA Ligase enzyme	400 U
Water	N/A

The ligation mix was transformed into BL21-CodonPlus® (DE3)-RIPL-competent cells and plated on to LB carbenicillin plates. Single colonies were picked and grown into 8 individual wells of a 12-well plate containing 1 mL of SB media with 100  $\mu$ g/mL carbenicillin. The clones were subcultured and induced O/N with 1 mM IPTG.

The expression of the peptides was checked by Western blotting analysis using HRP-labelled anti-His antibody. The peptides were later on checked by blotting against 3.G9 scFv (section 2.2.1.22) in order to determine the epitopes.



## **2.2.4 Rabbit anti-cTnI scFv library**

### **2.2.4.1 Immunisation of rabbit with cTnI and determining the serum titre**

A New Zealand white rabbit was immunised with 200 µg of cTnI in PBS, mixed in 1:1 ratio with Freund's complete adjuvant (FCA). Two subsequent boosts were given, at three week intervals, with 200 µg of cTnI (the material for immunisation had the same composition except Freund's incomplete adjuvant (FICA) was used). Three additional boosts with 50 µg of cTnI were given at intervals of 3 weeks and the serum titre was then determined.

Approximately 10 days after each boost, blood was collected from the rabbit and the serum was separated, aliquoted and stored in -20°C. The antibody titre developed against the antigen was determined as per section 2.2.1.1.

### **2.2.4.2 Characterisation of rabbit serum against different cTnI epitopes**

The reactivity of the serum was checked against different peptides, representing specific cTnI epitopes (Table 2.13), for characterising the population of polyclonal antibodies developed in the animal. This was carried out by indirect ELISA. The serum titre was determined and analysed for binding to the peptides coated on a plate. The N-terminal and C-terminal biotinylated peptides (1 µg/mL) were captured on the plate by coating 5 µg/mL neutravidin to the surface along with 1 µg/mL of peptides conjugated with KLH (Keyhole Limpet Hemocyanin). cTnI was also coated on the ELISA plate at similar concentration of peptides to compare the response obtained for whole molecule versus the peptides. Neutravidin was used as control. The ELISA procedure was similar to the titration ELISA in section 2.2.1.14, which included blocking, primary antibody, and secondary antibody and TMB substrate addition steps.

**Table 2.25: *cTnI* specific peptide composition and properties**

Peptide	MW (Da)	Theoretical pI	Amino Acid Sequence
DUSH1 (39-50)	1372	11.26	KISASRKLQLKT
DUSH2 (24-40)	2005	10.00	NYRAYATEPHAKKSK
DUSH3 (79-93)	1485	3.79	QPLELAGLGFAELQ
DUSH4 (169-180)	1452	9.70	RAHLKQVKKEDT

#### **2.2.4.3 Anti-cTnI polyclonal antibody purification from rabbit serum**

The anti-cTnI rabbit polyclonal was purified using protein-A immobilised on Sepharose 4B resin. A 1 mL suspension of immobilised protein A was equilibrated in a column with 30 mL of sterile-filtered PBS (150 mM, pH 7.4). One mL of rabbit serum was diluted with 9 mL of sterile-filtered PBS and passed through the column. The eluant was collected and passed through the column one more time. The column was washed with 30 mL of sterile-filtered PBS. The retained protein, binding to the protein A, 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 antibody fraction. The fractions were quantified on a NanoDrop™ ND-1000 using the pre-programmed 'IgG' option (mass extinction coefficient of 13.7 at 280 nm). The fractions containing high concentrations of anti-IgA were pooled and desalted using a PD10™ (particle size range: 85-260 µm, bed volume: 8.3 mL, bed height: 5 cm) size exclusion column. The antibodies were buffer exchanged against PBS and a small aliquot was taken for analysis on SDS-PAGE before storing the remainder at -20°C. All purification and desalting procedures were conducted at 4°C to minimise denaturing of antibody.

#### **2.2.4.4 Checkerboard ELISA of purified anti-cTnI polyclonal antibody**

A 96 well plate was coated with varying concentrations of the polyclonal antibody in 1x PBS (150 mM, pH 7.4) and left O/N at 4°C. The neutral pH of the buffer is optimal for stability of the antibodies due the physiological pH of the host animal. The following day, the antibody-coated plate was blocked with 200 µL per well 2.5% (w/v) BSA-milk-PBS solution for 1 hour at 37°C. After blocking the plate was washed 3 x 3 times with PBST and PBS and 100 µL of 1 µg/mL solution of the cTnI antigen was added to each well. Serial dilutions of anti-cTnI scFv ('in-house' developed) were made in 0.5% (w/v) BSA-milk-PBST, and added to the wells. The plate was incubated at 37°C for 1 hour. Following incubation, the plates were washed three times with both PBST and PBS followed by detection of the cTnI-pAb complex with 1:2,000 dilution of goat anti-rabbit-HRP-conjugated antibody. After 1 hour incubation at 37°C, the plate was washed again and developed with TMB substrate for 20 minutes. The reaction was quenched by the addition of 50 µL per well of 10% (v/v) HCl, and the absorbance was then determined at 450 nm on a Safire 2 plate reader (Tecan).

#### **2.2.4.5 RNA extraction and cDNA preparation**

The animal was sacrificed by neck dislocation after mild anaesthetic injection and RNA extraction along with cDNA preparation carried out as in sections 2.2.1.2 and 2.2.1.3, for both bone marrow and spleen. The prepared cDNA was used further for variable gene amplifications, after quantification on Nanodrop™ ND1000.

#### **2.2.4.6 PCR primers for amplification of rabbit scFv (pComb series)**

##### V\_5 Sense Primers

RSCVK1

5 GGG CCC AGG CGG CCG AGC TCG TGM TGA CCC AGA CTC CA 3

RSCVK2

5 GGG CCC AGG CGG CCG AGC TCG ATM TGA CCC AGA CTC CA 3

RSCVK3

5 GGG CCC AGG CGG CCG AGC TCG TGA TGA CCC AGA CTG AA 3

V\_3 antisense Primers, Long Linker

(Linker amino acid sequence: SSGGGGSGGGGGGSSSRSS):

RKB9J1o-BL

5 GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC  
GCC ACC AGA GGA TAG GAT CTC CAG CTC GGT CCC 3

RKB9Jo-BL

5 GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC  
GCC ACC AGA GGA TAG GAT CTC CAG CTC GGT CCC 3

RKB42Jo-BL

5 GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC  
GCC ACC AGA GGA TTT GAC SAC CAC CTC GGT CCC 3

V\_for 5 Sense Primer

RSC 1

5 GGG CCC AGG CGG CCG AGC TCG TGC TGA CTC AGT CGC CCT C 3

V\_rev 3 Anti-sense Primer, Long Linker

(linker amino acid sequence: SSGGGGSGGGGGGSSSRSS):

RJ o-BL

5 GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC  
GCC ACC AGA GGA GCC TGT GAC GGT CAG CTG GGT CCC 3

### VH 5 Sense Primers

RSCVH1

5 GGT GGT TCC TCT AGA TCT TCC CAG TCG GTG GAG GAG TCC RGG 3

RSCVH2

5 GGT GGT TCC TCT AGA TCT TCC CAG TCG GTG AAG GAG TCC GAG 3

RSCVH3

5 GGT GGT TCC TCT AGA TCT TCC CAG TCG YTG GAG GAG TCC GGG 3

RSCVH4

5 GGT GGT TCC TCT AGA TCT TCC CAG SAG CAG CTG RTG GAG TCC GG 3

### VH 3 Reverse Primers

RSCG-B

5 CCT GGC CGG CCT GGC CAC TAG TGA CTG AYG GAG CCT TAG GTT GCC  
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 (anti-sense)

5 GAG GAG GAG GAG GAG GAG CCT GGC CGG CCT GGC CAC TAG TG 3

### pComb rabbit anti-CRP scFv primer combinations

Various primer combinations were used for amplifying the variable genes. Each combination was labelled specifically to avoid any confusion with the product and the primers used to amplify the product.

#### Variable kappa light chain combinations

<u>Label:</u>	<u>Forward:</u>		<u>Reverse:</u>
VK1	RSCVK1	-	RKB9J10-BL
VK2	RSCVK1	-	RKB9J0-BL
VK3	RSCVK1	-	RKB42J0-BL
VK4	RSCVK2	-	RKB9J10-BL
VK5	RSCVK2	-	RKB9J0-BL
VK6	RSCVK2	-	RKB42J0-BL
VK7	RSCVK3	-	RKB9J10-BL
VK8	RSCVK3	-	RKB9J0-BL
VK9	RSCVK3	-	RKB42J0-BL

#### Variable lambda light chain combinations

<u>Label:</u>	<u>Forward:</u>		<u>Reverse:</u>
VL	RSC 1	-	RJ o-BL

#### Variable heavy chain combinations

<u>Label:</u>	<u>Forward:</u>		<u>Reverse:</u>
VH1	RSCVH1	-	RSCG-B
VH2	RSCVH2	-	RSCG-B
VH3	RSCVH3	-	RSCG-B
VH4	RSCVH4	-	RSCG-B

#### 2.2.4.7 Amplification of antibody variable genes using pComb3X series primers

PCR was carried out for both spleen and bone marrow cDNA separately. Ten different combinations were used for light chain amplification and four different combinations were used for heavy chain amplification.

**Table 2.26: PCR mix for heavy and light chain amplification of rabbit antibodies.**

Components	Conc. in 50 $\mu$ L Volume
GoTaq Buffer (5x)	1x
Sense Primer	60 pM
Anti-sense Primer	60 pM
cDNA	0.5 $\mu$ g
dNTP	0.2 mM
MgCl <sub>2</sub>	1.5 mM
H <sub>2</sub> O	N/A
GoTaq <sup>TM</sup> Polymerase	2.5 U

The PCR for the amplification of the rabbit antibody variable domain genes was performed in the PX2 thermal cycler (Thermo Electron Corporation) under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	94	300	1
2	94	15	30
	56	30	
	72	60	
3	72	600	1

Except for two combinations of variable kappa light chains the above conditions and mixture composition yielded the desired results. An alternative polymerase enzyme was used for amplification of the products that were not amplifying using conditions described previously.

**Table 2.27: PCR mix for light chain amplification of rabbit antibodies for VK3 and VK6.**

Components	Conc. in 50 $\mu$ L Volume
Phusion GC Buffer (5x)	1x
Sense Primer	60 pM
Anti-sense Primer	60 pM
cDNA	0.5 $\mu$ g
dNTP	0.2 mM
H <sub>2</sub> O	N/A
Phusion <sup>TM</sup> Taq*	1 U

\*Phusion is added to PCR tube after a 5 minutes hot start at 98°C for specific amplification of products.

Following conditions were used:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	98	360	1
2	98	15	30
	56	30	
	72	90	
3	72	600	1

#### 2.2.4.8 Splice-by-overlap extension (SOE) PCR

The ~ 750 bp amplicon of the SOE- PCR products were purified (section 2.2.1.6) using the Qiagen gel extraction Kit. The V<sub>H</sub> and V<sub>L</sub> purified fragments from each of the various combinations were pooled into equimolar concentrations and were used for PCR.

**Table 2.28: PCR mix for SOE-PCR of rabbit heavy and light chain domains.**

Components	Conc. in 50 $\mu$ L volume
HiFi buffer (10x)	1x
RSC-F Sense Primer	60 pM
RSC-B Anti-sense Primer	60 pM
V <sub>H</sub> chain mix	100 ng/rxn
V <sub>L</sub> chain mix	100 ng/rxn
dNTP	0.2 mM
MgSO <sub>4</sub>	2 mM
H <sub>2</sub> O	N/A
Platinum HiFi <sup>TM</sup> Taq	2.5 U



The SOE-PCR was performed in the PX2 thermal cycler (Thermo Electron Corporation) under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	94	120	1
2	94	30	30
	56	30	
	72	120	
3	72	600	1

#### 2.2.4.9 Digestion of vector and purified SOE-PCR product and cloning

A detailed procedure for the digestion of pComb3XSS vector and the SOE-PCR product is explained in section 2.2.1.8. For both bone marrow and spleen 10 µg of the SOE-PCR product was digested along with 50 µg of the vector.

The digested vector and the SOE-PCR products were gel-purified and ligated using the following conditions:

**Table 2.29: Ligation mix for cloning rabbit SOE-product into pComb3XSS vector.**

Components	Conc. in 200 µL Volume
10x Ligase buffer	1x
Digested pComb3XSS vector	1.4µg/rxn
Digested scFv gene	0.7 µg/rxn
T <sub>4</sub> DNA Ligase enzyme	400 U
Water	N/A

Two ligations were performed for bone marrow and four for spleen. The ligations were allowed to take place O/N at RT. On the following morning the ligation was inactivated, by heating the mix at 65°C for 15 minutes, and it was then ethanol precipitated (section 2.2.1.8) to concentrate the DNA.

#### **2.2.4.10 Transformation of cloned gene into *E. coli* by electroporation**

Ligated product for both bone marrow and spleen was resuspended by adding 20µL and 40 µL water, respectively. Ten µL of ligated product was electroporated into 100µL of commercial XL1-Blue cells by pulsing at 200 , 25 µF and 1.25 kV. The detailed procedure for transformation was explained in section 2.2.1.9. Six transformations were carried out for both bone marrow and spleen. After transformation the cells were resuspended in 3mL of SOC media and propagated for an hour at 37°C while shaking at 250 rpm.

Following incubation, the 7 x 5 mL cultures for both bone marrow and spleen were pooled and plated on LB agar plates supplemented with 100 µg/mL carbenicillin and 2% (w/v) glucose. The plates were left to grow O/N at 37°C and then scraped into SB medium supplemented with 20% (v/v) glycerol for long-term storage at -80°C.

#### **2.2.4.11 Rescue and subsequent precipitation of scFv-displaying phage**

The transformants were propagated by inoculating 150 µL of the library stock into 400 mL of SB media, supplemented with 100 µg/mL carbenicillin and 10 µg/mL tetracycline, followed by shaking at 250 rpm at 37°C. At mid-exponential phase of growth (O.D. ~ 0.6 @ 600 nm), the library was rescued by infecting with 400 µL of commercial M13K07 helper phage. After incubating the library with phage for 30 minutes, while static, it was transferred to a 37°C shaking incubator for 2 hours at 250 rpm. Subsequently, kanamycin was added to a final concentration of 70 µg/mL and the culture was incubated O/N at 37°C at 250 rpm. Phage precipitation was carried out as per section 2.2.1.10.

#### 2.2.4.12 Biopanning the library against cTnI for enrichment of specific clones

An immunotube was coated with 500  $\mu$ L of cTnI antigen overnight at 4°C. The tube was blocked for 1.5 hours at RT with 3% (w/v) BSA-PBS followed by washing once with both PBST and PBS. Precipitated phage (500  $\mu$ L) was added to a blocked tube and the tube was incubated (section 2.2.1.11). The tube was washed 5 times with both PBST and PBS. Specific phage were eluted by adding 500  $\mu$ L of 10  $\mu$ g/mL trypsin solution in PBS and incubating the tube at 37°C for 30 minutes. All of the eluted phage were then infected into 4 mL of XL1-Blue cells (O.D. ~ 0.6) and the cells were left static at 37°C for 30 minutes. The infected cells were plated on LB agar plates containing carbenicillin and left to grow overnight at 37°C (as rabbit clones are not very high expressers). Plating out allows weakly expressing clones, with high affinity, to grow and be enriched in subsequent rounds rather than facilitating the biased selection of clones owing to their high expression.

The following day the plates were scraped off and the phage was rescued. Five rounds of biopanning were carried out with repeating cycles of selection, enrichment and rescue with few variations in the parameters used (Table 2.30). The parameters that were varied in each round are listed below:

**Table 2.30: Variable parameters of panning of rabbit anti-cTnI scFv library.**

	PAN 1	PAN 2	PAN 3	PAN 4	PAN 5
Culture vol.	400 mL	150 mL	150 mL	150 mL	150 mL
cTnI coated	10 $\mu$ g	5 $\mu$ g	2.5 $\mu$ g	1 $\mu$ g	1 $\mu$ g
Blocking used	3% (w/v)	3% (w/v)	3% (w/v)	3% (w/v)	3% (w/v)
	BSA-PBS	Milk-PBS	BSA-PBS	Milk-PBS	BSA-PBS
Washes	5 x PBST	10 x PBST	10 x PBST	15 x PBST	15 x PBST
	5 x PBS	5 x PBS	10 x PBS	10 x PBS	10 x PBS

#### **2.2.4.13 Polyclonal phage ELISA and soluble expression of pan 5 outputs**

Polyclonal phage ELISA was carried out on panned outputs as in section 2.2.1.12. The ELISA plate was coated with 1 µg of cTnI O/N followed by blocking with 2.5% (w/v) BSA-milk-PBS for an hour. A 1:3 dilution of phage outputs in 0.5% (w/v) BSA-milk-PBS were added to the plate in duplicates and the plate was incubated for 1 hour at 37°C followed by washing thrice with PBST and PBS. A 1:2,000 dilution of HRP-conjugated mouse anti-M13 monoclonal antibody was added to the plate for 1 hour at 37°C. The plate was washed as before and developed and the absorbance was read. Milk and BSA were used as controls in this ELISA to detect any binding of phage to blocking solutions used.

A 10 µL aliquot of pan 5 output was infected into 4 mL of Top10F cells (O.D. ~ 0.6) and incubated while static at 37°C for 30 minutes. The infected cells were diluted starting from  $10^{-1}$ - $10^{-8}$  in SB media and 100 µL of each dilution was plated on to SB plates containing carbenicillin. The plates were incubated O/N at 37°C. Single colonies were picked into 3 x 96 well plates containing 200 µL SB media supplemented with 100 µg/mL carbenicillin and grown overnight. The cells were subcultured and stocked by adding 20% (v/v) final concentration of sterile glycerol. The subcultured cells were induced at O.D. ~ 0.6 with 1 mM final concentration of IPTG and expressed O/N at 30°C.

#### **2.2.4.14 Screening cTnI clones by indirect ELISA**

The induced cultures were subjected to three cycles of freeze-thawing and the lysates were collected by centrifugation. An indirect ELISA was carried out on a cTnI-coated (1 µg/mL) ELISA plate as per section 2.2.1.13.

#### **2.2.4.15 Cross-reactivity analysis of positive clones with skeletal troponin I (sTnI)**

ScFvs binding to the cTnI (as detected by indirect ELISA in section 2.2.4.14) were selected and analysed for cross-reactivity against skeletal troponin I (sTnI), which is a similar isoforms sharing high sequence homology with cTnI.

Plates were coated O/N with 1 µg/mL of both cTnI and sTnI. ELISA was carried out using 1:3 dilutions of the clones in 0.5% (w/v) BSA-milk marvel-PBST. The procedure followed was similar to that of section 2.2.1.13. The clones were analysed for binding the cTnI in comparison to sTnI, to determine their specificity. Clones specific for cTnI were selected for further studies.

#### **2.2.4.16 Competitive analysis of cTnI positive clones**

cTnI positive-scFvs were checked further to establish their competitiveness against free cTnI in solution. Plates were coated with 1 µg/mL of cTnI and blocked with 2.5% (w/v) BSA-milk-PBS. The lysates were diluted 1:3 in 0.5% (w/v) BSA-milk-PBS and mixed 1:1 ratio with 5 µg/mL with cTnI in solution. One hundred µL per well of this suspension was added to the plate and incubated for 1 hour at 37°C. The plate was washed 3 times with both PBST and PBS and 1:2,000 dilution of HRP-conjugated-anti-HA was added and the plates were left incubating for 1 hour at 37°C. The plates were washed as before, developed with TMB substrate and the absorbance was read at 450 nm.

#### **2.2.4.17 Ranking of clones by ‘off-rate’ analysis using Biacore 3000**

Ranking of clones were carried out as in section 2.2.1.17. A 30 nM concentration of cTnI was used. The data were evaluated by Biaevaluation 4.1 software. Ten clones with more than 99% ‘stability-left’ were sent for sequencing analysis.

#### **2.2.4.18 Restriction mapping of the clones**

Plasmid extraction of the clones selected from section 2.2.1.18 was carried out. The SOE-PCR product was amplified from respective plasmids using RSC-F (sense) and RSC-B (anti-sense) primers, using conditions similar to those described in section 2.2.3.8. The amplified products were subjected to digestion by *Bst*NI and *Alu*I enzymes (section 2.2.1.18). The digested products were analysed by gel electrophoresis on a 3% agarose gel.

#### **2.2.4.19 Cloning and expression of cTnI gene into pET-32b(+) vector**

Amplification of cTnI gene was attempted from human heart cDNA using specific primers. The primers were designed using a cTnI gene sequence published in pubmed accession no. AAH96166.

Primers for cTnI gene:

CTNI1FOR (sense)

5 CATGCCATGGCCAACTACCGCGCTTATGCCAC 3

CTNI2FOR (anti-sense)

5 CCTTTAGCGGCCGCGCTCTCAAACCTTTTCTTGC 3

The composition of the reaction mix and the conditions were optimised for amplifying the cTnI gene from the cDNA. However, there was amplification of several non-specific bands close to the cTnI band, when the PCR product was resolved on agarose gel.

Due to persisting problems with amplifying the cTnI gene from the cDNA, a truncated version of the cTnI gene cloned in pDream vector was synthesised from GenScript USA Inc. The plasmid was digested along with pET-32b(+) vector using *Nco*I and *Hind*III enzymes. Conditions used for both vector and insert are given below:

**Table 2.31: Reaction mix for digesting cTnI gene from pDream vector along with digestion of pET-32b(+) vector.**

Components	Conc. in 50 $\mu$ L volume	Components	Conc. in 50 $\mu$ L volume
10x buffer II	1x	10x buffer II	1x
pET-32b(+) vector	5 $\mu$ g	pDream vector	5 $\mu$ g
<i>Nco</i> I enzyme	100 U	<i>Nco</i> I enzyme	100 U
<i>Hind</i> III	200U	<i>Hind</i> III	200U
100x BSA	1x	100x BSA	1x
Water	N/A	Water	N/A

The digested products were gel purified and ligated O/N.

**Table 2.32: Ligation mix for cloning cTnI gene into pET-32b(+) vector.**

Components	Conc. in 10 $\mu$ L volume
10x Ligase buffer	1x
Digested pET-32b(+) vector	100 ng
Digested cTnI gene	60 ng
T <sub>4</sub> DNA Ligase enzyme	400 U
Water	N/A

The ligation mix was transformed into BL21-CodonPlus® (DE3)-RIPL-competent cells and plated on to LB carbenicillin plates (section 2.2.2.4). Single colonies were picked and grown into 8 individual wells of a 12-well plate containing 1 mL of SB media with

100 µg/mL carbenicillin. The clones were subcultured and induced O/N (section 2.2.2.5) with 1 mM IPTG.

#### **2.2.4.20 SDS-PAGE and Western blot analysis of the lysates**

cTnI expression was checked by SDS-PAGE and Western blot analysis. The lysates were treated with 4x sample treatment buffer followed by denaturing protein at 95°C for 10 minutes. Two SDS-PAGE gels were carried out as in section 2.2.1.21. A total of nine samples were analysed which included 8 induced clones and one un-induced cell control. The protein was transferred from gels to nitrocellulose membrane at 15 V for 20 minutes (section 2.2.1.22).

The procedure performed for blotting was similar to that of section 2.2.1.22. One membrane was detected with anti-His-HRP-conjugated antibody and the other was detected with anti-peptide 3-HRP-cTnI antibody. The positive clone was further screened by Western blotting using anti-peptide 1, 2 and 4, cTnI antibodies.

### **2.2.5 Formatting of Rabbit anti-cTnI Antibodies for Optimal Expression and Conjugation**

#### **2.2.5.1 Modification of pMoPac 16 vector**

The pMoPac 16 vector was modified for optimal expression analysis replacing the human constant domain in the vector to that of rabbit and chicken antibodies. This study will enable the analysis of the effect of insertion of a constant domain, from a different host, on antibody expression. This was carried out by amplifying the constant domains from the host species with primers containing RE sites similar to the ones at the end of the vector's human constant region. The amplified constant domains were then cloned into the pMoPac vector (obtained from Dr. Conor Hayes, Applied Biochemistry Group,



School of Biotechnology, Dublin City University, Ireland), with anti-Prostate specific antigen (PSA) scFv cloned in it.

#### **2.2.5.1.1 Primers for amplification of rabbit and chicken constant regions**

Primers were designed for amplifying rabbit kappa ( ) and chicken lambda ( ) chains from the cDNA of respective animals. The primer sequences are shown below:

*Primers for rabbit constant region*

RABCLAMBFOR 5 sense primer

5 ATA AGA ATG CGG CCG CTG GTG ATC CAG T 3

RABCLAMBREVtrp 3 antisense primer

5 CCG GCA ATG GCC GAC GTC GAC CCA GTC A 3

*Primers for chicken constant region*

CLAMBFOR 5 Sense primer

5 ATA AGA ATG CGG CCG CTC AGC CCA AGG TGG CCC CCA C 3

CLAMBREV 3 Reverse primer

5 CCG GCA ATG GCC GAC GTC GAC GCT CTC GGA CCT CTT CAG GG 3

#### **2.2.5.1.2 Amplification of rabbit and chicken constant regions**

Both the rabbit and chicken constant kappa and lambda light chains were amplified in a Biometra T<sub>GRADIENT</sub> PCR machine using the following reaction mixture and conditions:

**Table 2.33: PCR mix for amplification of chicken and rabbit constant light chains.**

Components	Conc. in 50 $\mu$ L volume
HiFi buffer (10x)	1x
RAB/CLAMBFOR	50 pM
RAB/CLAMBREV	50 pM
cDNA	20 ng
dNTP	0.2 mM
MgSO <sub>4</sub>	1 mM for C / 4 mM for C
H <sub>2</sub> O	N/A
Platinum HiFi <sup>TM</sup> <i>Taq</i>	2.5 U

The only differences in amplification of both the constant light chains were the use of different concentrations of MgSO<sub>4</sub>. 1 mM was found to be optimum for chicken C , whereas 4 mM MgSO<sub>4</sub> was used for rabbit C .

Conditions used:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	94	300	1
2	94	30	30
	61	30	
	72	60	
3	72	600	1

#### 2.2.5.1.3 Digestion and cloning of the constant regions into pMoPac vector and its transformation in *E. coli* cells

The PCR product was checked on 1% (w/v) agarose gel for both the light chain amplicons (~390 bp). The amplified products were gel-purified and digested with *NotI* and *SalI* restriction enzymes along with the pMoPac 16 vector, which was digested sequentially.

**Table 2.34: Digestion of pMoPac 16 vector, chicken and rabbit constant light chains.**

Components	Conc. in 150 $\mu$ L volume	Components	Conc. in 100 $\mu$ L volume
10x buffer III	1x	10x buffer II	1x
pMoPac vector	30 $\mu$ g	Insert (C /C )	10 $\mu$ g
<i>NotI</i> enzyme	180 U	<i>NotI</i> enzyme	100 U
100x BSA	1x	<i>SaII</i> enzyme	200U
Water	N/A	100x BSA	1x
		Water	N/A

The reaction was incubated at 37°C O/N followed by purifying the digested product by gel extraction (section 2.2.1.6). *NotI* digested vector was further digested with *SaII* enzyme O/N at 37°C using the following composition of reaction mixture:

**Table 2.35: Digestion of pMoPac vector with *SaII* enzyme.**

Components	Conc. in 250 $\mu$ L volume
10x buffer III	1x
pMoPac vector	9 $\mu$ g
<i>SaII</i> enzyme	180 U
100x BSA	1x
Water	N/A

The vector was treated with Antarctic phosphatase<sup>TM</sup> enzyme to prevent self-ligation. Both the digested vector and the insert was gel purified and ligated O/N at room temperature.

**Table 2.36: Ligation mix for cloning constant light chains into of pMoPac vector.**

Components	Conc. in 100 $\mu$ L volume
10x ligase buffer	1x
Digested pMoPac vector	100 ng
Insert (C /C )	56 ng
T <sub>4</sub> DNA Ligase enzyme	400 U
Water	N/A

The ligation mix was transformed into chemically competent TOP10F cells (section 2.2.2.4) and plated onto LB agar plates supplemented with 100 µg/mL carbenicillin and incubated O/N at 37°C. The transformed colonies were cherry picked into a 96 well plate containing SB media with 100 µg/mL carbenicillin and grown overnight. This plate was stored in -80°C as a back up, by adding glycerol to a final concentration of 20% (v/v). These colonies were subcultured and induced O/N (section 2.2.1.13) and screened on a plate coated with 1 µg/mL PSA. Indirect ELISA was performed similar to that of section 2.2.1.13, with the only exception that HRP-conjugated-goat anti-rabbit (whole molecule) antibody for rabbit C and HRP-conjugated-goat anti-chicken IgY antibody for chicken C, were used as secondary antibodies for detection of clones containing respective constant domains. Using PSA as capturing antigen enabled the selection of clones expressing functional scAbs, whereas, domain specific secondary antibodies selected the scAbs with rabbit and chicken constant regions from the population of native human constant region clones.

#### **2.2.5.2 Changing variable domain orientation**

The variable heavy chain and the light chain domains were reamplified from the cTnI MG4 scFv using primers designed for reversing the orientation. The heavy chain sense primer and the light chain antisense primer contained the *Sfi*I sites. The linker was coded by heavy chain antisense primer and the light chain sense primer, thus altogether reversing the orientation of heavy and light chains while assembling the gene by SOE-PCR.

### **2.2.5.2.1 Primers for reversing the orientation**

#### Primers for amplification of the variable domains

RABVH For 5 sense primer

5 GCG GCC CAG CCG GCC ATG GCG CAG CAG CAG CTG ATG GAG TCC GG  
3

RABVH Rev 3 antisense primer

5 GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC  
AGA GGA ACT AGT GAC TGA TGG TGG AGC CTT AGG TTG CCC 3

RABVK For 5 sense primer

5 GGT GGT TCC TCT AGA TCT TCC GAG CTC GAT CTG ACC CAG ACT CCA  
3

RABVK Rev 3 antisense primer

5 GGC CCC CGA GGC CGC TTT GAC GAC CAC CTC GGT CCC 3

#### Primers for SOE-PCR

RabSWSOE2 For 5 sense primer

5 GGA ATT CGC GGC CCA GCC GGC CAT GGC GCA G 3

RabSWSOE1 Rev 3 antisense primer

5 TTA CTC GCG GCC CCC GAG GCC GCT TTG 3

### **2.2.5.2.2 Amplification of heavy and light variable domains**

The heavy and light chains were amplified from the MG4 plasmid using the same mixture composition and PCR conditions as that of chicken C (section 2.2.4.1.2). The only difference was the annealing temperature used. An annealing temperature of 56°C was found to be optimum for both heavy and light chain amplification.

### 2.2.5.2.3 SOE-PCR for assembling the variable domains

SOE-PCR was used to assemble both variable heavy and light domains. The PCR was carried out in a Biometra T<sub>GRADIENT</sub> PCR machine using equimolar ratios of both the domains. The reaction mixture and the conditions used are represented below:

**Table 2.37: SOE-PCR for assembling anti-cTnI scFv genes in V<sub>H</sub>-V<sub>L</sub> orientation.**

Components	Conc. in 50 $\mu$ L volume
HiFi buffer (10x)	1x
RabSWSOE1	50 pM
RabSWSOE2	50 pM
Heavy chain cDNA	100 ng
Light chain cDNA	100 ng
dNTP	0.2 mM
MgSO <sub>4</sub>	1mM
H <sub>2</sub> O	N/A
Platinum HiFi™ Taq	2.5 U

The PCR was carried out in Biometra T<sub>GRADIENT</sub> PCR machine under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	94	300	1
2	94	30	30
	56	30	
	72	60	
3	72	600	1

The PCR product was analysed by agarose gel electrophoresis for amplification to the correct size (~750bp) and was scaled up to be used for cloning purposes.

### **2.2.5.3 Changing the *Sfi*I sites in the primers for intercloning into pComb and pMoPac vectors**

#### **2.2.5.3.1 Primers for changing the *Sfi*I sites for cloning into different vectors**

The *Sfi*I sites in the existing SOE primers were changed in order to clone the amplified scFv product from one vector into the other, without the need for amplifying the variable genes again and reassembling them.

##### Primers for changing *Sfi*I sites of V<sub>L</sub>-V<sub>H</sub> domain SOE product for pMoPac cloning

RAB VL-VH PAK\_ for 5 sense primer

5 GGA ATT CGC GGC CCA GCC GGC CAT GGC GGA GCT C 3

RAB VL-VH PAK\_ rev 3 antisense primer

5 TTA CTC GCG GCC CCC GAG GCC GCA CTA GTG 3

##### Primers for changing *Sfi*I sites of V<sub>H</sub>-V<sub>L</sub> domain SOE product for pComb cloning

RAB VH-VL pcomb\_ for 5 sense primer

5 GGA ATT CGC GGC CCA GGC GGC CAT GGC GCA G 3

RAB VH-VL pcomb\_rev 3 antisense primer

5 TTA CTC GCC TGG CCG GCC TGG CCG CCT TTG 3

#### **2.2.5.3.2 Amplification of SOE products for cloning into different vectors**

The V<sub>L</sub>-V<sub>H</sub> domain and the V<sub>H</sub>-V<sub>L</sub> scFv domains were reamplified from the MG4 scFv and MB3 scAb SOE products by using the primers from section 2.2.4.3.1. The V<sub>L</sub>-V<sub>H</sub> scFv domain amplified from the MG4 had *Sfi*I sites modified for cloning into the pMoPac vector. Similarly, the V<sub>H</sub>-V<sub>L</sub> scFv domain amplified from MB3 scAb had *Sfi*I sites for cloning into the pMoPac vector.

**Table 2.38: PCR mix for changing the *SfiI* sites in the SOE-products, with different  $V_H$  and  $V_L$  orientations, for cloning into different vectors.**

Components	Conc. in 50 $\mu$ L volume	Components ( $V_L$ - $V_H$ )	Conc. in 50 $\mu$ L volume
HiFi buffer (10x)	1x	GC buffer (5x)	1x
RAB VH-VL pcomb_ for	50 pM	RAB VL-VH PAK_ for	50 pM
RAB VH-VL pcomb _rev	50 pM	RAB VL-VH PAK_ rev	50 pM
$V_H$ - $V_L$ SOE product	20 ng	MG4 SOE product	20 ng
dNTP	0.2 mM	dNTP	0.2 mM
MgSO <sub>4</sub>	1mM	H <sub>2</sub> O	N/A
H <sub>2</sub> O	N/A	Phusion <i>Taq</i>	1 U
Platinum HiFi <sup>TM</sup> <i>Taq</i>	2.5 U		

The conditions used for  $V_H$ - $V_L$  scFv gene amplification from the SOE product were similar to those of section 2.2.4.2.3. However, the amplification of  $V_L$ - $V_H$  scFv gene was optimised for MgCl<sub>2</sub> concentration and annealing temperature. Finally, Platinum *Taq* HiFi was substituted with Phusion *Taq* polymerase for the gene amplification from the MG4 SOE product.

The scFv gene was amplified using the following PCR conditions:

Stage	Temperature ( $^{\circ}$ C)	Time (seconds)	No. of cycles
1	98	360	1
2	98	30	30
	56	30	
	72	60	
3	72	600	1

#### 2.2.5.4 Cloning of scFv gene with different orientation and *SfiI* sites into pComb and pMoPac vectors

The antibody genes, with different orientations and scFv size, were cloned into both the pComb 3XSS and pMoPac vectors. The  $V_H$ - $V_L$  gene was cloned into three pMoPac vector constructs with rabbit, chicken and human constant regions along with the same gene with pComb-compatible *SfiI* sites cloned into pComb3XSS vector.



The  $V_L$ - $V_H$  gene was already cloned into the pComb3XSS vector (MG4 scFv) so it was modified by changing *Sfi*I sites compatible to the pMoPac vector and cloned into three vector constructs with different constant regions.

**Table 2.39: Digestion of different vector constructs along with SOE products (with different  $V_H$  and  $V_L$  orientations) by *Sfi*I enzyme.**

Components	Conc. in 40 $\mu$ L volume	Components	Conc. in 40 $\mu$ L volume
10x buffer III	1x	10x buffer II	1x
pMoPac vector / pComb3XSS	7 $\mu$ g	Insert ( $V_L$ - $V_H$ / $V_H$ - $V_L$ )	7 $\mu$ g
<i>Sfi</i> I enzyme	120 U	<i>Sfi</i> I enzyme	120 U
100x BSA	1 x	100x BSA	1 X
Water	N/A	Water	N/A

The reaction was incubated at 50°C O/N followed by purifying the digested product by gel extraction (section 2.2.1.6). The *Sfi*I digested vector and the product were ligated O/N at 37°C using the following composition for the reaction mixture:

**Table 2.40: Ligation mix for cloning different SOE-products into different vector constructs.**

Components	Conc. in 10 $\mu$ L volume
10x Ligase buffer	1x
pMoPac vector/pComb3XSS	100 ng
Insert (C /C )	112 ng
T <sub>4</sub> DNA Ligase enzyme	400 U
Water	N/A

The ligation mix was transformed into chemically competent TOP10F cells by the heat shock method (section 2.2.2.4), followed by selecting the transformants on LB carbenicillin agar plates. The colonies were grown, subcultured and expressed overnight using a standard protocol (section 2.2.1.13). The transformants were screened by assaying them against cTnI and detecting the binders with anti-constant chain specific antibody (section 2.2.4.13), in an ELISA-based method.

#### **2.2.5.5 Expression analysis of anti-cTnI antibody in different formats**

The 8 different formats of the antibody (6 scAb and 2 scFv) were analysed for expression. Five mL O/N cultures of the antibody formats were carried out in SB carbenicillin media. These cultures were further subcultured into 20 mL SB carbenicillin media supplemented with 1x 5O5 medium. The cultures were propagated at 37°C while shaking at 200 rpm. All the cultures were induced at an O.D. ~ 0.8 (Abs @ 600 nm), with a 1 mM final concentration of IPTG followed by an O/N incubation at 30°C at 200 rpm. While expressing antibody formats, all the cultures were treated similarly and identical conditions for growth and expression were used. On the following day, the cells were centrifuged down at 3,220 x g for 30 minutes. The supernatant was decanted and the pellet was resuspended in 500 µL PBS. The cells suspension was transferred into a sterile 1.5 mL microfuge tubes. The cells in the suspension were lysed by alternating freeze-thaw cycles and the debris from the lysis was removed by centrifugation at 22,000 x g for 15 minutes. The supernatants containing the antibodies were transferred to a clean sterile 1.5 mL tubes.

The expression of the antibody in 8 different formats was checked by performing an indirect ELISA on the lysates containing the antibodies. The lysates were assayed in duplicate in three different plates coated with 1 µg/mL of cTnI. The assay was carried out using a range of different antibody dilutions (1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000) to accommodate all the antibody formats on one plate for comparison of expression. The dilutions of the antibody were made in 0.5% (w/v) BSA-milk marvel-PBST to reduce any background signal. HRP-conjugated-anti-His monoclonal antibody (1:2,000) was used for probing the antibody bound to the antigen at each dilution. His-tag was the only common tag in both the pMoPac and pComb vectors. Hence, an anti-His tag was ideal as secondary antibody in this assay. The ELISA was developed by

adding TMB substrate. The reaction was allowed to proceed for only 5 minutes due to rapid colour development. This was followed by quenching the reaction with 50  $\mu$ L of 10% (v/v) HCl. The signal was assumed to be a measure of good antibody expression.

The scFvs (with different domain orientations) were analysed separately for expression. The procedure followed was similar to the one described above except that a range of antibody dilutions were used, starting from 1:10 to 1:51,200 and HRP-conjugated-anti-HA monoclonal antibody (1:2,000) was used for probing the antigen-scFv complex.

#### **2.2.5.6 Purification analysis of anti-cTnI antibody in different formats**

The scFvs with  $V_L$ - $V_H$  orientation and  $V_H$ - $V_L$  orientation were expressed in 250 mL of SB carbenicillin media and purified by IMAC (section 2.2.1.20). The purified protein was buffer-exchanged and the efficiency of purification was analysed by SDS-PAGE (section 2.2.1.21).

#### **2.2.5.7 Site-directed mutagenesis of the MG4 scFv**

By using molecular biological techniques mutations can be induced at specific sites in the antibody. PCR is one such molecular tool which can be used for site-directed mutagenesis. Specific primers were designed for mutating the Cys 80 residue in the framework region of MG4 scFv for expression analysis. The Cys 80 was substituted with two amino acids Ala and Ser. Both the forward and the reverse primer contained the sequence of the amino acid (Ala/Ser) to be substituted in place of Cys along with an overlapping codon in the scFv sequence for proper priming and specific amplification of the product.

#### 2.2.5.7.1 Primers for site-directed mutagenesis of MG4 scFv

##### Primers for changing Cys 80 to Ser 80

Serine for 5 sense primer

5' GTG GAG AGT GAC GAT GCT GCC 3'

Serine rev 3 antisense primer

5' GTC ACT CTC CAC GCC GCT GAT G 3'

##### Primers for changing Cys 80 to Ala 80

Alanine for 5 sense primer

5' GTG GAG GCT GAC GAT GCT GCC 3'

Alanine rev3 antisense primer

5' GTC AGC CTC CAC GCC GCT GAT G 3'

#### 2.2.5.7.2 Site-directed mutagenesis of Cys 80 to Ser 80 and Ala 80 by PCR

PCR was carried out to replace Cys 80 with Ser 80 and Ala 80 by amplifying the scFv gene along with the whole plasmid. Phusion *Taq* is ideal for such amplifications due to a lower error rate. PCR was carried out using the MG4 scFv plasmid as template.

**Table 2.41: PCR mix for site-directed mutagenesis of anti-cTnI scFv.**

Components (V <sub>L</sub> -V <sub>H</sub> )	Conc. in 50 $\mu$ L volume
GC buffer (5x)	1x
Serine for/ Alanine for	50 pM
Serine rev/Alanine rev	50 pM
MG4 plasmid	10 ng
dNTP	0.2 mM
H <sub>2</sub> O	N/A
Phusion <i>Taq</i>	1 U

The PCR was carried out in Biometra T<sub>GRADIENT</sub> PCR machine under the following conditions:

Stage	Temperature (°C)	Time (seconds)	No. of cycles
1	98	360	1
2	98	30	30
	60	30	
	72	180	
3	72	600	1

The PCR products were analysed on a 0.8% agarose by gel electrophoresis. The plasmid amplified was gel-purified, digested with *Sfi*I and cloned into fresh pComb3XSS vector, to avoid any unwanted mutations during PCR amplification.

**Table 2.42: Mix for digestion of SOE-products obtained from site-directed mutagenesis of anti-cTnI scFv into pComb3XSS vector.**

Components	Conc. in 10 $\mu$ L volume	Components	Conc. in 40 $\mu$ L volume
10x buffer III	1x	10x buffer II	1x
pComb3XSSvector	2 $\mu$ g	Insert ( $V_L-V_H / V_H-V_L$ )	2 $\mu$ g
<i>Sfi</i> I enzyme	40 U	<i>Sfi</i> I enzyme	40 U
100x BSA	1x	100x BSA	1x
Water	N/A	Water	N/A

The reaction was incubated at 50°C O/N. The digested product was purified and cloned into the *Sfi*I digested vector by ligating the reaction O/N at 37°C. The composition of the ligation reaction is given below:

**Table 2.43: Ligation mix for cloning of SOE-products obtained from site-directed mutagenesis of anti-cTnI scFv into pComb3XSS vector.**

Components	Conc. in 10 $\mu$ L volume
10x Ligase buffer	1x
Digested pComb3XSS vector	100 ng
Digested scFv gene (mutated)	120 ng
T <sub>4</sub> DNA Ligase enzyme	400 U
Water	N/A

### **2.2.5.7.3 Transformation and screening of clones**

Transformation of the cloned gene was carried out into chemically competent TOP10F cells by heat shock method (section 2.2.2.4), followed by plating out the transformants on LB carbenicillin agar plates. Screening of the transformants was carried out by indirect ELISA (section 2.2.1.13), using cTnI as coating antigen and HRP-conjugated-anti-HA as detecting antibody. The confirmation of mutation was carried out by sequencing the clones with SOE primers for V<sub>L</sub>-V<sub>H</sub> domain orientation.

### **2.2.5.7.4 Expression of mutated scFvs with MG4**

The expression analysis was carried out (as in section 2.2.5.5) by growing and expressing 20 mL cultures under similar conditions. The amount of expression was deduced from the titre of antibody obtained at end point analysis by ELISA.

The lysates obtained were further purified using 1 mL Ni-NTA resin following a standard IMAC protocol (section 2.2.1.20). The concentration of purified protein was quantified on NanoDrop<sup>TM</sup> spectrophotometer ND-1000. The purified antibodies, mixed with 4x sample treatment buffer, were analysed for purification by SDS-PAGE (section 2.2.1.21) and Western blot analysis (section 2.2.1.22).

# **Chapter 3**

## **Generation of a Chicken Anti-hFABP scFv**

### 3.1 Introduction

#### 3.1.1 Chicken as a host

Chickens are widely used animals for development of specific antibodies against conserved mammalian proteins as they evoke a stronger immune response with low doses of mammalian antigens (Larsson *et al.*, 1998), due to the large phylogenetic difference between chickens and mammals (Gassmann *et al.*, 1990). In past 50 years, the chicken immune system was extensively studied in comparison to other avian species (Kothlow *et al.*, 2005), providing extensive fundamental information on antibody development.

A chicken contains single functional  $V_L J_L$  and  $V_H J_H$  genes resulting in limited diversity generated due to the rearrangement of these genes to produce heavy and light chains. This results in the chicken antibody repertoire being a derivative of single  $V_H$  and  $V_L$  germ line sequences (McCormack *et al.*, 1993). This makes the avian immune system better for simplified recombinant antibody library generation, as it utilises only a small set of primers for PCR amplification of chicken V genes (van Wyngaardt *et al.*, 2004). Consequently, this saves time and resources (Andris-Widhopf *et al.*, 2000; Yamanaka *et al.*, 1996), and also reduces the loss of rare transcripts due to differences in primer efficiencies (Barbas *et al.*, 2001).

Avian immunoglobulin development consists of three phases: pre-bursal, bursal and post-bursal phases (Sayegh *et al.*, 2000). In the prebursal stage the antibody precursors are produced in bone marrow and rearrangement of both Ig heavy and light chain variable genes takes place in a non-sequential order. Pre-bursal B-cells with rearranged heavy and light chain enter the Bursa of Fabricius. The Bursa of Fabricius is an epithelial and lymphoid organ in birds with a major role in hematopoiesis. The Bursa-



derived B-cells undergo massive diversification by gene conversion followed by selection of B-cells. The complete process of B-cell selection is unclear. Approximately 95% of the B-cells are deleted by apoptosis allowing only 5% of the B-cells to move to the peripheral lymphoid organs where they encounter antigens (Kohonen *et al.*, 2007). The migration of B-cells from the Bursa of Fabricius may be attributed to the changes in surface glycosylation of the B-cell at different stages of B-cell development (Masteller *et al.*, 1995; Masteller *et al.*, 1997).

Then Bursa of Fabricius plays an important role in the generation of an avian immunocompetent antibody repertoire. A highly diverse chicken antibody repertoire is generated primarily by a somatic gene conversion process (despite only one functional  $V_L$  and  $V_H$  genes). This process involves exchange of  $V_L$  and  $V_H$  sequences of the functional gene sequences with that of pseudogene sequences, generating enormous diversity. However, evidence shows that processes such as somatic hypermutation are also involved in the generation of antibody diversity in avian species (Arakawa *et al.*, 2002).

Three classes of immunoglobulins are found in chicken: IgA, IgM, and IgY (Sayegh *et al.*, 2000) which are distinguishable in concentration, structure, and immunochemical function. The majority of antibody belongs to the IgY class representing 75% of the immunoglobulin population (Chalghoumi *et al.*, 2009) which corresponds to mammalian IgG in structure and function (Warr *et al.*, 1995).

The antibodies produced in chickens have certain advantages to those produced in mammalian hosts. The first advantage is that chickens are very economical to use due to low rearing and maintenance costs. Egg-laying hens can provide high yields of antibody

as compared to mammals (Schade *et al.*, 1994). The phylogenetic distance also ensures that mammalian IgG do not cross-react with chicken IgY (Hadge and Ambrosius, 1984). Thus, the problems of interference in immunological assays for detecting mammalian antigens are reduced by use of IgY (Larsson and Mellstedt, 1992). In addition, IgY antibodies do not bind to bacterial Fc receptors, such as staphylococcal protein-A or streptococcal protein-G and do not activate the mammalian complement system (Schade *et al.*, 1996; Zhang, 2003). IgY is known to be more stable over a wider pH and temperature range, and can be stored for longer periods in suitable buffers (Olovsson and Larsson, 1993; Shimizu *et al.*, 1992; Shimizu *et al.*, 1994; Zhang, 2003). Chickens are also reported to be suitable hosts for immunisation with multiple antigens (Hof *et al.*, 2008).

Chicken provides a versatile source of antibodies for both diagnostics and research (van Wyngaardt *et al.*, 2004). Numerous high-affinity chicken scFvs have been successfully used for diagnostic applications (Fehrsen *et al.*, 2005; Park *et al.*, 2005; Finlay *et al.*, 2006).

### **3.1.2 Heart-fatty acid binding protein as an analyte**

Cardiac fatty acid binding protein is a cytosolic protein that plays an important role in maintaining myocardial lipid homeostasis (Fournier and Richard, 1990). This protein belongs to a family of intracellular fatty-acid binding proteins exhibiting high affinity for small lipophilic ligands, along with eight other mammalian isoforms (Storch and Corsico, 2008; Wang *et al.*, 2008). FABP family members have highly conserved three dimensional structures and 22-70% amino acid sequence similarity. Each isoform differs from the other isoform in terms of tissue specificity, tissue content, and isoelectric point (Glatz and van der Vusse, 1996; Storch and Corsico, 2008).

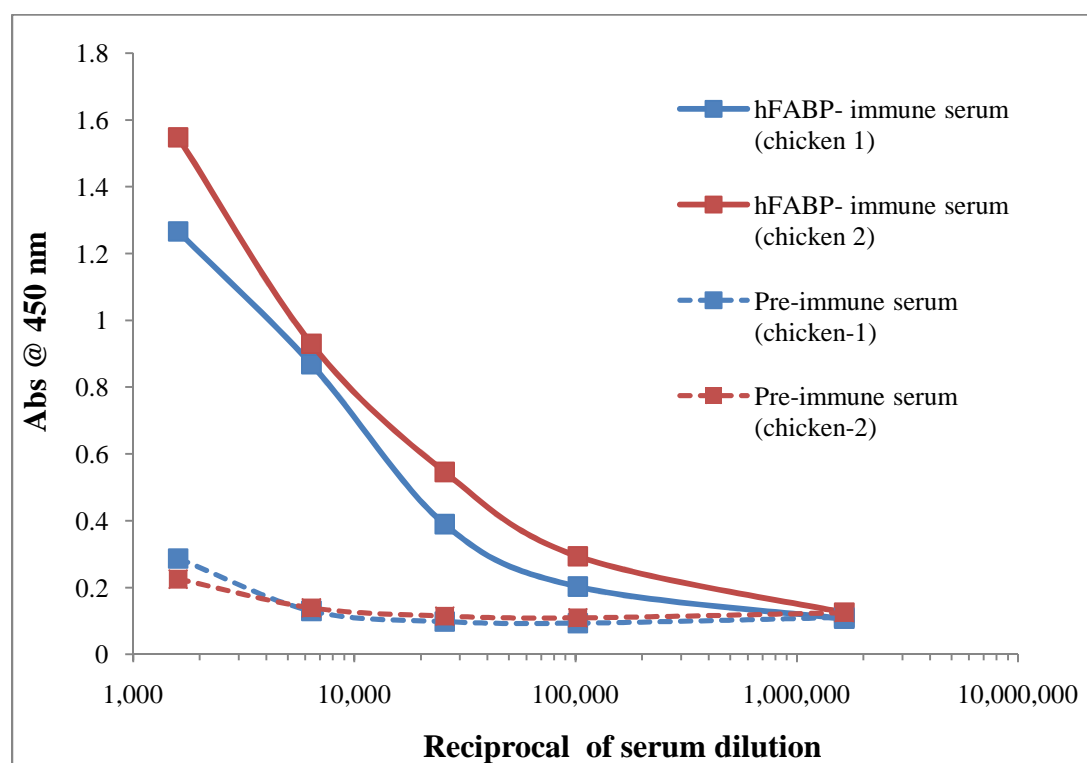
HFABP is a small, hydrophilic, low molecular mass (15 kDA), slightly acidic protein (pI 5.3) (Nielsen *et al.*, 1990) with a high concentration in the myocardium but is found to a lesser extent in skeletal muscles. HFABP concentration in the heart is 0.57 mg/g which is approximately 50 times more than in skeletal tissue (Cavus *et al.*, 2006). Its confinement to the cytoplasm, in high concentrations, is responsible for elevated release of hFABP in response to minor myocardial injury which makes it a suitable early marker (Pelsers *et al.*, 2005).

The current study exploits the chicken immune system for the generation and characterisation of specific antibodies to hFABP.

### **3.2 Construction of Chicken anti-hFABP scFv Library and Biopanning for Selection of Specific Clones**

A panned chicken anti-hFABP library was provided by Dr. Stephen Hearty (Applied Biochemistry Group, School of Biotechnology, Dublin City University, Ireland). Details on the immunisation of chicken and antibody library construction were provided in the section 2.2.1.1.

The serum antibody titres of chickens, immunised with hFABP, were greater than 1:300,000, making them ideal candidates for the construction of an antibody library. The hFABP immune serum from a chicken along with the pre-immune serum was assayed by ELISA (section 2.2.1.1) to estimate the specific immune response against hFABP (Figure 3.1).

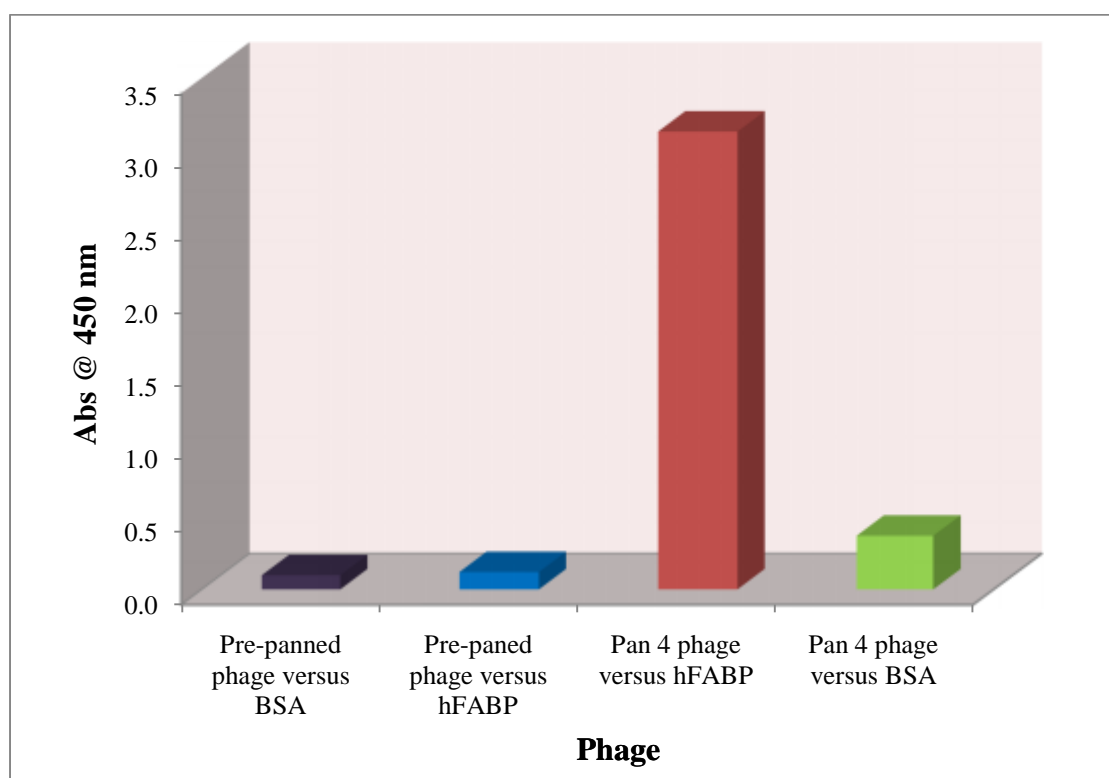


**Figure 3.1: Titres of chicken immune sera against hFABP.**

*Solid lines represent the immune serum whereas the dotted line represents the pre-immune serum. A serum titre of 1:300,000 against hFABP was obtained.*

### 3.3 Screening of hFABP-Specific Clones

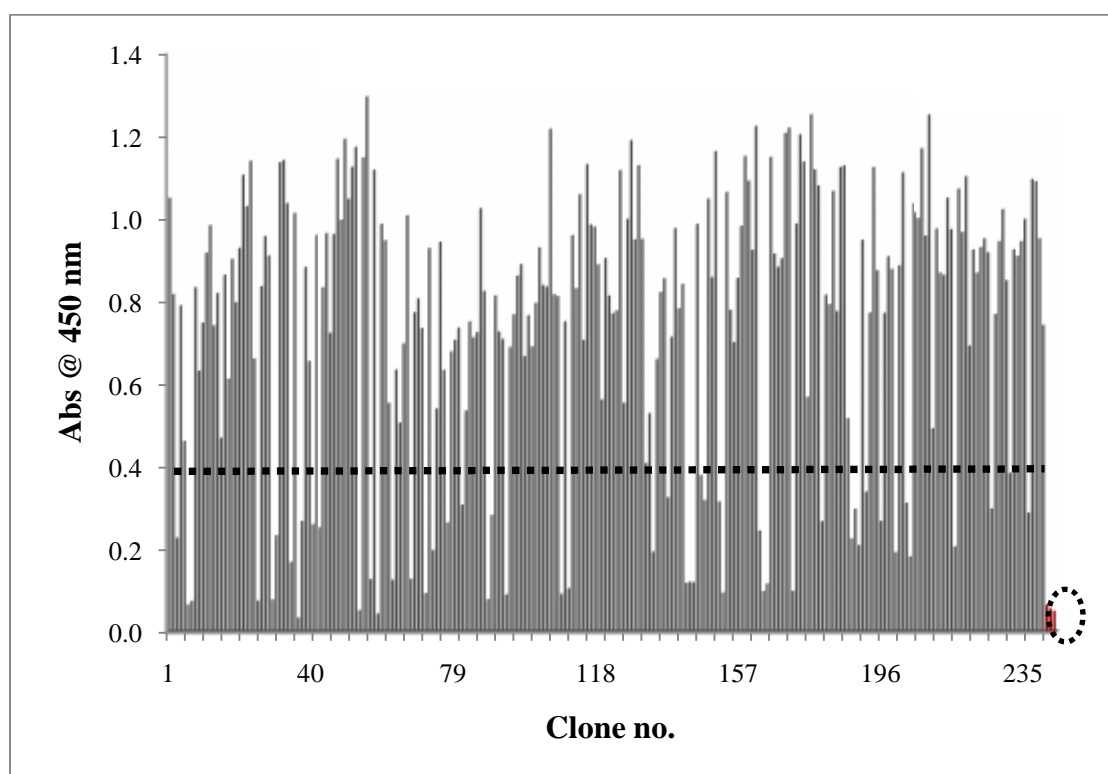
Phage outputs from prepan and round 4 of biopanning were checked by indirect ELISA against hFABP and the blocking solution (BSA) (Figure 3.2). The phage was diluted 1:5 in 0.5% (w/v) BSA-milk marvel in PBS to avoid non-specific binding. The scFvs displaying phage, specific to hFABP, were detected using a HRP-conjugated mouse anti-M13 antibody. The ELISA was developed with TMB substrate after 20 minutes of incubation and the absorbance was read at 450 nm.



**Figure 3.2: Polyclonal phage ELISA of a chicken anti-hFABP library**

*There is a strong specific signal in round four phage outputs, compared to the pre-panned phage, showing a significant enrichment of hFABP-specific scFvs. There is also a slight increase in signal against BSA.*

Round 4 outputs were solubly expressed in TOP10F' cells and plated on SB-carbenicillin plates to isolate single colonies. Two hundred and forty colonies were picked, grown and expressed O/N. Solubly expressed monoclonal scFvs were screened against hFABP by indirect ELISA. A HRP-conjugated-anti-HA antibody was used as secondary antibody. Approximately 90% of the clones screened were identified to be positive to hFABP by ELISA (Figure 3.3). A reference level was set (twice the O.D. obtained against the negative controls), above which 80 clones were selected for further analyses based on their high absorbance values.

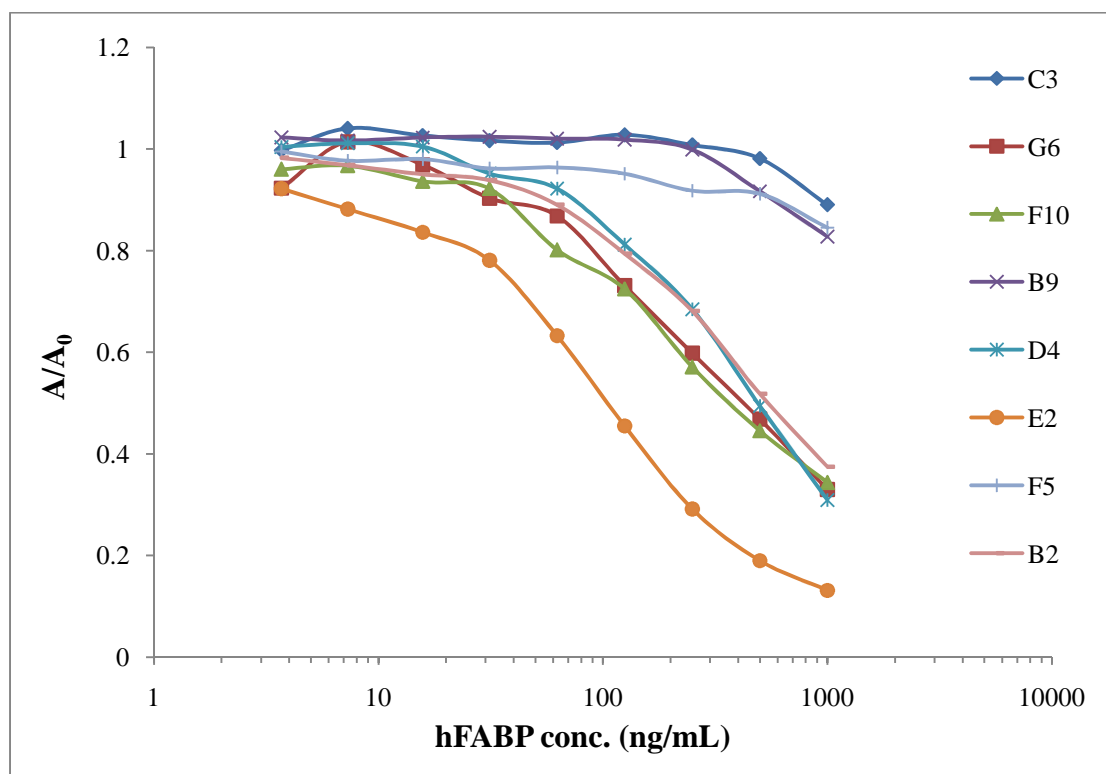


**Figure 3.3: Monoclonal ELISA of solubly expressed scFvs against hFABP**

*A high percentage of clones were selected from the screening of the panned hFABP library. The broken line indicates the reference limit above which the clones were selected for subsequent ranking analysis. A total of 80 clones were selected for ranking analysis. Dotted circle indicates reagent controls used in screening*

An inhibition ELISA was performed for eight positive clones selected randomly, in order to check their binding against free hFABP in solution. For the inhibition ELISA the clones were titred against hFABP using lysates diluted serially ranging from 1:2 to 1:1024. The midpoint of the titration curve was identified for each clone from the linear region of the titration curve. Each clone was diluted appropriately, depending on the results from titration ELISA, and mixed with different concentrations of free hFABP in 0.5% (w/v) BSA-milk-PBS. The mixtures were incubated for 30 minutes at 37°C and added to an ELISA plate coated with 0.5 µg/mL of hFABP. For each antibody, an  $A_0$  control was used, where  $A_0$  is the absorbance of final antibody dilution used in the assay without antigen inhibition. The absorbance value obtained for each dilution was divided

by the  $A_0$  absorbance value to get the degree of inhibition. The ELISA analysis showed varying degrees of binding indicating different  $IC_{50}$  values as shown in Figure 3.4.



**Figure 3.4: Inhibition ELISA of anti-hFABP scFvs.**

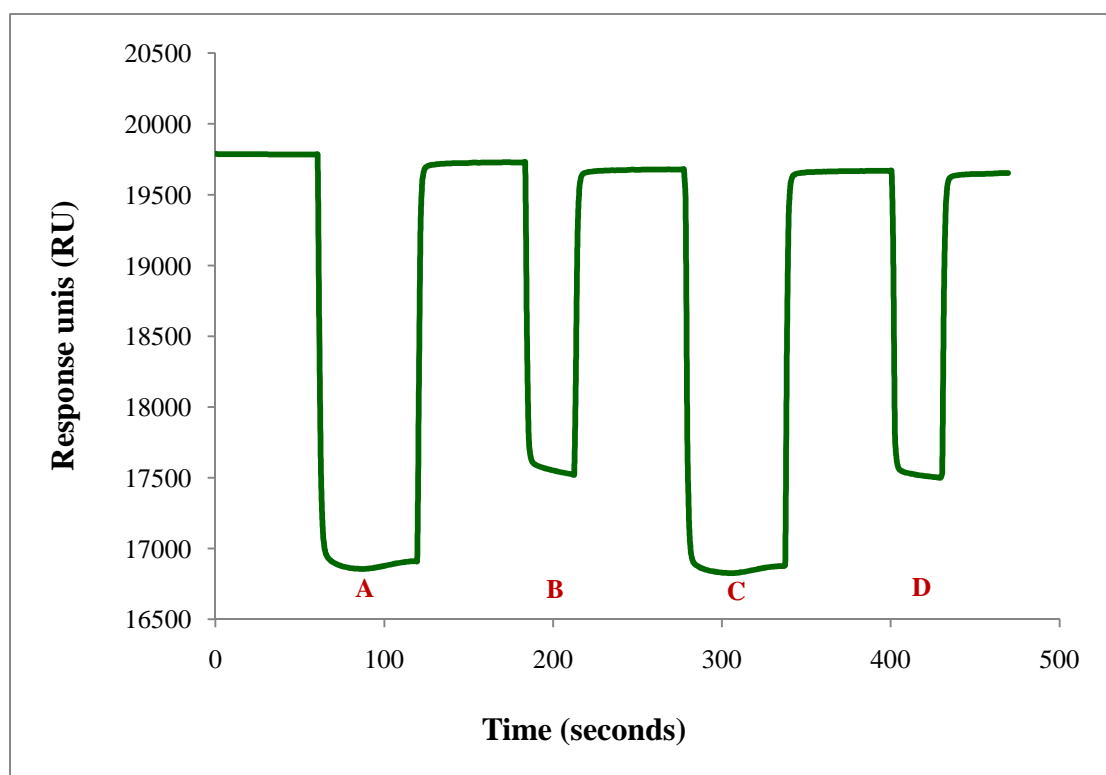
*This ELISA was carried out using midpoint values from the linear range of titration curves of each antibody against different concentrations of free hFABP antigen (1,000, 500, 250, 125, 62.5, 31.25, 15.75, 7.3, and 3.7 ng/mL). The antigen-antibody mixture was assayed against bound hFABP. The absorbance values obtained for the antibody inhibited against different antigen concentrations (A) were normalised against the absorbance of the antibody without any inhibition ( $A_0$ ). This was carried out by dividing the A value by the  $A_0$  value and plotting it against the antigen concentration. The clones analysed were all competitive against free hFABP in solution.*

### 3.4 Ranking of Clones by Biacore 3000

#### 3.4.1 Preparation of chip for ranking analysis of anti-hFABP scFvs

The Biacore instrument was cleaned of any impurities in the flow channel by running a standard desorb program. A CM5-dextran chip was used for this study. The dextran

layer on the chip surface was preconditioned prior to immobilisation. Figure 3.5 shows an ideal preconditioning sensorgram using 0.5% (v/v) SDS and 50 mM NaOH.



**Figure 3.5: Preconditioning of the CM5 dextran chip surface.**

*Alternating cycles of SDS and NaOH solutions were used for hydrating and cleansing the chip surface before immobilisation of the antibody.*

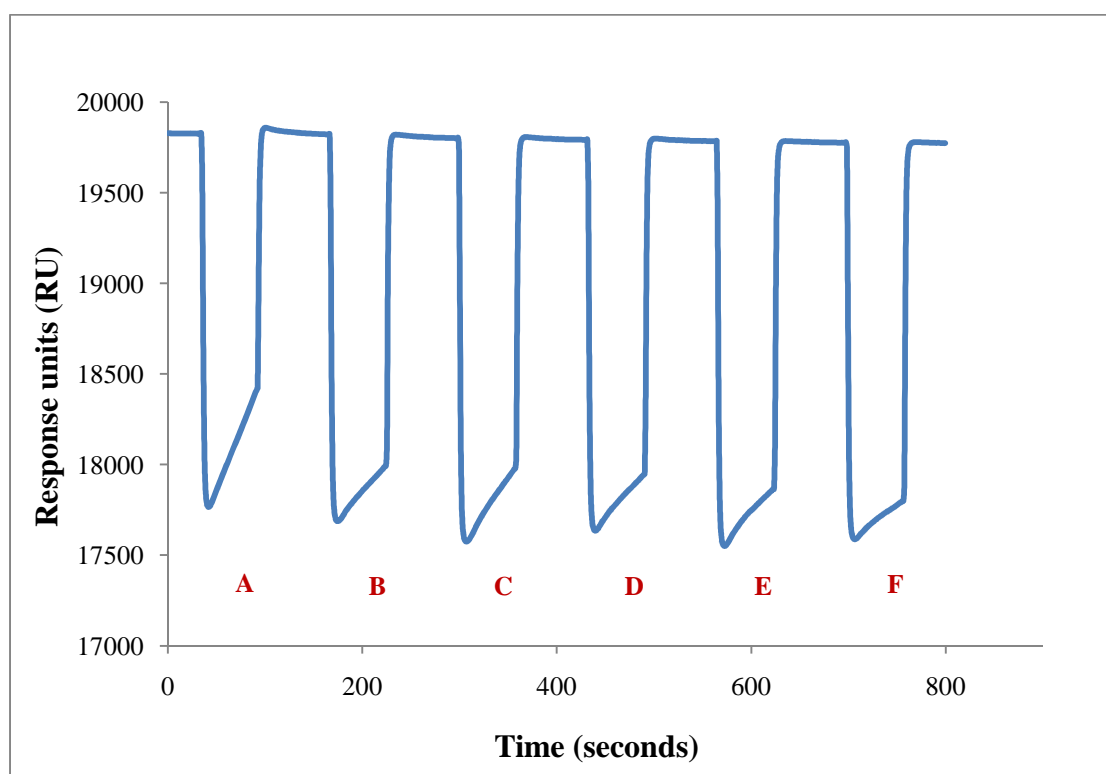
*A & C: 0.05% (w/v) SDS; B & D: 50 mM NaOH*

In ranking analysis, the scFvs are selected based on their ‘off-rates’. This involved immobilising the scFvs on the chip surface and passing the analyte over them to study the biomolecular interaction. Direct immobilisation of the scFv on the chip surface can result in several issues such as inactivation of the scFv, hindering antigen-antibody interaction due to inaccessibility of scFv paratope, and problems associated with the reproducibility of chip surface for analyses of multiple scFv clones etc. In order to circumvent these problems associated with the direct immobilisation of scFv, an alternate approach of indirectly immobilising the antibodies was used. It involved capturing the scFvs on the chip surface by an antibody that acted as a primary binder.



These capture antibodies can be chosen depending on the species in which the scFv is generated or based on the tag, expressed along with the scFv. It is an efficient method that ensures better orientation of the scFv to facilitate antigen binding. Such an approach allows the analysis of multiple scFv clones in the same program without the need for immobilising each clone individually one at-a-time thus making the chip reproducible for several analyses.

Anti-HA antibody was used as the standard capture antibody for the scFvs expressed using the pComb3X phagemid vector system, containing the HA tag. To achieve the maximum immobilisation of the capture antibody preconcentration analysis was performed with the antibody dissolved in sodium acetate buffer at different pHs (section 2.2.1.15). At pHs lower than the isoelectric point (PI), the protein attains a net positive charge resulting in the binding of protein to the negatively charged dextran matrix. The pH at which maximum binding is observed is selected for immobilising the protein (Figure 3.6).



**Figure 3.6: Preconcentration analysis for anti-HA immobilisation.**

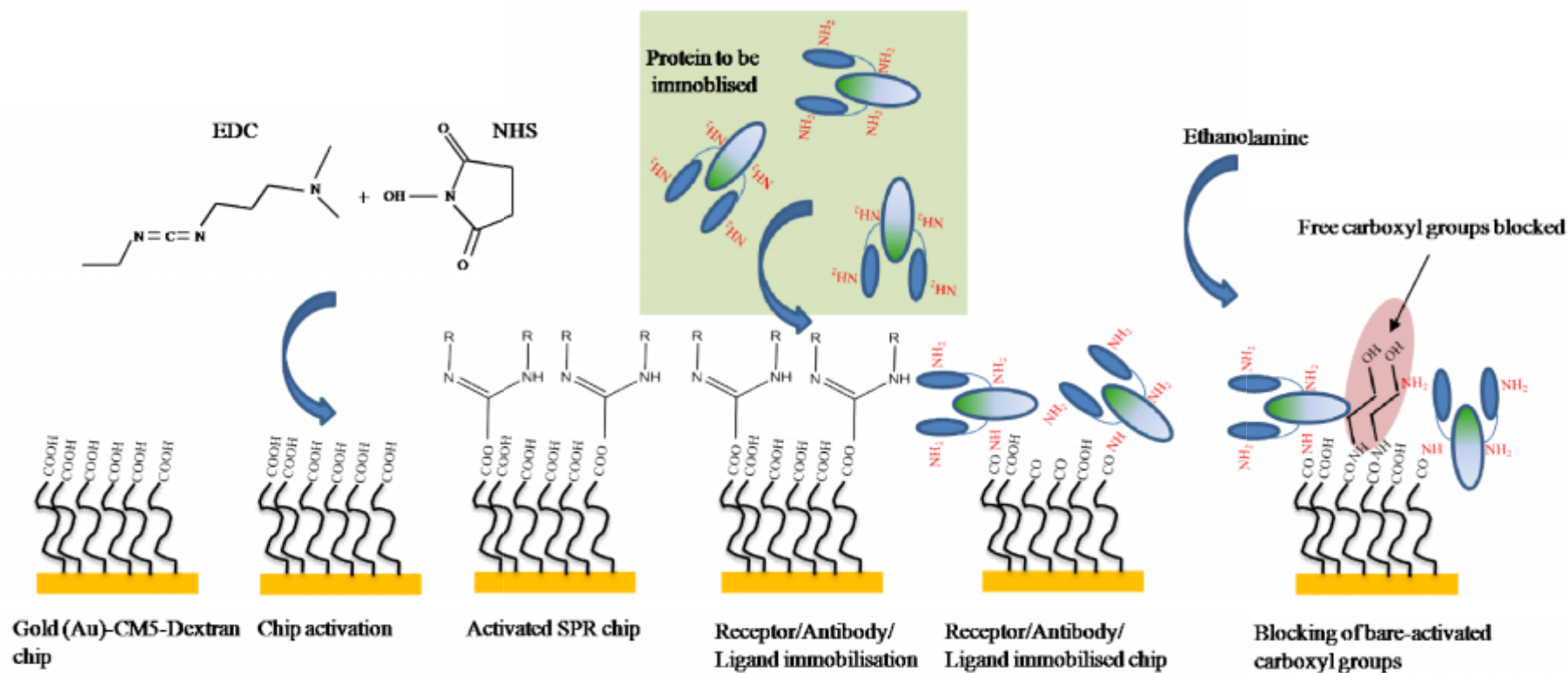
*This figure shows a typical preconcentration sensorgram obtained on analysing anti-HA antibody in 10 mM sodium acetate buffer with different pHs between 4.0-5.0. Five  $\mu\text{g/mL}$  of commercial anti-HA antibody was used for this analysis, which is very low concentration of the antibody to bind the surface, resulting in inverse sensorgram peaks. The sensorgram below the baseline is due to the difference in the refractive index of the running (HBS) and the sample buffer (sodium acetate) used for the analysis. Maximum binding was observed at pH 4.0 making this pH optimal for immobilisation of the antibody on the chip.*

*A: 10 mM sodium acetate (pH 4.0); B: 10 mM sodium acetate (pH 4.2); C: 10 mM sodium acetate (pH 4.4); D: 10 mM sodium acetate (pH 4.6); E: 10 mM sodium acetate (pH 4.8) and F: 10 mM sodium acetate (pH 5.0).*

### **3.4.2 Immobilisation of capture antibody**

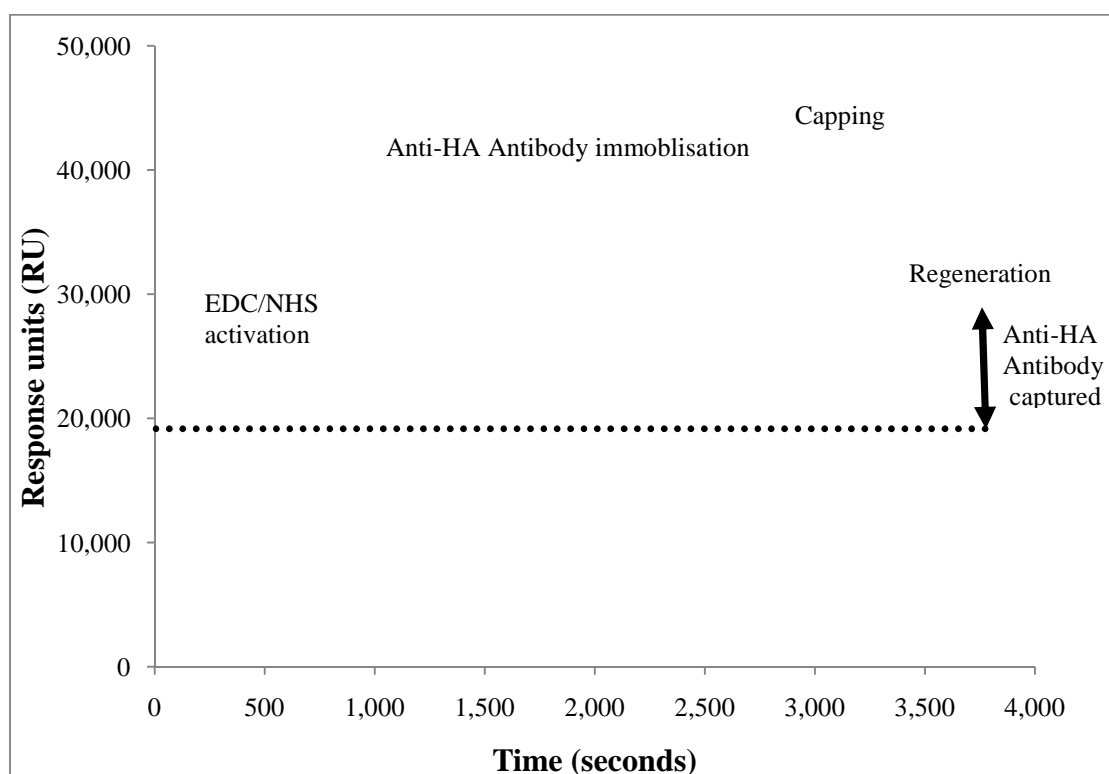
The surface chemistry of the CM5 dextran hydrogel on the gold surface of the sensor chip provides several possible approaches to couple the protein covalently to the surface. Amine coupling is the most commonly used method of coupling in which protein is covalently bound to the surface *via* primary amine groups. In our studies anti-

HA antibody was used as capture antibody for high throughput screening of the scFvs. To activate the surface, equal volumes of 400 mM EDC (N-ethyl-N'-(dimethylaminopropyl) carbodiimide hydrochloride) and 100 mM NHS (N-hydroxysuccinimide) were mixed and passed over the dextran surface. Approximately 40% of surface is activated on passing EDC and NHS over the surface of the chip. Anti-HA antibody was passed over the activated surface at a concentration of 50  $\mu\text{g/mL}$  (150 $\mu\text{L}$ ) in acetate buffer with the pH lower than the pI value. Due to the low pH of the buffer, the antibody becomes positively charged and becomes attached to the negatively charged surface *via* amine bond formation. Any free reactive amine group on the surface were capped using 1 M ethanolamine hydrochloride. Antibody loosely bound to the charged surface was removed by regenerating the surface with 20 mM NaOH. This provides a stable baseline value for further analysis. Figure 3.7 represents the changes in surface chemistry during immobilisation. A typical sensorgram obtained during an immobilisation procedure is shown in Figure 3.8.



**Figure 3.7: Surface modification and immobilisation on CM5 dextran chip.**

EDC causes a dehydration reaction between the carboxyl group of CM5 dextran chip and the NHS hydroxyl group, resulting in the formation of NHS-ester-activated molecule. This activated molecule is highly reactive to any primary amine group-containing molecule. Anti-HA antibody displaces the NHS with the amine groups present in the protein forming an amide bond. Any activated carboxyl group not engaged in covalent coupling to anti-HA antibody is blocked by ethanolamine, which provides primary amine groups for amide bond formation.



**Figure 3.8: Immobilisation of anti-HA antibody on a CM5 dextran surface activated by EDC/NHS.**

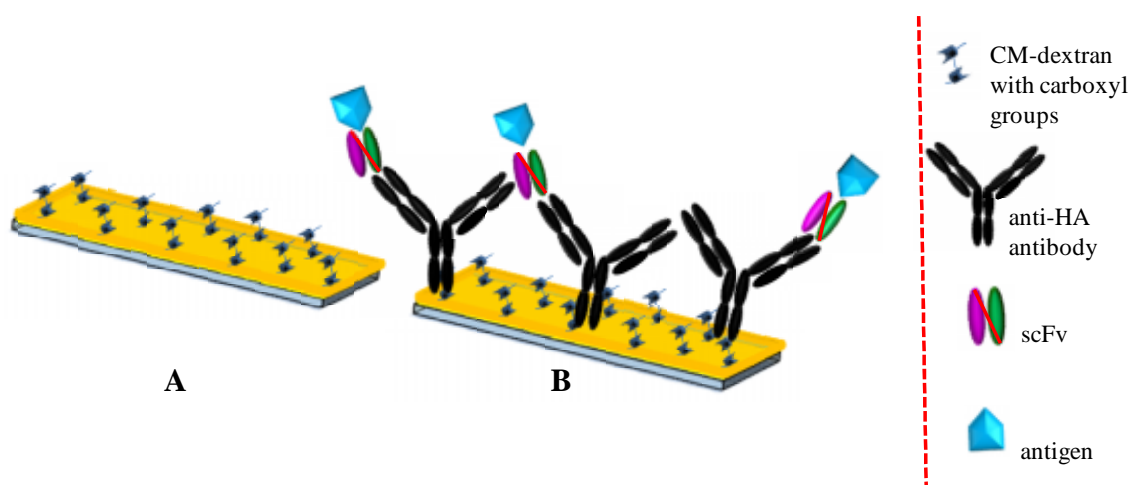
The dotted line indicates the baseline. The solid line indicates the sensorgram reflecting the change in the mass on the surface of the chip, while passing over different solutions. By calculating the difference in mass on the chip (after regeneration) from the baseline, the amount of anti-HA antibody captured on the surface can be calculated. About 9554.3 RU ( $\sim 9.55 \text{ ng/mm}^2$ ) of anti-HA antibody was captured by passing 100  $\mu\text{L}$  of antibody at a concentration of 50  $\mu\text{g/mL}$  over the chip surface.

### 3.4.3 ‘Off-rate’ analysis of clones

The scFvs were primarily characterised based on their ‘off-rates’, by calculating antibody/antigen stability, using an antibody capture assay (Figure 3.9). Such a method enables a quick ranking of multiple clones based on their  $K_{\text{off}}$ , independent of their concentration. In this assay an antibody against HA-tag was covalently immobilised on the CM5 chip surface and crude bacterial lysates with 12 mg/mL CM dextran-BSA (section 2.2.1.17) were passed over the surface, specifically capturing HA-tagged scFvs. The analyte, hFABP, was passed over the captured scFvs at a constant concentration (30

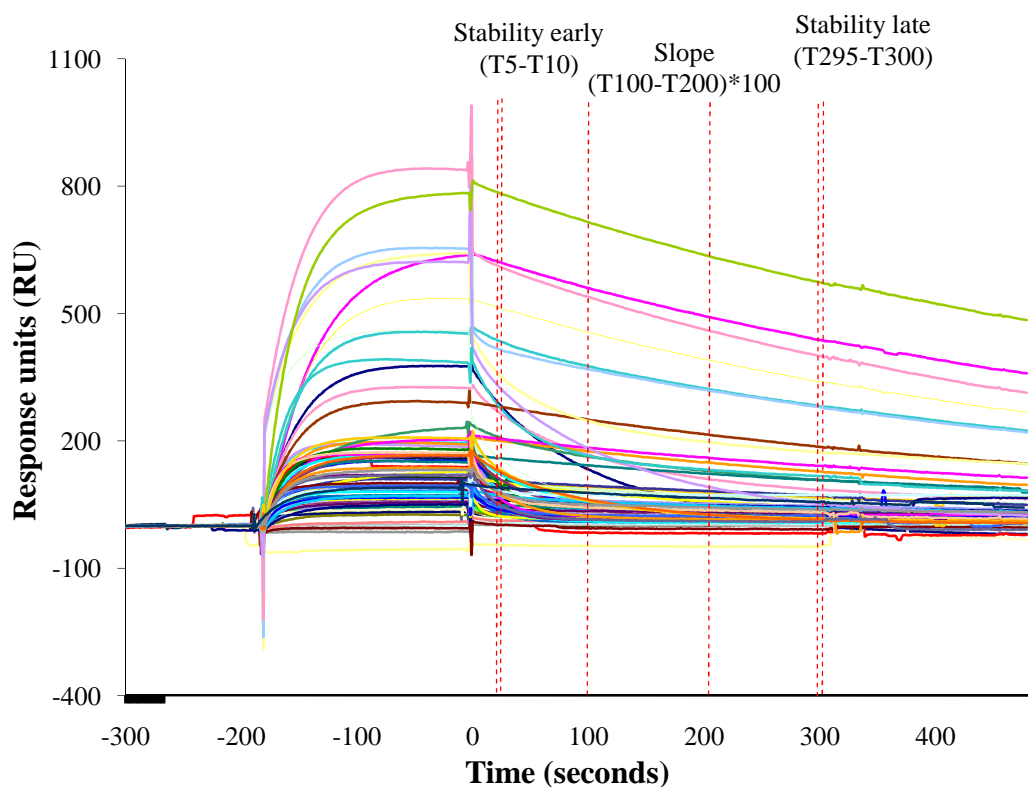
nM). Any systematic artefacts were removed by double referencing the data obtained for each antibody, against reference flow cell and the buffer response (zero analyte concentration). The sensorgrams were analysed on BIAevaluation 4.1 software and the data was exported to an excel sheet for evaluation.

The slope of the sensorgram for each individual clone along with stability early, late and percentage left were calculated. Each slope was calculated for a 100 second period, 100 seconds after the initiation of hFABP dissociation, by calculating the difference in the response units (RU) and multiplying it by 100. The antibody/antigen complex stability (% left) was calculated by expressing the ‘stability late’ (calculated from the average response from 295 to 300 seconds after buffer injection) as a percentage of ‘stability early’ (calculated from the average response from the first 5 seconds after buffer injection).



**Figure: 3.9: The format used for the ‘off-rate’ analysis of the clone.**

**A** represents the reference cell surface and **B** is the flow cell where the anti-HA antibody is immobilised. The scFvs captured on the anti-HA immobilised surface in turn bind the antigen, when it is passed over the surface.



**Figure 3.10: Ranking of anti-hFABP scFv.**

Sensorgrams of 80 clones were overlayed in BIAevaluation 4.1 software. Each sensorgram represents the binding of hFABP to a captured scFv. The dotted line indicates the report points used for calculating the slope along with stability early and stability late which was further used for the calculation of percentage stability.

The sensorgrams obtained for each of the 80 scFvs were overlayed (Figure 3.10) and the capture levels of these clones on the anti-HA surface was checked. Twenty five scFvs with a % left value greater than 60% were selected (Table 3.1). Due to an identical % left value exhibited by many scFvs, fingerprinting analysis was carried out to determine the diversity of the selected clones.

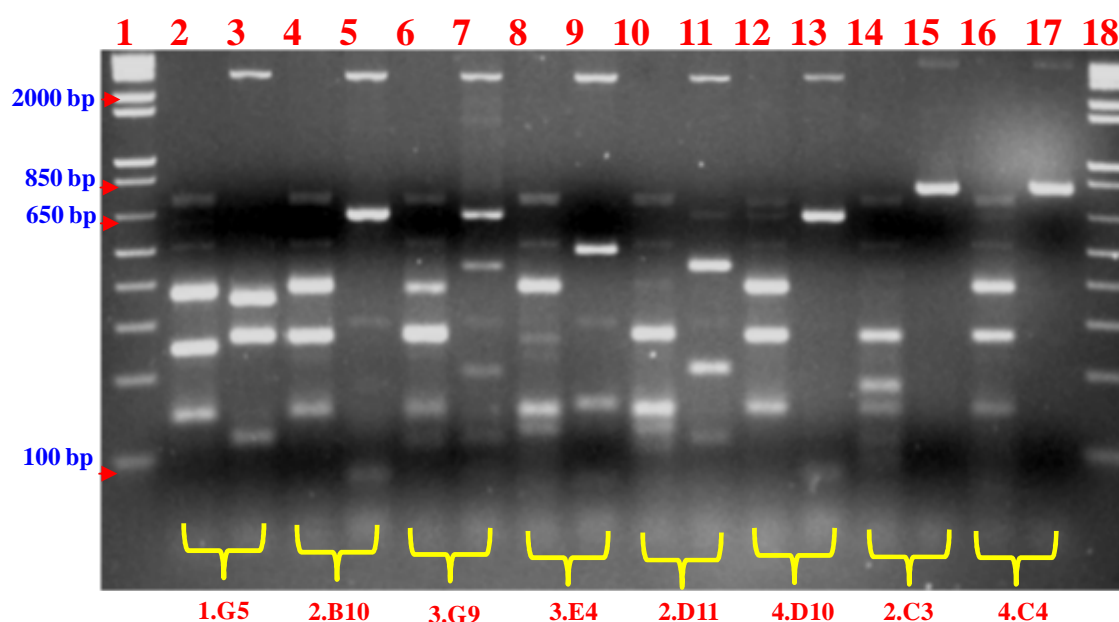
**Table 3.1: Percentage stability calculated for each clone.**

Clone	% Retained	Clone	% Retained	Clone	% Retained
1.B2	43	4.B5	63	2.C6	63
1.F10	65	2.C3	79	2.D11	60
1.E2	64	4.C4	63	2.G4	22
1.D4	14	4.C5	6	4.G9	10
1.F11	27	4.C8	24	4.G6	62
1.G2	34	4.C9	*NM	3.B5	*NM
1.G3	34	4.C11	7	3.B8	97
1.G5	72	4.D2	48	3.B10	70
1.G6	10	4.D5	3	3.C10	10
1.G8	6	4.D6	1	3.D6	62.19
2.B7	70	4.D8	65	3.D9	63
2.B10	65	4.D9	40	3.D11	64
2.D7	5	4.D10	66	3.E4.	64
2.D8	44	4.D11	18	3.E9	15
2.D11	20	4.B7	4	3.E10	36
2.E7	15	4.B11	*NM	4.B6	71
2.E8	24	4.D7	16	4.C3	18
2.F3	30	4.E2	31	3.F2	40
2.F6	39	4.E5	40	3.F6	11
2.G2	111	4.E6	68	3.F10	16
2.G6	48	4.E8	15	3.F11	*NM
2.G7	28	4.E10	39	3.G4	66
2.G8	7	4.F2	7	3.G7	36
2.G11	39	4.F6	9	3.G8	53
4.B2	12	4.F10	10	3.G9	79
4.B3	63	4.G3	35		
4.B4	50	4.G8	60		

\*NM: Not measurable

Restriction mapping of clones was carried by amplifying the scFvs using overlap primers and digesting the PCR products with two different enzymes, *Bst*NI and *Alu*I. *Bst*NI recognises CC/WGG and *Alu*I recognizes AG/CT both of which occur commonly in the antibody sequence. The resulting pattern following the digestion with these enzymes, gives a good indication of antibody diversity.





**Figure 3.11: Restriction mapping analysis of anti-hFABP scFvs.**

Lane 1 and 18: 1 kb DNA ladder plus; even lanes (2, 4, 6, 8, 10, 12, 14 and 16): *AluI* digested scFvs and odd lanes (3, 5, 7, 9, 11, 13, 15 and 17): *BstNI* digested scFvs.

Clones exhibiting similar DNA fingerprints were grouped into categories. Seven different categories of scFvs were obtained (Figure 3.11), showing a diverse panel of high affinity antibodies as indicated by ranking analysis. The clones of the same group were analysed by comparing different parameters from the ‘off-rate’ data, in order to check if they are similar clones (Table 3.2). The diverse panels of selected antibodies were subjected to kinetic analysis based on their association and dissociation behavior against the hFABP antigen.

**Table 3.2: Grouping of antibodies in different profiles based on fingerprinting analysis.**

Clone no.	Capture level	R-early (T5-10s)	R-late (T290-295s)	% Retained	
4.D8	798.10	287.54	188.20	65	Fingerprint profile 1
2.B10	1432.70	526.30	342.73	65	
3.G9	577.10	92.25	72.43	79	
4.D10	309.30	108.99	71.91	66	
3.G4	428.70	162.95	108.26	66	
4.G8	281.90	93.27	56.32	60	
3.D6	931.30	19.32	12.01	62	
4.B6	988.00	62.24	43.92	71	
1.E2		631.90	404.28	64	
1.G5	2022.30	799.95	577.58	72	Fingerprint profile 2
2.B7	1779.90	634.43	441.31	70	
4.E6	643.50	210.00	142.17	68	
3.B10	227.90	66.99	46.73	70	
4.G6	188.70	50.14	31.21	62	
3.D9	824.30	7.69	4.88	63	
2.C3	1386.50	27.34	22.26	81	Fingerprint profile 3
4.C4	540.20	204.98	129.01	63	
4.B3	1326.20	455.19	284.83	63	
4.B5	315.50	107.45	67.56	63	
					Fingerprint profile 4
3.E4	598.00	15.16	9.77	64	
3.B8	851.60	7.18	6.98	97	
					Fingerprint profile 5
2.C6	857.80	20.69	13.10	63	
3.D11	637.70	46.36	29.58	64	Fingerprint profile 6
2.D11	516.70	113.11	68.22	60	Fingerprint profile 7
1.F10	879.90	436.38	281.94	65	

#### 3.4.4 Kinetic analysis of scFvs

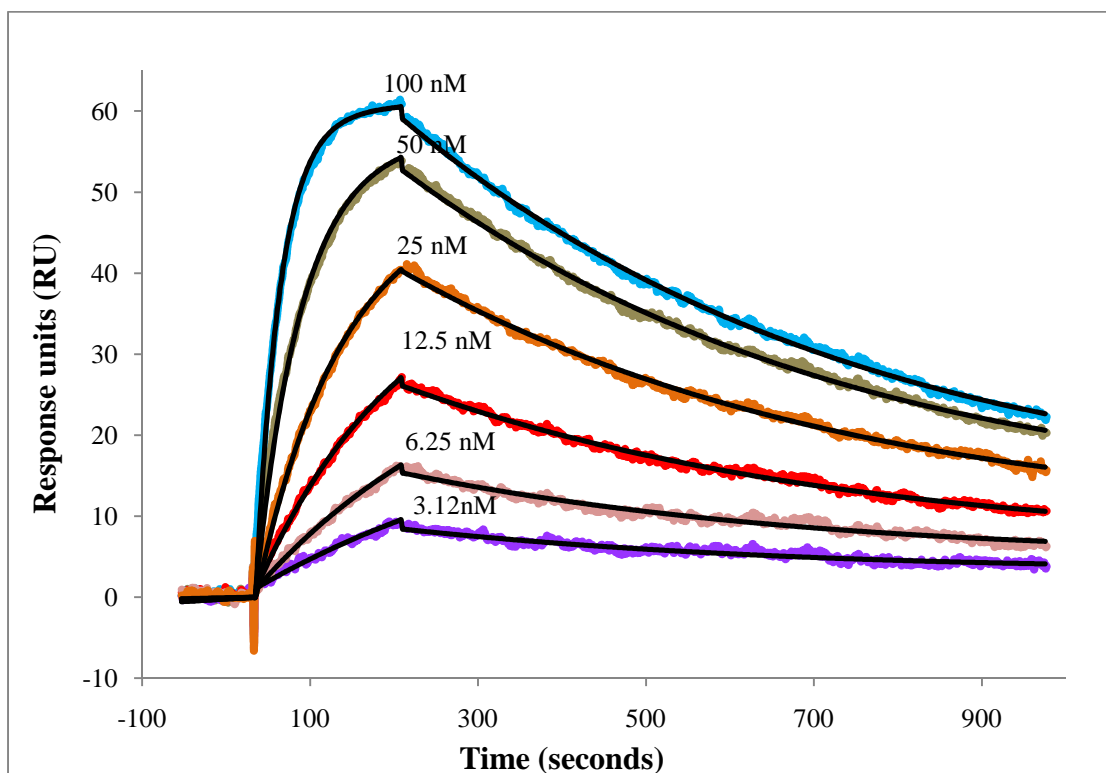
Kinetic analysis was performed with different concentrations of hFABP, diluted in HBS. Initially, the scFv was manually analysed on Biacore to estimate appropriate dilution for capturing 100-200 RU of scFv, in order to get clean and reliable data on

association and dissociation rates. Each scFv was checked manually before running the program, as the optimum dilution to be used varied depending on scFv expression levels. A kinetic wizard was set up to calculate the affinity of the individual scFv clones. The hFABP samples were injected over the captured scFv surfaces for 3 minutes and the dissociation phase monitored for 12 minutes. The dataset was evaluated using BIAevaluation 4.1 software. Data obtained was double referenced by subtracting the buffer response (zero analyte concentration) and the response from reference flow channel for each antibody tested. The affinities of the clones were determined by fitting the curves in BIAevaluation 4.1 software (Figure 3.12). The fitting of curves determines the  $\text{Chi}^2$ , which should be as low as possible indicating the quality of the data.

Table 3.3 shows the association and dissociation rates of all the selected clones along with their affinities. Three clones (marked \*) with low nanomolar affinity were selected for further purification and specificity studies.

**Table 3.3: *Biacore data on different affinity parameters of selected clones.***

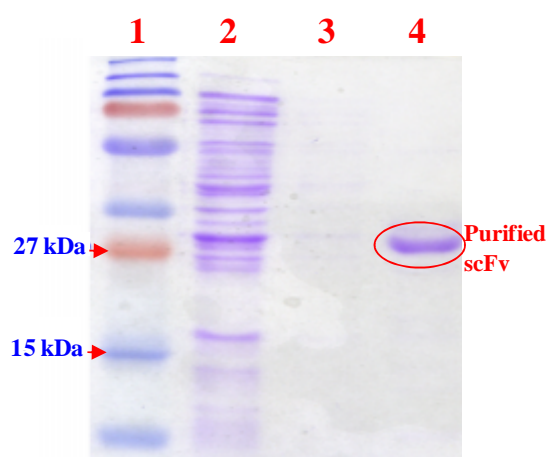
Clone no.	$k_a=(M^{-1}s^{-1})$	$k_d=(s^{-1})$	$K_A(1/M)$	$K_D(M)$	
4.D8	$3.63 \times 10^5$	$1.53 \times 10^{-3}$	$2.33 \times 10^8$	$4.3 \times 10^{-9}$	Fingerprint profile 1
*2.B10	$3.9 \times 10^5$	$9.58 \times 10^{-3}$	$4.07 \times 10^8$	$2.46 \times 10^{-9}$	
*3.G9	$5.46 \times 10^5$	$4.57 \times 10^{-4}$	$1.29 \times 10^9$	$8.36 \times 10^{-10}$	
4.D10	$3.05 \times 10^5$	$1.35 \times 10^{-3}$	$2.25 \times 10^8$	$4.44 \times 10^{-9}$	
3.G4	$3.74 \times 10^5$	$1.41 \times 10^{-3}$	$2.64 \times 10^8$	$3.78 \times 10^{-9}$	
4.G8	$4.48 \times 10^5$	$1.59 \times 10^{-3}$	$2.82 \times 10^8$	$3.58 \times 10^{-9}$	
3.D6	Unreliable data				
4.B6	$3 \times 10^5$	$1.37 \times 10^{-3}$	$2.35 \times 10^8$	$4.25 \times 10^{-9}$	Fingerprint profile 2
1.E2	$3.12 \times 10^5$	$1.42 \times 10^{-3}$	$2.19 \times 10^8$	$4.56 \times 10^{-9}$	
1.G5	$3.72 \times 10^5$	$1.07 \times 10^{-3}$	$2.95 \times 10^8$	$3.43 \times 10^{-9}$	
2.B7	$2.66 \times 10^5$	$1.13 \times 10^{-3}$	$2.36 \times 10^8$	$4.24 \times 10^{-9}$	
4.E6	$2.96 \times 10^5$	$1.12 \times 10^{-3}$	$2.64 \times 10^8$	$3.78 \times 10^{-9}$	
3.B10	$3.16 \times 10^5$	$1.57 \times 10^{-3}$	$2.01 \times 10^8$	$4.97 \times 10^{-9}$	
4.G6	$2.55 \times 10^5$	$1.56 \times 10^{-3}$	$1.64 \times 10^8$	$6.10 \times 10^{-9}$	Fingerprint profile 3
3.D9	Unreliable data				
2.C3	Unreliable data				
4.C4	$2.38 \times 10^5$	$1.39 \times 10^{-3}$	$1.72 \times 10^8$	$5.83 \times 10^{-9}$	
4.B3	$2.68 \times 10^5$	$1.75 \times 10^{-3}$	$1.53 \times 10^8$	$6.54 \times 10^{-9}$	Fingerprint profile 4
4.B5	$2.5 \times 10^5$	$1.94 \times 10^{-3}$	$1.29 \times 10^8$	$7.76 \times 10^{-9}$	
3.E4	Unreliable data				Fingerprint profile 5
3.B8	Unreliable data				
					Fingerprint profile 6
2.C6	$1.04 \times 10^4$	$1.13 \times 10^{-5}$	$9.23 \times 10^8$	$1.08 \times 10^{-9}$	
*3.D11	$3.25 \times 10^5$	$8.97 \times 10^{-4}$	$3.66 \times 10^8$	$2.73 \times 10^{-9}$	Fingerprint profile 7
2.D11	$3.33 \times 10^5$	$4.81 \times 10^{-3}$	$6.92 \times 10^7$	$1.45 \times 10^{-8}$	
					Fingerprint profile 7
1.F10	$2.81 \times 10^5$	$1.27 \times 10^{-3}$	$2.21 \times 10^8$	$4.52 \times 10^{-9}$	



**Figure 3.12: Kinetic analysis of anti-hFABP scFv.**

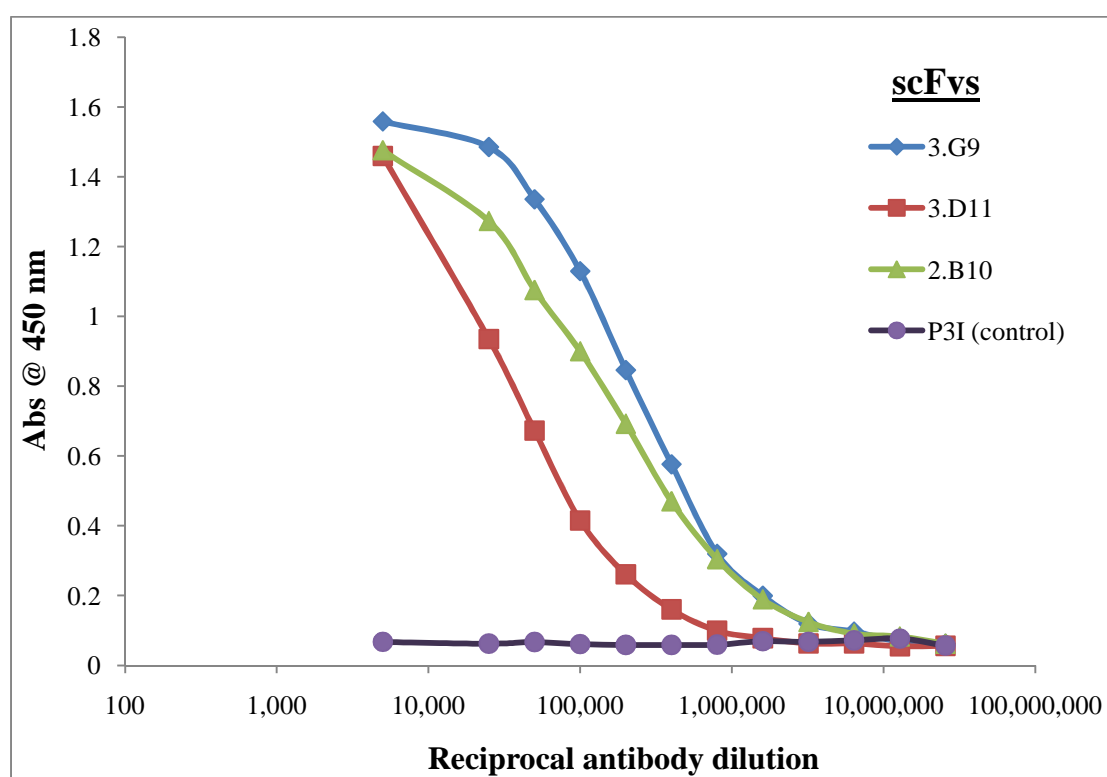
*This figure represents the local fit of a 1:1 binding with drifting baseline model to the response data obtained from the Biacore 3000 for clone 2.B10 with multiple analyte concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0 nM). The black lines represent the local fit to the response data (coloured lines). The residual plot shows that the data points, distributed randomly above and below the zero line, are within acceptable limits.*

Three scFvs (2.B10, 3.G9 and 3.D11) were selected based on their  $K_D$  values after kinetic analysis. The scFvs were expressed in large-scale (500 mL) and purified using an IMAC column (section 2.2.1.20). The eluted protein was buffer exchanged with PBS and the purity of protein was analysed by SDS-PAGE. The titre of the purified antibody against hFABP was calculated by ELISA. A high concentration of pure scFv was obtained (Figure 3.13 and 3.14). Kinetic analysis (in triplicate) was performed on Biacore 3000 to determine the affinity of purified scFvs. Table 3.4 shows the dissociation constant of the three scFvs analysed and the standard deviations obtained in three successive analyses.



**Figure 3.13: SDS-PAGE gel analysis of the IMAC-purified protein.**

Lane 1: Fermentas prestained protein marker; lane 2: flow through obtained after passing the lysate through IMAC; lane 3: wash containing any non-specific protein weakly bound to the column and lane 4: purified scFv.



**Figure 3.14: Titration of purified anti-hFABP scFvs by ELISA.**

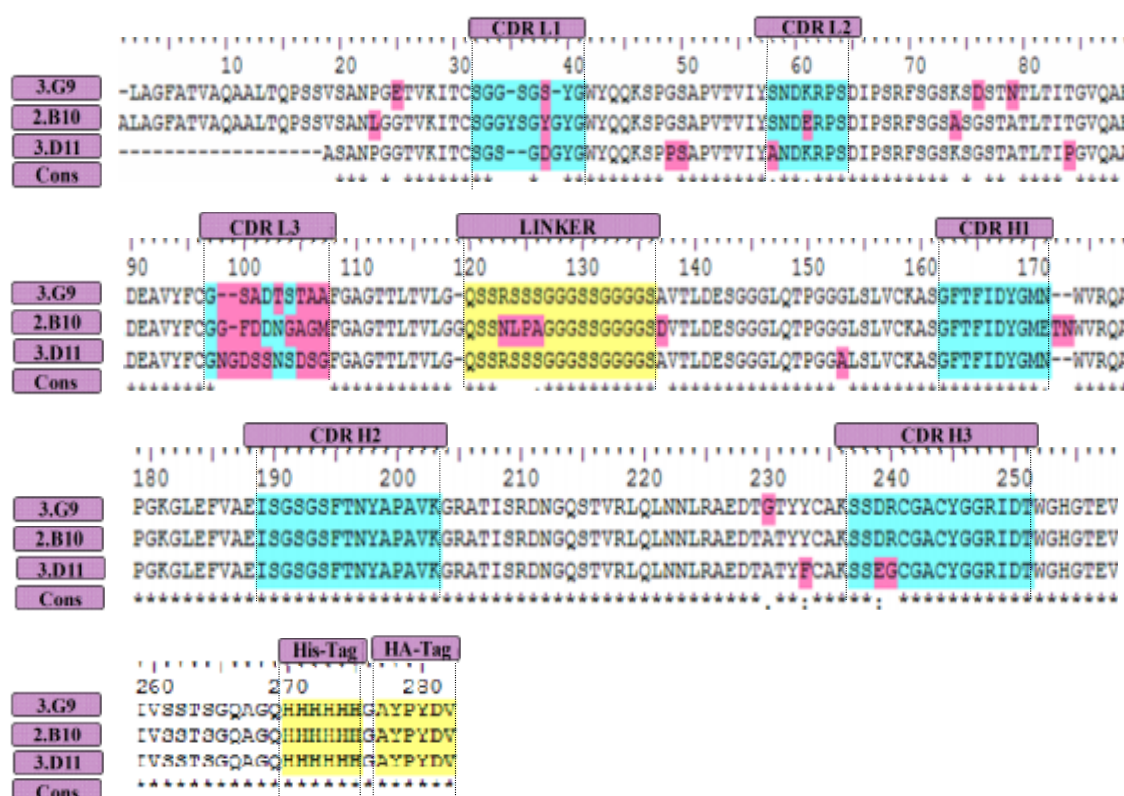
The scFvs were titred against hFABP antigen using dilutions from 1:5000 to 1:25600000. P3I is a chicken anti-troponin antibody which was used as control. ScFv 3.G9 and 2.B10 were titred out to more than a million whereas 3.D11 was more than 100,000, against hFABP. High scFv titres were obtained ensuring efficient protein expression and purification.

**Table 3.4: Dissociation constants of anti-hFABP scFvs.**

Clone	$K_D$ ( $\pm$ SD) (M)
3.G9	$1.53 (\pm 0.25) \times 10^{-9}$
2.B10	$4.86 (\pm 0.56) \times 10^{-9}$
3.D11	$4.04 (\pm 0.14) \times 10^{-9}$
n=3 independent analysis	

### 3.5 Sequence Analysis of scFvs

Sequencing of scFvs were carried out in triplicate. The sequences were aligned using ClustalX software. The CDR's and other regions (linker and vector tags) were identified and highlighted using BioEdit 7.0 software.

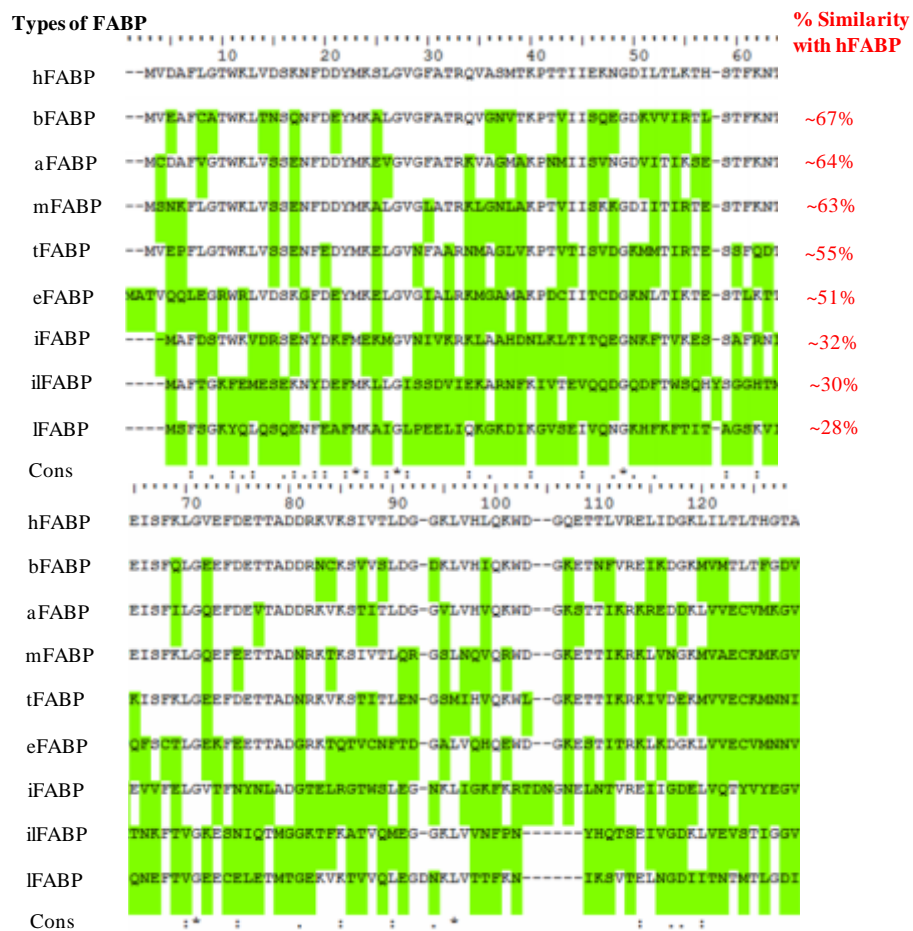


**Figure 3.15: Analysis of anti-hFABP scFv sequences.**

The CDR's are highlighted in blue and the linker and tag regions are highlighted in yellow. The sequence which is different from the consensus is highlighted in pink. There are little dissimilarity in the sequence of framework region of the scFvs. Minor diversity is observed in CDR L1, L2 and H3 regions whereas CDR L3 shows major diversity. Interestingly 2.B10 has a mutation in the linker sequence which is uncommon.

### 3.6 Cross-Reactivity Analysis of the scFvs

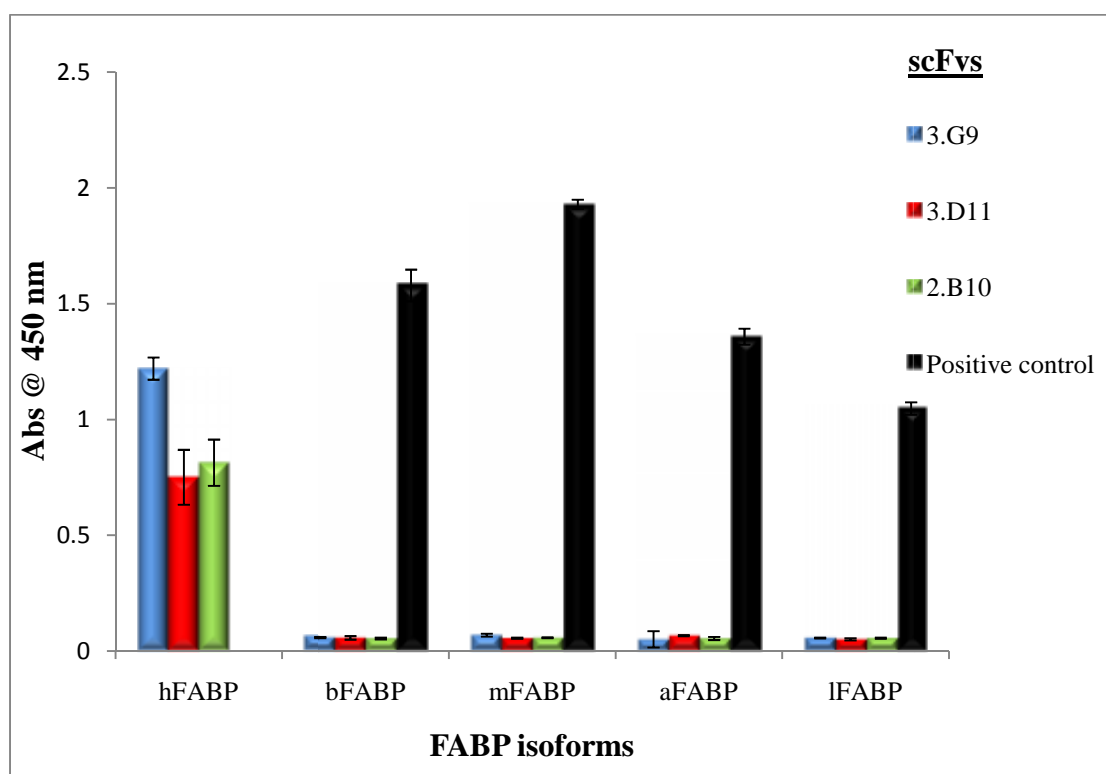
A total of nine different isoforms of FABP are found in humans (Storch and Corsico, 2008). FABP isoforms were aligned using ClustalX and closely related isoforms to hFABP were identified (Figure 3.16). The scFvs were checked against three closely related isoforms (bFABP, mFABP, and aFABP) along with one distant isoform (lFABP) of hFABP, as negative control, to analyse the specificity of the antibodies heart isoform. The analysis was carried out both by ELISA and Western blotting.



**Figure 3.16: Sequence alignment and percentage similarity of FABPs.**

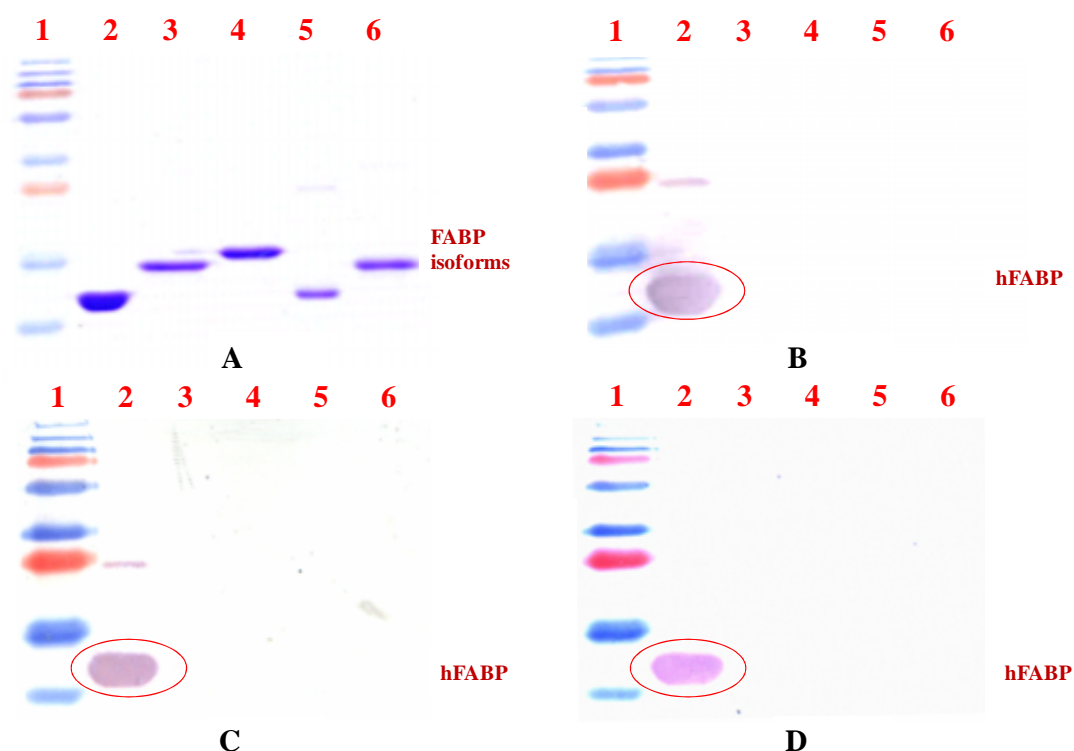
Sequences of nine different FABP isoforms were aligned and analysed for percentage similarity. The sequences which were different from hFABP were highlighted green. Among the different isoforms, bFABP, mFABP and aFABP were highly similar to hFABP sharing 67%, 64%, 63% similarity, whereas, lFABP was least similar showing 28% similarity in sequence. hFABP: heart FABP; bFABP: brain FABP; aFABP: adipocyte FABP; mFABP: myelin FABP; tFABP: testis FABP; eFABP: epidermal-type FABP; iFABP: intestinal FABP; ilFABP: ileal FABP and lFABP: liver FABP.





**Figure 3.17: Cross-reactivity analysis of anti-hFABP scFvs by ELISA.**

Highly similar (bFABP, mFABP, aFABP) and the least similar hFABP isoforms (lFABP) were coated on an ELISA plate along with hFABP. The scFvs, selected after kinetic analyses were checked against them. The black bar shows the positive control for each of the antigen used which is the commercially purchased antibody for respective antigen (anti-bFABP, anti-mFABP, anti-aFABP, anti-lFABP antibody). The graph shows that all the three scFvs (3.G9, 2.B10 and 3.D11) are highly specific to the cardiac isoform of FABP.



**Figure 3.18: Cross-reactivity analysis of anti-hFABP scFvs by Western blotting.**

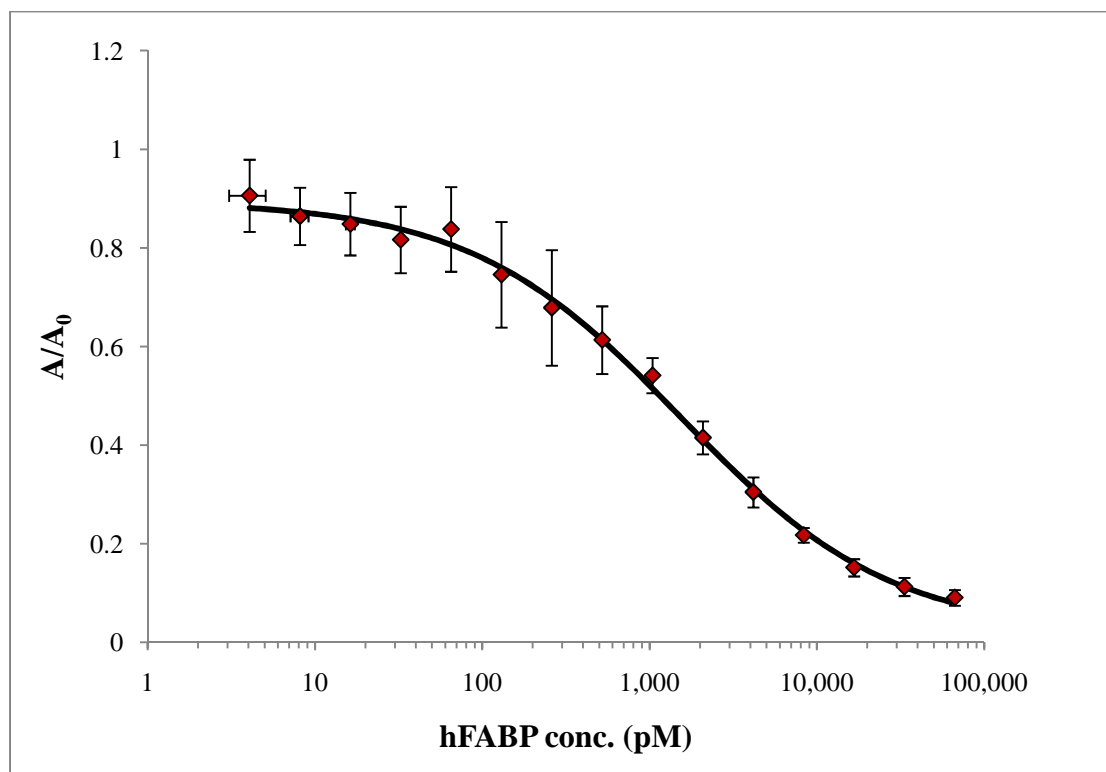
Different isoforms of FABP were run on SDS-PAGE gel and blotted with three different scFvs (3.G9, 2.B10 and 3.D11). Only hFABP reacted with the scFvs which was developed using anti-HA antibody. This bolt confirms the specificity of the scFvs as determined by ELISA.

**A:** SDS-PAGE gel; **B:** blot developed with 3.G9 scFv; **C:** blot developed with 2.B10 scFv and **D:** blot developed with 3.D11 scFv. In all the parts, lane M: Fermentas prestained protein marker; lane 1: hFABP; lane2: bFABP; lane3: mFABP; lane 4: aFABP and lane 5: lFABP.

### 3.7 Inhibition assays

The scFv 3.G9, due to its low nanomolar affinity towards hFABP, was selected for inhibition studies to calculate the limit of detection (LOD) and  $IC_{50}$  values. Inhibition ELISA of the purified 3.G9 scFv was carried out using midpoint from the linear range of the titration curve obtained during the titration ELISA of the purified scFv (section 3.4.4). Intraday (three assays on the same day) and interday (three assays on different days) validations were carried out to evaluate experimental variations. The standard deviation was calculated for the  $A/A_0$  value of each antigen concentration representing

the inhibition of the scFv to free antigen in solution. The calibration curves were fitted to the data set with a four-parameter equation using BIAevaluation 4.1 software. The calibration curve facilitates the back calculation of antigen concentration at the given dilution which can be further verified for accuracy. The scFv was found to have very low LOQ value of 1.90 ng/mL (130 pM) with an  $IC_{50}$  of 15.62 ng/mL (1,040 pM).



**Figure 3.19: Inhibition ELISA of 3.G9 scFv.**

*This assay shows that the back calculated values are close to the report points (red dots) showing good accuracy of the assay. Standard deviation was within acceptable limits.*

All the values were very reproducible with a standard deviation of less than 0.2 and less than 20% coefficient of variation (Findlay *et al.*, 2000). Antigen concentrations were back calculated, using BIAevaluation 4.1 software, from the calibration curve plotted using antigen concentration in pM and  $A/A_0$  values (Figure 3.19). The values were further used in determining the % recovery for each concentration of antigen to

determine the accuracy of the data. The % recovery obtained was found to be  $100 \pm 20\%$  determining the assay range of the curve (Table 3.5).

**Table 3.5: Parameters obtained for different antigen concentrations in inhibition assays.**

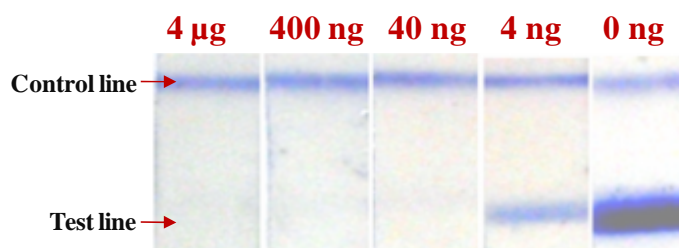
<b>hFABP (ng/mL)</b>	<b>Average (interday)</b>	<b>Standard deviation</b>	<b>% Coefficient of variation</b>	<b>% Recovery</b>
<b>1,000.00</b>	0.09	0.01	17.53	77
<b>500.00</b>	0.11	0.01	16.19	100
<b>250.00</b>	0.15	0.01	11.56	112
<b>125.00</b>	0.21	0.01	6.87	109
<b>62.50</b>	0.30	0.03	10.01	105
<b>31.25</b>	0.41	0.03	8.06	97
<b>15.62</b>	0.54	0.03	6.57	84
<b>7.81</b>	0.61	0.06	11.20	100
<b>3.90</b>	0.67	0.11	17.23	117
<b>1.90</b>	0.74	0.10	14.37	119
<b>0.95</b>	0.83	0.08	10.24	50
<b>0.47</b>	0.81	0.06	8.24	165
<b>0.23</b>	0.84	0.06	7.47	147
<b>0.11</b>	0.86	0.05	6.71	159
<b>0.05</b>	0.90	0.07	8.06	Low

### 3.8 Lateral flow immunoassay

An ELISA-based assay was successfully developed using the 3.G9 scFv. However, a lateral flow immunoassay (LFIA) was investigated using this scFv to reduce time-consuming steps in ELISA. These include an hour long incubations, wash steps and addition of labelled antibody. A proof-of-concept LFIA was standardised. This comprised of a number of steps similar to ELISA. An inhibition assay format was tested for LFIA development using the 3.G9 scFv.

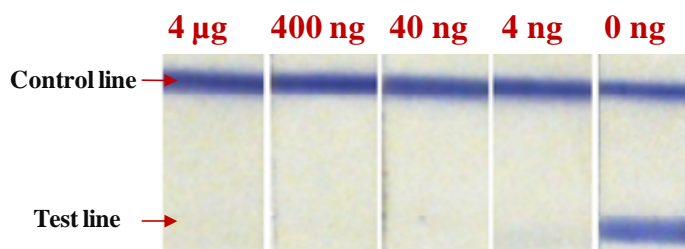
In an inhibition assay format, the test line colour intensity is inversely proportional to the concentration of the hFABP in the antigen-antibody mixture. A good correlation between the intensity of the line and the concentration of hFABP was obtained (Figure 3.20). The assay was further validated using hFABP-spiked sera (Figure 3.21). The

analysis showed that the LFIA was more sensitive in serum when compared to phosphate buffer. However, the performance of LFIA can be further improved by conjugating the scFv to coloured beads or colloidal gold particles and, thus, further reducing the overall turn-around time (TAT).



**Figure 3.20: Lateral flow immunoassay development using phosphate buffer.**

A range of hFABP concentrations were used (4 µg/mL to 4 ng/mL). The assay was shown to be highly sensitive, showing inhibition at a 4 ng/mL concentration of hFABP which was below the cut-off hFABP levels required for clinical analyses.



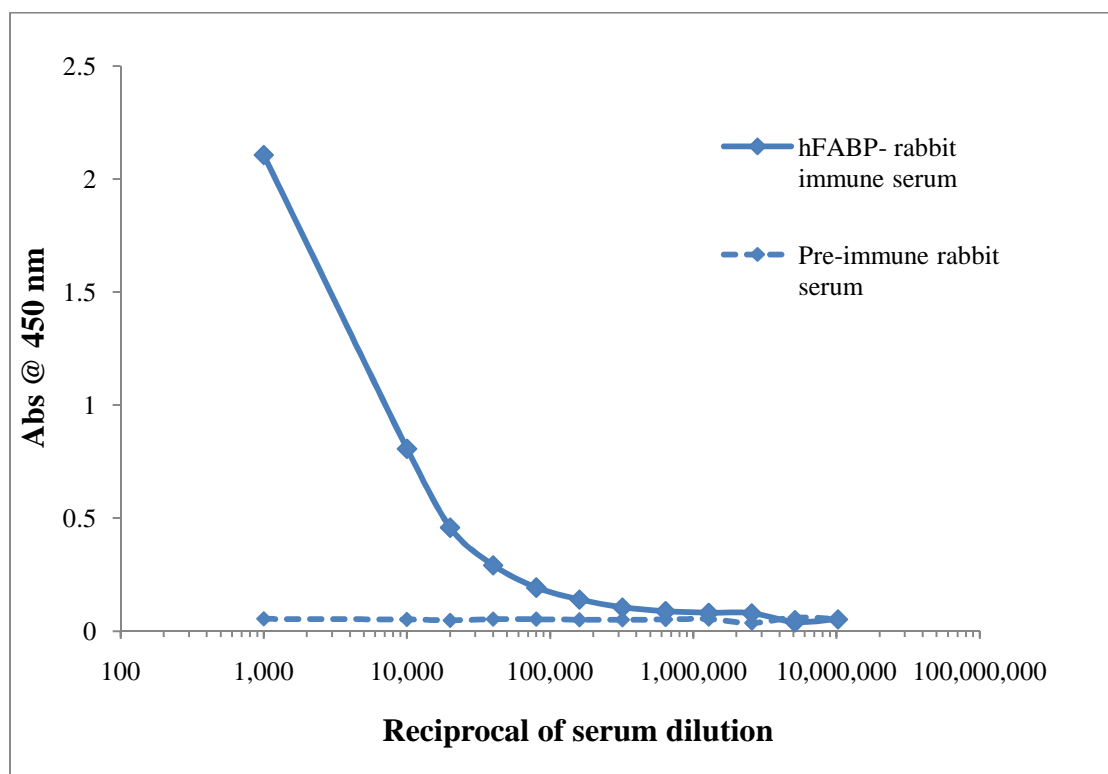
**Figure 3.21: Lateral flow immunoassay validation using hFABP-spiked human serum.**

The binding of scFv to the test line was completely inhibited at a 4 ng/mL hFABP concentration in serum, where no band was visible. The assay to be more sensitive in serum than buffer, where the scFv binding to the test line was partially inhibited, producing a weak band.

### 3.9 Rabbit Polyclonal Serum Titre

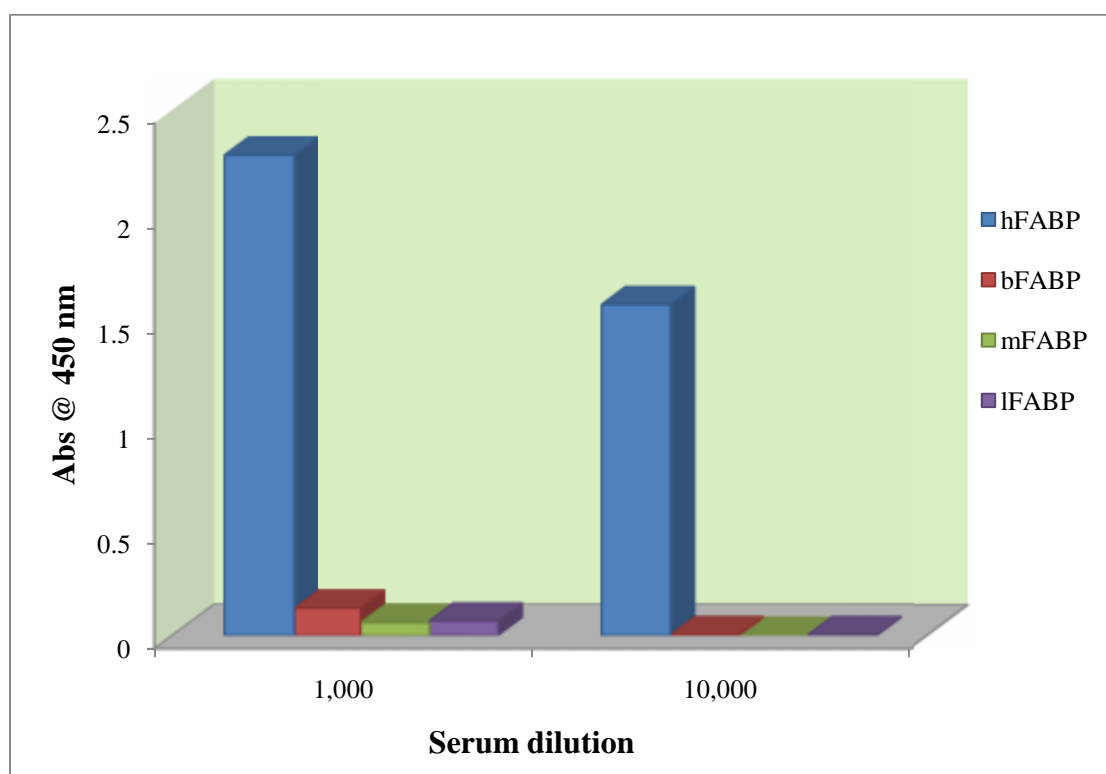
A New Zealand white rabbit was immunised with hFABP to obtain polyclonal antibodies. The serum titre was analysed for a range of dilutions by indirect ELISA. The serum titre was approximately 1:100,000 (Figure 3.22). The serum was checked against

other isoforms of FABP to characterise the specificity of polyclonal antibodies produced by the animal (Figure 3.23).



**Figure 3.22: Rabbit serum titre against hFABP antigen.**

*A range of serum dilutions was analysed starting from 1:1,000 to 1: 10,240,000. Pre-immune serum was used as control against the immune serum to detect the animal response against the hFABP antigen.*



**Figure 3.23: Analysis of rabbit sera against FABP isoforms.**

*Rabbit polyclonal sera were checked against different isoforms of FABP. The result indicates that the antiserum is specific to hFABP epitopes.*

### 3.10 Discussion

“Time saves muscle” is a common saying for cardiologists. During a heart attack every minute counts. All the guidelines from different medical and scientific bodies, lay stress on the use of early biomarkers in diagnostic assays so that proper care and treatment can be instituted for the patient as quickly as possible (Thygesen *et al.*, 2007).

The cardiac isoform of FABP, hFABP, is an early marker of myocardial ischemia. It appears in blood as early as 1 hour after the onset of MI (Glatz *et al.*, 1998; Chan *et al.*, 2005 and Hou *et al.*, 2006). In many respects, hFABP resembles myoglobin, a recommended early biomarker in recent guidelines (NACB writing group members *et al.*, 2007). However, on comparing both the markers, hFABP was reported to offer

advantages over myoglobin in terms of diagnostic efficacy (Okamoto *et al.*, 2000; Pelsers *et al.*, 2005; Cavus *et al.*, 2006; and Zaninotto *et al.*, 2006).

Several commercial immunoassays rely on utilising monoclonal antibodies (MAbs) for detection of hFABP in patient's blood (Chan *et al.*, 2005). However, despite the high specificity offered by MAbs, they suffer from some disadvantages such as high cost of production, longer production time, and interference in assays due to the presence of anti-animal antibodies (Kricka, 1999; Emanuel *et al.*, 2000 and Smith *et al.*, 2004). ScFvs are the most commonly used recombinant antibody fragment format owing to their small size, ease of production, manipulation by tailoring valency and specificities (Holliger and Hudson, 2005) and large-scale expression in prokaryotes (Martin *et al.*, 2006) and eukaryotes (Gasser and Mattanovich, 2007; Tang *et al.*, 2007b). ScFvs can be genetically modified allowing for generation of novel and rare functionalities (Alcocer *et al.*, 2000), e.g. tagging of fusion proteins to the antibody produced (Terpe, 2003) making it extremely useful for immunoassay development employing multiple immunoassay platforms.

A diverse panel of anti-hFABP scFvs were obtained by using chickens as the hosts for antibody generation. High proportions of specific binders were obtained on screening the panned outputs representing an effective enrichment of hFABP-specific clones characterised initially using the SPR-based 'ranking' approach. Characterisation of the antibodies using high throughput SPR-based systems (Biacore A100) is a versatile technique that eases the task of selecting high affinity binders from a large population of clones (Leonard *et al.*, 2007). However, the format of analysis should be selected appropriately for the characterisation of clones. It was previously shown that the analysis of recombinant fragments by immobilising analyte could be misleading due to



multimerisation effects (Townsend *et al.*, 2006). Despite this, determination of the competitive efficiency of the antibody to the analyte in solution phase is useful in assessing its efficiency to detect the antigen in clinical samples. Therefore, all the analyses were carried out using a capture method in which the haemagglutinin (HA)-tagged scFv was captured with the help of an anti-HA-specific antibody and passing the analyte over it in solution. Such a method allows proper orientation of the scFv to enable efficient detection of the analyte in solution along with rapid concentration-independent ranking of multiple clones based on their  $K_{\text{off}}$  values.

Initially, 80 scFvs were affinity-ranked by monitoring antigen dissociation in early and late phase and calculating the stability of antigen/antibody complex from the % left value. This method offered a rapid characterisation of clones based on their 'off-rates' irrespective of antibody expression and concentration. Twenty five scFvs with antigen capture levels above 60% after 10 minutes of dissociation in buffer were chosen from the initially selected 80 clones and carried forward for further studies.

The scFvs selected by ranking, due to their similar % left values, were characterised by fingerprinting analysis. It is a simple method to study the homology in sequence of multiple clones based on their digestion profile. The enzymes *Bst*NI and *Alu*I were used for this study in order to provide a detailed profiling of the antibody which sometimes is not possible by using one alone while differentiating between highly related antibody sequences (Hawlsch *et al.*, 2000). The enzymes *Bst*NI and *Alu*I divided the selected scFvs into 7 distinct profiles, confirming diversity in their sequence. This diversity of scFv sequence after ranking shows that high affinity clones are not restricted to a particular sequence of heavy and light chains. The affinity of clones was further calculated by performing kinetic analysis by passing different concentrations of hFABP

antigen over the anti-HA-captured scFvs. Binding characteristics of each scFv, such as association and dissociation constants, were calculated by fitting the sensorgrams with BIAevaluation 4.1 software<sup>TM</sup>. Three scFvs with high affinity were selected from the data and purified for kinetic evaluation.

The scFvs were solubly expressed in large-scale yielding high quantities of pure and stable protein without any need for further optimisation of purification. The specificities of the scFvs were checked against closely related FABP isoforms, sharing a homology of 63-67%. The analyses confirmed the high specificity of the selected scFvs for the cardiac isoform of FABP making them ideal biorecognition reagents for diagnosis of cardiac diseases.

Sequencing analysis of the scFvs revealed different lengths of the light chain CDRL1 and L3, along with point mutations in the other CDRs and framework regions. CDRL3 was highly diverse in terms of amino acid sequence which can be expected due to combinatorial V-J joining. The change in the CDR length can be attributed to the joining of random segments of variable pseudogenes, resulting in gene conversion and altering the length of the variable chains. This process is responsible for converting most of the non-functional rearranged variable gene in the chicken immune system by changing the many out of frame codons into correct ORFs (Ratcliffe, 2006). Sequencing analysis also revealed that estimation of diversity only based on fingerprinting, using restriction enzymes, may not provide sufficient information, as the sequence of two clones (2.B10 and 3.G9) with a similar restriction profile had sequence variations.

Kinetic evaluation of the purified scFvs was carried out to verify the kinetic data obtained from impure lysates and to determine its reproducibility. Kinetic evaluation of

the purified 3.G9 scFv showed 0.5 times decrease in the affinity data ( $8.36 \times 10^{-10}$  to  $1.53 \times 10^{-9}$  M) as compared to that obtained from the crude bacterial lysate, which may be attributed to the influence of other bacterial proteins present in the crude lysate inducing non-specific binding to the chip surface. However, the kinetic evaluations of all the scFvs were reproducible when carried out in triplicate with low standard deviations.

ScFv 3.G9 was identified to have high specificity and a low nanomolar affinity towards hFABP making it an ideal candidate for assay development. However, it was necessary to determine the LOQ and  $IC_{50}$  of the scFv to determine if genetic engineering was necessary to enhance specificity/sensitivity, prior to its utilisation in various assays. A reproducible standard curve was obtained with an assay range of 1.90-500.00 ng/mL. The LOQ for the scFv was found to be satisfactory and compared favourably with most of the commercial immunoassays (Azzazy *et al.*, 2006). A lateral flow immunoassay was developed to reduce overall TAT, using enzymatic inhibition assay format. Proof-of-concept was established using an additional secondary antibody to probe the antigen-antibody interaction followed by addition of substrate. The assay was highly sensitive detecting upto 4 ng/mL which is within the normal hFABP concentrations in healthy individuals (1.6-19 ng/mL) (Kleine *et al.*, 1992; Chan *et al.*, 2003; [http://www.signosisinc.com/PDF/hFABP\\_User\\_manual\\_020208.pdf](http://www.signosisinc.com/PDF/hFABP_User_manual_020208.pdf)). However, this assay needs further optimisations to further reduce the TAT, in order to be used as a rapid point-of-care assay.

PAbs are routinely used in immunoassays for many decades owing to their ease of production, and low cost and labour requirements. They have the advantage of providing stable, multi-specific populations of antigens. Rabbits are usually the animal

of choice in laboratories, due to their convenient size which provides ease of handling, relatively long life-span, adequate production of high-titre, high-affinity antibodies, and ease of withdrawal of large volumes of blood (Cooper and Paterson, 2009). In the present study, pAbs were obtained by immunising a New Zealand white rabbit. The primary objective of pAb development was to obtain antibodies against certain epitopes of hFABP different from that of already existing recombinant avian antibodies

High titres of pAbs obtained in the rabbit sera, against the hFABP antigen, indicated that human hFABP is immunogenic in rabbits increasing the probability of obtaining specific antibodies against the antigen. Characterisation of pAbs against different FABP isoforms suggested the polyclonal antibodies produced were specific against the cardiac isoform of the FABP and can be further incorporated in sandwich assay formats.

# **Chapter 4**

## **Expression of Human hFABP**

## **4.1 Introduction**

### **4.1.1 *E. coli* as an expression system**

Expansion of biotechnology industry has led to an increased demand of recombinantly expressed proteins for a variety of applications. A variety of expression systems (prokaryotic, eukaryotic, mammalian, viral, insect and plant) were studied for the expression of heterologous proteins. The choice of expression system depends on several features such as cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications, and biological activity of the protein of interest (Goeddel, 1990).

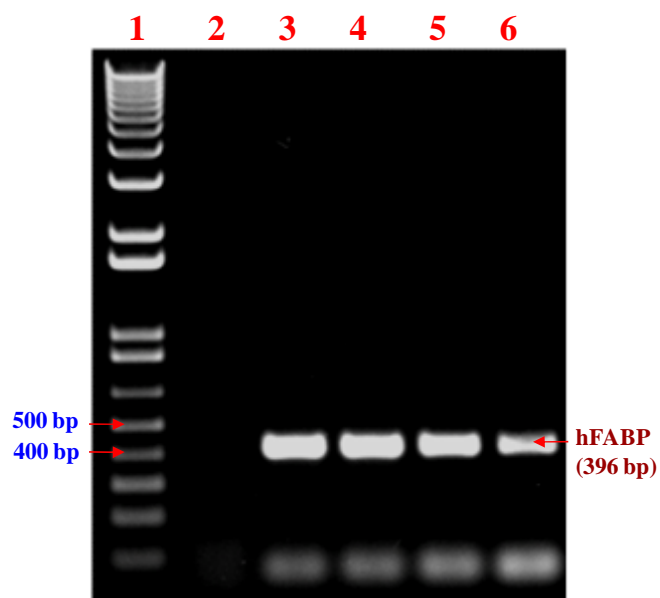
Among the existing expression systems, *E. coli* is the most popular and routinely used host for heterologous protein expression (Hannig and Makrides, 1998; Ratelade *et al.*, 2009) due to its relative simplicity, low cost and rapid high-density cultivation. Its well-characterised genetics provide ease of manipulation that facilitates the production of protein with the desired characteristics. An array of vector systems and cell-strain, providing various useful features, are available commercially.

For many years, heterologous gene expression in *E. coli* was hampered by issues including inefficient translation of eukaryotic mRNAs due to major differences in codon usage between the foreign gene and native *E. coli*, failure of *E. coli* to completely remove amino-terminal initiator methionine residues, the formation of insoluble aggregates of the expressed proteins called inclusion bodies in *E. coli* cytoplasm, degradation of the protein by host cell proteases and problems with protein purification (LaVallie and McCoy, 1995). However, recent advances in understanding of the function, regulation and interactions of cellular gene products led to the development of new components such as vectors, recombinant fusion partners and mutant strains

(Sørensen and Mortensen, 2005). Their availability circumvented most of the issues by providing useful approaches such as tight regulation of expression, expression of protein with fusion tags, expression of difficult genes containing rare codons, extracellular protein expression and ease of purification, thus, making *E. coli* an attractive host for protein generation.

## **4.2 Amplification of hFABP from Human Heart cDNA**

Human fatty acid binding protein (hFABP) was expressed 'in-house' exploiting current knowledge of recombinant DNA technology. The sequence of hFABP was studied and primers were designed to amplify the gene. HFABP shares high homology with few other isoforms of FABP which makes the amplification of specific gene a critical point in expression. FABPs are characterised into nine isoforms based on the tissue of their expression (Storch and Corsico, 2008) making their origin highly confined to a particular tissue. Human heart cDNA was used as the template for amplifying the gene due to the expression of hFABP in myocardial muscles. The PCR was optimised for annealing temperature and  $MgCl_2$  concentration. The PCR products were analysed for amplification by agarose gel electrophoresis.



**Figure 4.1: Amplification of hFABP gene from human heart cDNA.**

*Lane 1: 1 kb DNA ladder plus; lane 2: negative control; lane 3: 1 mM MgCl<sub>2</sub>; lane 4: 2 mM MgCl<sub>2</sub>; lane 5: 3 mM MgCl<sub>2</sub> and lane 6: 4 mM MgCl<sub>2</sub>. The annealing temperature of 61°C was found to be optimum for hFABP gene amplification. No major difference in the amplification was observed by increasing MgCl<sub>2</sub> concentration so 1 mM MgCl<sub>2</sub> was used for all subsequent hFABP gene amplifications.*

### **4.3 Cloning of hFABP Gene in pET-26b(+) Vector**

The amplified gene was purified by gel extraction of the specific PCR product at 396 bp, resolved by agarose gel electrophoresis. The gene, amplified with primers containing restriction sites which were compatible to the sites of the vector, was digested with *NcoI* and *NotI* enzymes, creating cohesive ends, which were then cloned into an expression vector, pET-26b(+). The digestion of the gene with two different enzymes ensures unidirectional cloning of the gene into the vector, reducing the background from non-functional clones arising from cloning of the gene in opposite direction. Restriction digestion with two enzymes also prevents re-ligation of vector causing only the vector with cloned gene to be expressed.



The ratio of vector to insert is critical for successful cloning. The ratio of vector to insert should be a minimum of 3:1 which is calculated according to their respective sizes. The most commonly used formula is:

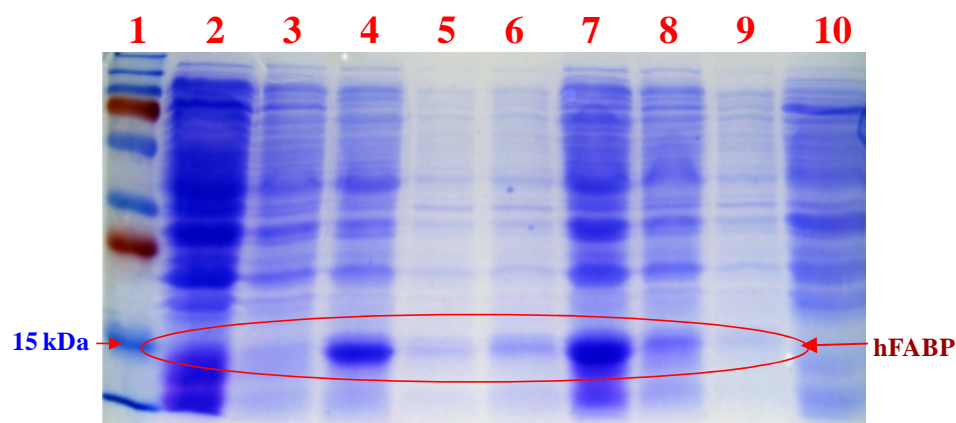
$$\text{Vector in ng} / \text{vector size (in bp)} = 3 \times (\text{insert in ng} / \text{insert size (in bp)})$$

The vector and the gene were mixed in the required ratios and the cohesive ends created by digestion were ligated by T4 DNA ligase enzyme.

#### **4.4 Transformation of the Cloned Gene in *E. coli* Cells and Expression of Protein**

A variety of techniques can be used for introducing DNA into *E. coli* cells. Heat shock and electroporation are the most commonly used methods. For gene cloning purposes, heat shock is the most convenient method providing an efficiency of  $10^5$  to  $10^6$  transformants per microgram of DNA. This method requires chemically competent cells with increased membrane permeability. This can be obtained by treating the cells with divalent cations like  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ .

The chemically competent BL-21 strain of *E. coli* cells was transformed with the cloned gene following a standard heat shock protocol (section 2.2.2.4). The transformed colonies were selected from the population taking advantage of the presence of the kanamycin resistance gene coded by pET-26b(+) vector. A total of 8 colonies were picked and grown O/N followed by subsequent subculture and induction with IPTG. The colonies were sonicated to achieve periplasmic extraction of the protein which was analysed by SDS-PAGE electrophoresis. The successful expression of hFABP can be clearly seen in the gel in the form of a clear band present in the transformed clones (Figure 4.2).



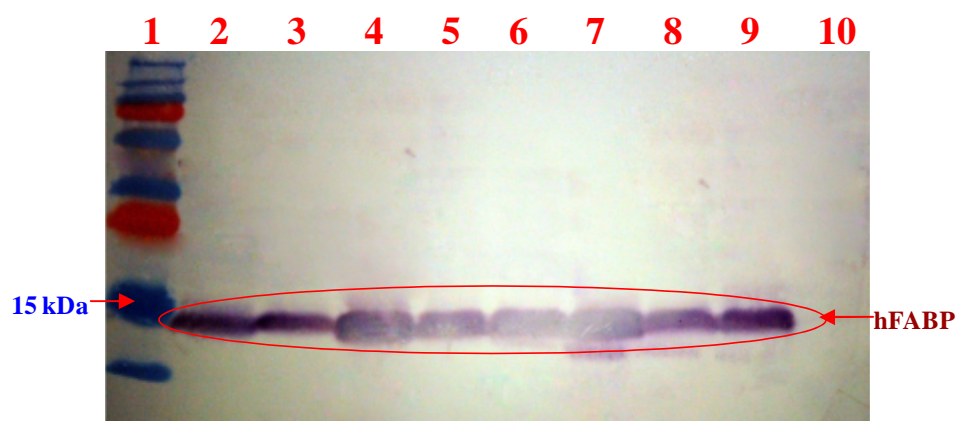
**Figure 4.2: SDS-PAGE analysis for expression of hFABP.**

*The lysates from the expressed clones were treated with sample treatment buffer and analysed on 12.5% SDS-PAGE along with a negative control. Lane 1: Fermentas prestained protein marker; lanes 2 to 9: different hFABP transformed clones; and lane 10: negative control {HEXA gene cloned in pET-26b(+ ) vector}. The gel shows a thick band of appropriate size for hFABP (~15 kDa) that can be observed clearly in several clones (2, 4, 7 and 8) which is absent in the control. In lanes 2, 5, 6 and 9 only a faint band was present but subsequently this was verified to be hFABP by immunoblot analysis.*

The clones analysed by SDS-PAGE electrophoresis were further checked by immunoblotting, using specific anti-hFABP antibody. The immunoblot analysis was carried out by probing the hFABP antigen protein with specific anti-hFABP antibody (3.G9) and detecting the antigen-antibody interaction with an anti-HA antibody (targeted against the HA tag in the scFv expressed using the pComb vector system). The 3.G9 antibody used for probing the protein is a highly sensitive anti-hFABP scFv, developed 'in-house' by immunising the chicken with commercial hFABP antigen.

The immunoblotting procedure is explained in section 2.2.1.22. Briefly, the proteins from the gel were transferred to the nitrocellulose membrane. The membrane was blocked with 2.5% (w/v) milk-BSA-PBS followed by incubating the membrane with

1:5,000 dilution of 3.G9 anti-hFABP scFv in 0.5% (w/v) milk-BSA-PBS. The reaction was detected using HRP-conjugated-anti-HA antibody using TMB as the substrate. Figure 4.3 represents the immunoblot developed to confirm the expression of hFABP antigen.



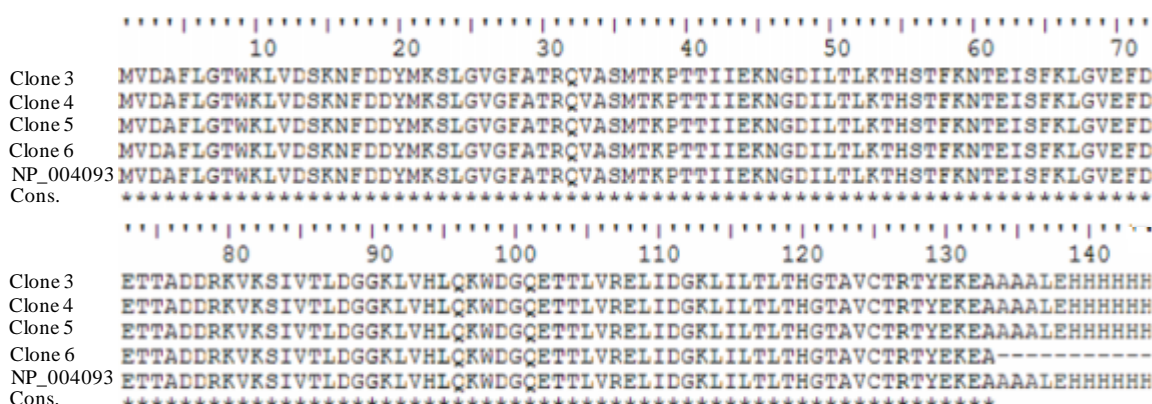
**Figure 4.3: Immunoblot analysis for expression of hFABP.**

*The results from SDS-PAGE were confirmed by immunoblotting the clones with anti-hFABP scFv. Lane 1: Fermentas prestained protein marker; lanes 2 to 9: different hFABP transformed clones; and lane 10: negative control (HEXA gene cloned in pET-26b(+)) vector). The blot shows a clear band of appropriate size (~15 kDa) for hFABP is developed for all the clones except the control, thus confirming the results from SDS-PAGE. It also verified that this recombinant antigen can be substituted for commercial antigen in our study as it can be detected with very high sensitivity with the scFv antibodies generated in this research.*

#### **4.5 Analysis of the hFABP Expressing Clones by Sequencing**

The hFABP clone sequences were determined by sequencing the cloned gene using primers specific to the T7 promoter and the terminator region of the pET vectors. The sequences were aligned with each other along with the published sequence, using sequence alignment bioinformatics software, ClustalX. The sequence analysis was necessary to ensure that the gene amplified is intact without any mutation in the sequence during the amplification and cloning processes. Figure 4.4 shows that the

hFABP gene amplified from human heart cDNA is completely identical to hFABP published sequence.



**Figure 4.4: Sequence analysis of hFABP clones.**

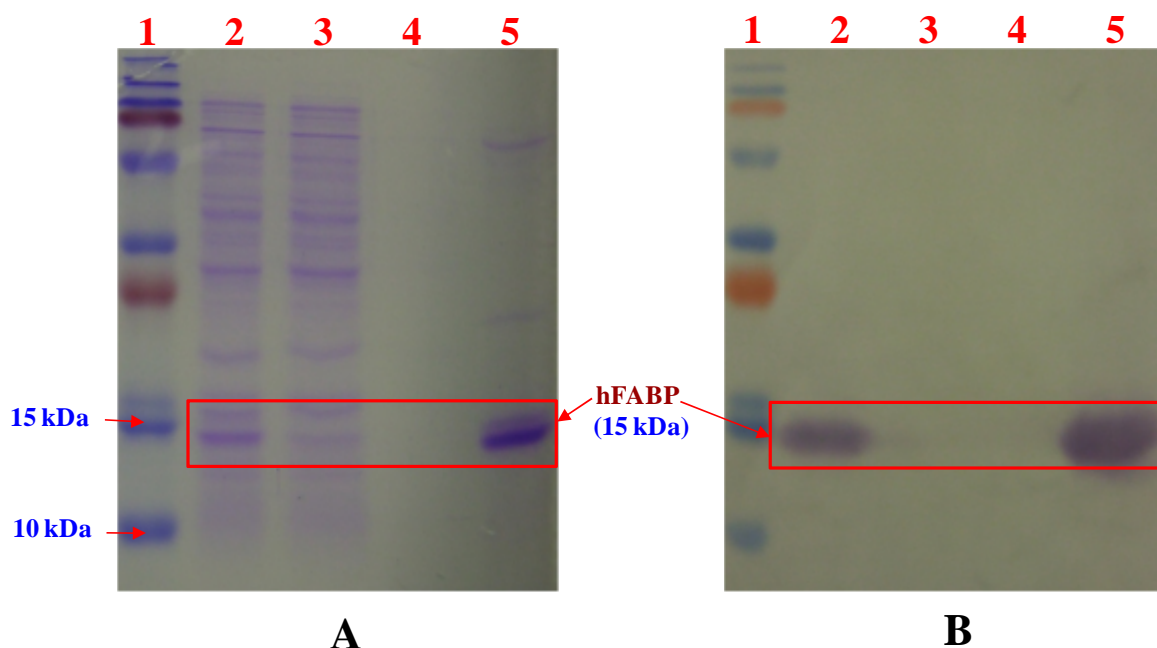
*Four clones were analysed by sequencing for the similarity of the amplified gene sequence with that to the published gene sequence NP\_004093. The sequences were 100% similar to the published sequence. It is also verified that the gene is in the correct frame to the His-Tags encoded by the vector.*

#### **4.6 Large-Scale Protein Expression and IMAC Purification of the Expressed Protein**

Among the hFABP-expressing clones clone 3 was further used for large-scale production of the protein. The hFABP protein was produced by growing up a large volume culture of clone 3 which was then expressed O/N. The bacterial cells were pelleted, resuspended in lysis buffer and periplasmic protein was extracted by sonication. Bacterial lysate preparation is an important step in protein purification. It is necessary to keep the recombinant protein intact by reducing unwanted proteolysis or degradation due to the contamination by host proteins after cell lysis. This can be ensured by maintaining the lysate on ice until purification, where the activity of these contaminating host proteins will be minimum. The protein lysate after sonication was centrifuged for removal of cell debris and the supernatant was purified using IMAC (section 2.2.1.20).

IMAC is a robust method for purification of polyhistidine tagged proteins. As the name suggests, it is based on the principle of purification of 6x His-tagged proteins owing to their affinity towards the nickel ions immobilised onto nitrilo-triacetic acid (Ni-NTA) matrices. These affinity tags are coded by the vector, which are expressed along with the protein thus, providing convenience in purification of expressed recombinant protein from the host proteins.

The protein bound to Ni-NTA can be eluted by using a low pH buffer (10 mM sodium acetate pH 4.4) which reduces the binding affinity between the  $\text{Ni}^{2+}$  and the His-tag. Purity of the eluted protein can be increased by reducing non-specific interactions of other host proteins containing His-residues with the Ni-NTA matrix. Non-specific binding of proteins can be eliminated in the early stages of purification, by adding moderate concentrations of imidazole to the lysis and running buffer. Imidazole forms the side chain of His amino acid which binds to the  $\text{Ni}^{2+}$  in the column. Addition of imidazole to buffers competes with other His-containing proteins, for binding the Ni-NTA column. Moderate imidazole concentration cannot inhibit 6x tagged recombinantly expressed protein from binding the column thus yielding a pure protein on elution. The efficiency of purification was analysed by performing SDS-PAGE and immunoblot analysis on the lysate, 'flow-through', and 'wash-through' obtained at different stages of purification along with the purified protein (Figure 4.5).



**Figure 4.5: Analysis of purification of hFABP.**

*A: SDS-PAGE*

*B: Immunoblot analysis.*

*High concentration of pure hFABP was obtained by IMAC purification. Lane 1: Fermentas prestained protein marker, lane 2: Lysate; lane 3: 'flow-through' obtained by passing lysate through the column; lane 4: 'wash-through' obtained by passing running buffer through the column and lane 5: IMAC-purified protein.*

## 4.7 Discussion

The availability of high quality of the antigen is a prerequisite for successful generation of antibodies. Antigen from its natural source is always preferred due to its proper folding and post translational modification options. Efficient antibody generation and its characterisation requires ample amount of antigen (Hust and Dübel, 2004). However, obtaining high amounts of antigen from natural sources is not always feasible.

In present study, the analysis of anti-chicken antibodies on different assay formats necessitates an excess of the hFABP antigen for both optimisation and verification steps. Purified commercial FABP is very expensive to be used for such analysis. Apart from cost, the development of assays requires a tight control of the quality of antigen,

e.g. batch variations and presence of cross contaminants such as myoglobin (Crisman *et al.*, 1987) from its natural source will result in variation in assay. Therefore, it was essential to find an alternate cost-effective source that can satisfy the needs of the current research.

For many years recombinantly expressed proteins have generated valuable reagents for biotechnology-based research including their use in various diagnostic and therapeutic applications (Chu and Robinson, 2001; Swartz, 2001; Andersen and Krummen, 2002). Recombinant expression of protein, using *E. coli* as expression host, is an attractive approach to satiate the need of antigen required for further analysis of the anti-hFABP antibodies. Additionally in-frame fusion of the protein to tag sequences can facilitate their folding, purification and their attachment to support materials in the correct orientation increasing the accessibility of epitope. Ease of antigen production, the availability of constant supply of antigen at low cost, and no/less batch to batch variability are additional highly valuable advantages.

Advances in genetic engineering have greatly facilitated the expression of mammalian proteins in prokaryotic systems such as *E. coli* (Makrides, 1996; Tolia and Joshua-Tor, 2006). The choice of vector plays an important role in expression of recombinant proteins. pET vectors are the most commonly used in *E. coli* cells owing to their tight regulation of protein expression due to the presence of T7 phage RNA polymerase promoter (Sørensen and Mortensen, 2005). In this study, pET-26b(+) and BL-21 (DE3) strain of *E. coli* were used. The decision to use BL-21 (DE3) was prompted by its outstanding performance in recombinant expression applications due to its ability to produce T7 polymerase, express rare codons, vigorous growth in minimal media conditions and its deficiency in Lon and OmpT proteases (Sørensen and Mortensen,

2005; Ratelade *et al.*, 2009). Many studies reported a similar combination of the pET vector with the BL-21 (DE3) *E. coli* strain for successful expression of proteins (Peeters *et al.*, 1991; Schreiber *et al.*, 1998; Richieri *et al.*, 2000).

Recombinant expression of mammalian proteins using a prokaryotic expression system is a difficult task due to the requirement of glycosylation and other post-translational modifications for biological activity (Makrides, 1996). On the other hand, many recombinantly expressed proteins retain their full biological activity in the absence of any post-translational modification (Liang *et al.*, 1985; Fuh *et al.*, 1990). Many studies have reported successful applications of recombinantly expressed FABP using *E. coli* for different analyses (Billichi *et al.*, 1988; Peeters *et al.*, 1991; Zanotti *et al.*, 1992; Makrides, 1996).

In line with the finding of this study ‘in-house’ hFABP was successfully expressed in the *E. coli* BL-21 (DE3) strain. The SDS-PAGE analysis revealed over-expression and hFABP was subsequently efficiently purified yielding sufficient quantities for further analyses. The protein was checked for its biological activity against specific hFABP antibodies by immunoblotting analysis. The results showed that epitope conformation was retained by the recombinantly expressed protein when compared with the native protein. These results were in agreement with previous studies (Makrides, 1996) and confirmed that recombinant hFABP can be used as a substitute for tissue-derived human hFABP in antibody-based assays.



# **Chapter 5**

## **Epitope Mapping of Anti-hFABP scFv**

## 5.1 Introduction

Antibodies are sought as immuno-recognition reagents for several purposes owing to their specificity against their targets (antigens). Every antibody is targeted to a particular region on the antigen, known as antigenic determinants or epitopes, with which it reacts specifically (Murphy *et al.*, 2007; Morris, 2008). Similarly, the part of antibody that reacts with a given epitope is known as a paratope. Antigens can have different chemical compositions (section 1.3.1). However, hFABP is protein in nature and, therefore, this section will focus only on protein antigens.

The epitopes can be either continuous or discontinuous. Continuous epitopes (also known as sequential or linear epitopes) comprise of linear amino acid sequences and are mostly resistant to protein denaturation, whereas, discontinuous epitopes (also known as assembled or conformational or non-linear epitopes) comprise of amino acids separated in the protein sequence, brought together by the protein folding. Discontinuous epitopes are dependent on the native configuration of the proteins and are destroyed by protein denaturation (Morris, 2001; Murphy *et al.*, 2007; van Regenmortel, 2009).

The procedure of locating these immuno-reactive sub regions on the antigens interacting with the antibodies, is termed as ‘epitope mapping’ (van Regenmortel, 2009) and is a critical step for many applications. Epitopes can be determined either based on their structural or functional significance. Structural epitope mapping is defined as identifying amino acids in the antigen binding region which are in contact with the antibody paratope. However, these amino acids may or may not be significant for antigen-antibody binding. On the other hand, functional epitopes are determined by identifying amino acid residues which are important for antigen-antibody binding.

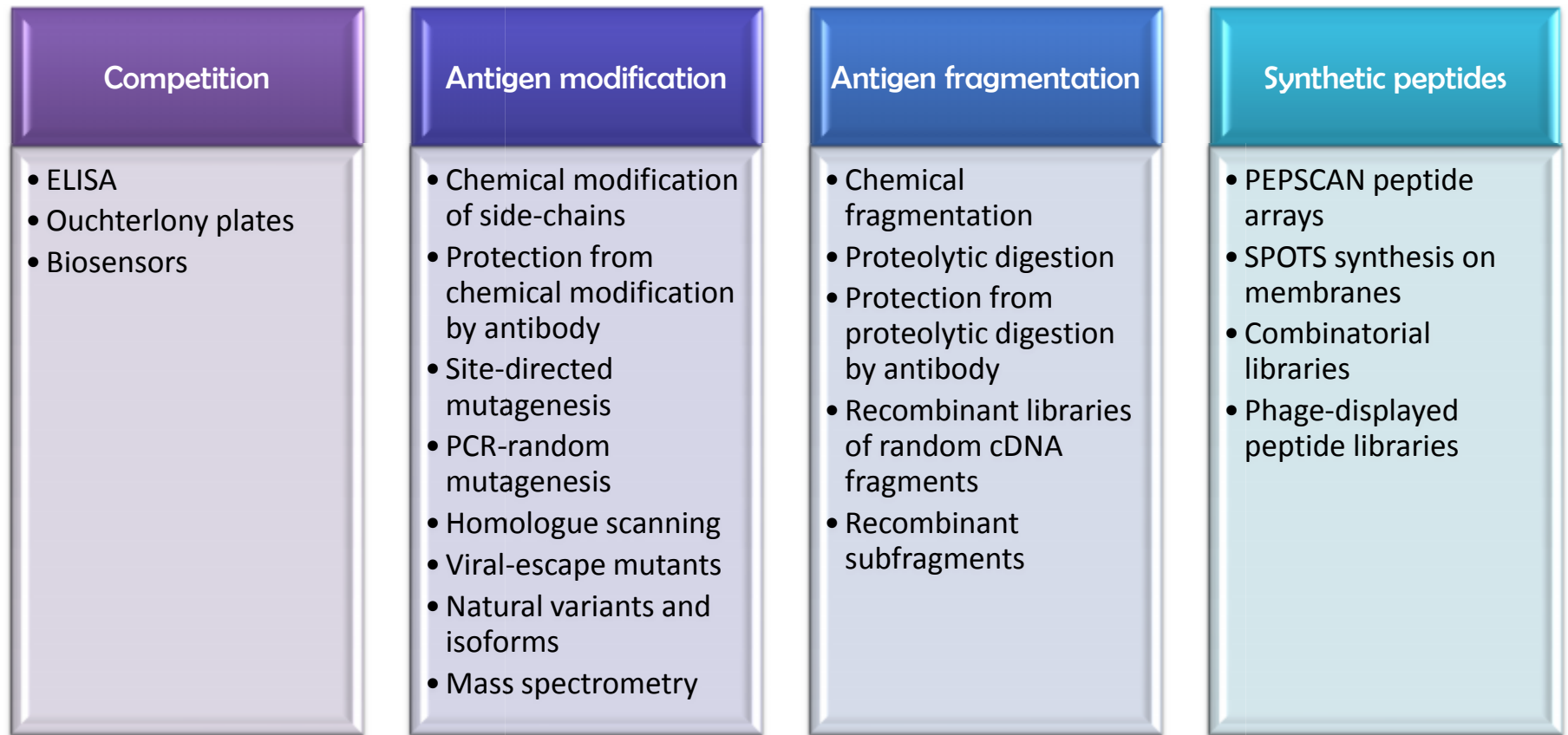
Epitope mapping is mainly focussed on identifying the functional epitopes (van Regenmortel, 1989).

Epitope mapping finds extensive use in studying antibody-antigen interactions, development of sensitive diagnostic assays, immunochemistry and synthetic vaccine designing (Beck-Sickinger and Jung, 1993; Morris, 2001). Recognition of epitope is also useful in development of diagnostic kits where the availability or the cost of the antigen is the limiting factor. The region of antigen recognised by the antibodies can be synthetically synthesised and utilised as antigen control. Similarly, the epitope can be expressed in a bacterial expression vector and fused with an affinity tag. The fusion protein thus obtained can be utilised for affinity purification of the antibodies (Maier *et al.*, 2010). Additionally, the recognition of epitope can provide valuable information of the specificity of the antibody and can be useful for describing antigen-antibody binding in patents (Ladner, 2007).

A variety of experimental approaches are used for identification of the antigenic epitopes binding to immunoglobulins. Methods such as X-ray crystallography, nuclear magnetic resonance (NMR) and electron microscopy are the methods employed for structural epitope mapping (Morris, 2008). They provide a three-dimensional (3D) insight in antigen-antibody binding. However, they are costly, time consuming and cumbersome approaches with relatively low success rates (Morris, 1996; Rockberg *et al.*, 2008). Various methods are reported for functional epitope mapping (Figure 5.1). However, the most widely employed strategies for epitope mapping are either based on fragmenting the antigen or using peptides as recognition elements. In the first approach the antigen is broken down in smaller and smaller fragments and the fragment/s reacting with the antibody provide an insight into the epitope being recognised by the antibody.

In the second approach, a pool of peptides is used as possible antigens for antibody recognition (Pettersson, 1992; Fack *et al.*, 1997). The peptide(s) binding to the antibody is recognised as epitope or mimotopes; where a mimotope is a peptide, not present in antigen sequence, but having the ability to bind to a particular antibody by mimicking the antigen epitope (Geysen *et al.*, 1986; van Regenmortel, 2009).

Recently, phage display has enabled researchers to utilise the second strategy (i.e. the strategy utilising peptides), more efficiently and swiftly by allowing the display of many peptides (a peptide library), on the surface of the phage and subsequently, selecting the peptide(s) reacting specifically with the antibody by biopanning. Certain random peptide (RPLs) libraries, displaying random, different sized peptides, are available commercially (<http://www.neb.com/nebecomm/products/category52.asp#126>; <https://commerce.invitrogen.com/index.cfm?fuseaction=viewCatalog.viewProductDetails&productDescription=723>). Alternatively, the gene (DNA) encoding the antigen of interest is PCR-amplified and partially digested with endonuclease (e.g. *DNaseI* or specific restriction enzymes) to generate 50-400 bp long fragments, which are used to construct a gene-fragment library (also known as antigen-fragment libraries (AFLs)) (van Zonneveld *et al.*, 1995; Gupta *et al.*, 1999; Irving *et al.*, 2001).



**Figure 5.1: Strategies for mapping of functional epitopes.**

*An array of techniques is reported for determination of antigenic epitopes. Commonly used methods include subfragmentation of the antigen or utilising peptides.*

*(Compiled from Morris, 2008).*

## 5.2 Epitope mapping using Ph.D.<sup>TM</sup>-12 Peptide Library

Epitope mapping of 3.G9 scFv was initially attempted using a commercial peptide library Ph.D.<sup>TM</sup>-12. This is a random peptide library, with a complexity in excess of two billion independent clones, constructed by fusing numerous dodecapeptide sequences to a minor coat protein (pIII) of the M13 phage. The displayed peptides (12-mer) are expressed as fusion products at the N-terminus of pIII separated by a short (G<sub>3</sub>S) spacer. The phage particles, expressing peptide(s) reacting specifically to the 3.G9 antibody, can be detected using biopanning.

### 5.2.1 Selection of anti-hFABP scFv specific peptides

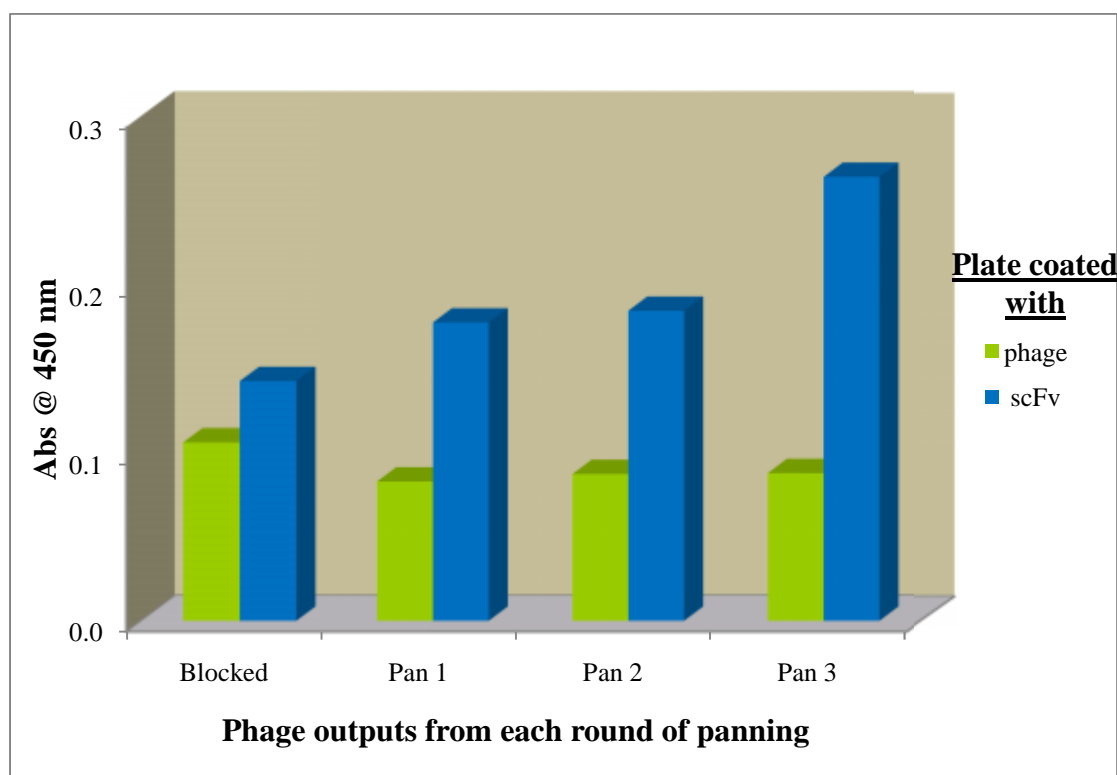
The library was depleted against the capture antibody immobilised on magnetic beads before panning against the scFv, for removal of any phage binding to the beads. Three rounds of panning against anti-hFABP scFv 3.G9 were then carried out using the commercial Ph.D.<sup>TM</sup>-12 peptide library. Anti-His and anti-HA-specific antibody-immobilised magnetic beads were used alternatively to capture the HA and His-tagged-3.G9 scFv, thus, ensuring proper orientation of scFv paratope. With each round of panning the concentration of scFv was decreased and the stringency of washing was increased. A subsequent increase in the titre of the output phage was obtained at each round of panning suggesting the enrichment of scFv-specific binding phage (Table 5.1).

**Table 5.1: Phage output and input titre obtained at each round of panning.**

Panning round	Output titre
Pan 1	4 x 10 <sup>10</sup>
Pan 2	8 x 10 <sup>10</sup>
Pan 3	2.4 x 10 <sup>11</sup>

### 5.2.2 Screening of anti-hFABP scFv specific peptides

Polyclonal phage ELISA (Figure 5.2) was carried out to assess the enrichment of phage specific to the 3.G9 scFv (section 2.2.1.12 and 2.2.3.13). Two approaches were used to find the best possible way to measure the scFv-peptide binding which may be problematic due to the small size of the expressed peptides and possible loss of epitope accessibility: (i) coating the phage directly on a plate and passing scFv over it and (ii) coating the scFv on a plate and passing phage over it, to detect the interaction between scFv and phage. Based on the analysis, Pan 3 output was used for further selection of clones expressing peptides for sequencing analysis.



**Figure 5.2: Polyclonal phage ELISA using phage outputs obtained from each round of panning.**

*A slight increase in the signal was observed on coating the scFv on the plate and assaying phage outputs (in duplicate) against it. However, on coating the phage outputs there was a low signal which was consistent in all the rounds which may be due to the selection of phage against anti-HA antibody, on magnetic beads, despite of the depletion step in panning.*

Blue/White screening was used for selecting the phage for sequencing analysis. It is a commonly used screening approach by which clones expressing the functional *lacZ* gene are identified by plating them on media containing X-gal-IPTG. The phage cloning vector, M13KE, is derived from the cloning vector M13mp19, which contains a *lacZ* gene. X-gal in the media is cleaved by  $\beta$ -galactosidase (expressed by the *lacZ* gene in the presence of IPTG) yielding galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter is then oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product, thus, differentiating the library phage plaques from the wild-type filamentous phage.

Twenty blue plaques from the plates were selected and infected into *E. coli* K12 ER2738 cells. The cells were grown for 4.5 hours and the cells were centrifuged to collect the supernatant containing the phage. The phage obtained was screened against 3.G9 scFv by ELISA. No signal was obtained against the scFv.

### **5.2.3 Sequencing of peptides selected after panning**

As no specific signal was obtained against the 3.G9 scFv in ELISA, the supernatant containing the phage was further processed for isolating single stranded DNA (section 2.2.3.1.4). The DNA which was sequenced and translated to determine the peptide sequences selected and enriched against 3.G9 scFv by panning. Out of 20, the majority of peptides were of no value as no specific peptide sequence related to the hFABP sequence was obtained. These peptides had a high proportion of the amino acid, histidine, which may have been selected due to the anti-His antibody immobilised on the magnetic beads. Three peptides representing HA-tag sequence were also obtained. These may have been selected by the anti-HA antibody immobilised on the magnetic beads, similar to anti-His antibody, which was used as capture antibody for the scFv. Only 3 peptides resembled a stretch of sequence (5 amino acids) from the hFABP,



underlined in Table 5.2. This sequence was checked in other isoforms of hFABP. As the scFv 3.G9 is highly specific to cardiac isoform (section 3.6), it is necessary that the obtained peptide sequence is not present in any other FABP isoform. The peptide sequence was found to be specific to the cardiac isoform of FABP (hFABP).

**Table 5.2: Peptide sequences obtained on biopanning commercial Ph.D.<sup>TM</sup>-12 Peptide Library.**

Type of peptide sequences obtained	No. of colonies obtained	Peptide sequence
Non-relevant sequences	14	QSPVNHYYHYHI SLRATPFHDQLA STHHRHYHDTLA STHHRHHHH KHMHWHPALNT
HA tag sequences	3	YPYDVDPDYA
hFABP-specific peptide	3	YPTTHAHT <u>TPVR</u>

One more round of panning was carried out to enrich for the hFABP-specific phage. The panning was carried out by coating the scFv on a plate, without any capture antibody, to ensure the selection of peptide-expressing phage against scFv paratope rather than the against the capture antibody immobilised on beads. Enrichment of the peptide in biopanning against the scFv will increase its probability of representing the antigenic epitope. Ten plaques were selected by blue/white screening and sequenced. Sequencing results yielded only one peptide sequence specific to hFABP. In the absence of conclusive data on possible epitope of 3.G9 scFv, hFABP-specific gene-fragment library was constructed.

### **5.3 Epitope mapping using gene-fragment library**

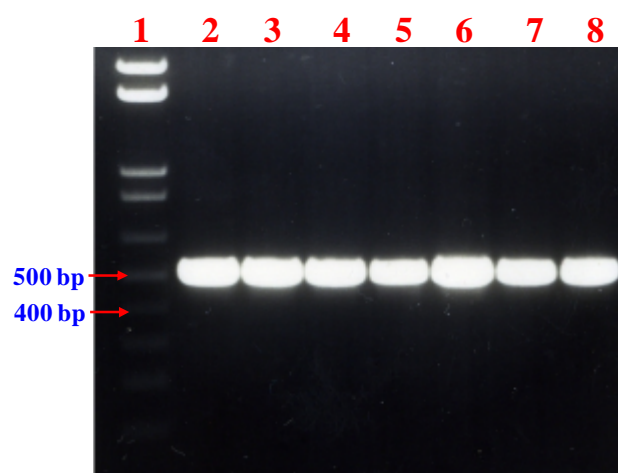
Gene-fragment library was constructed to overcome the problems encountered with commercial Ph.D.<sup>TM</sup>-12 Peptide Library. This library was constructed from specific gene of the protein against which antibody was generated, thus, the chances of getting peptides specific to the epitope are more when compared to commercial peptide libraries.

#### **5.3.1 Construction of gene-fragment library**

Gene-fragment library construction involved 3 important steps: (i) modification of vector, (ii) generation of gene-fragments and (iii) its processing for cloning into the vector and cloning and transformation into *E. coli*.

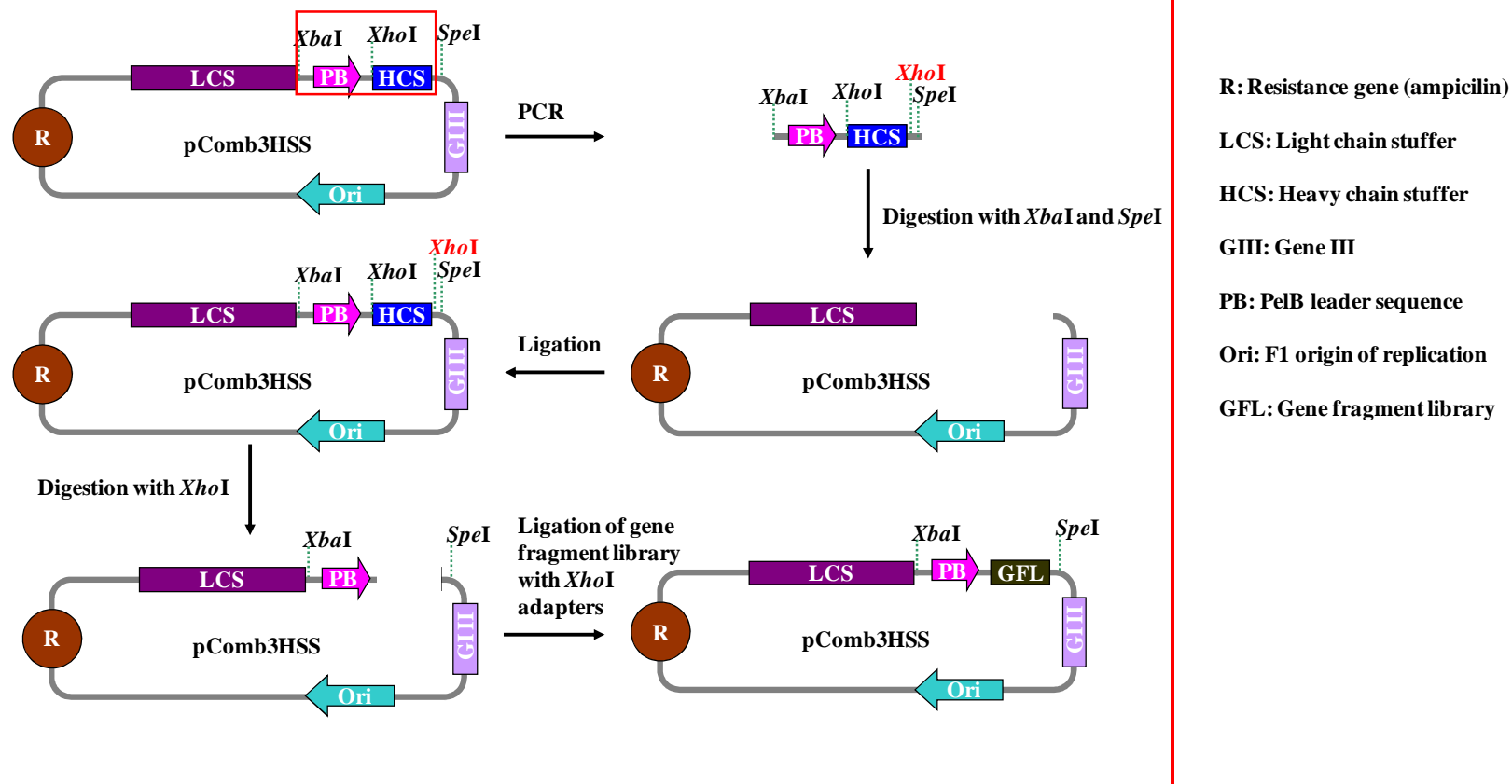
#### **5.3.2 Modification of vector**

Construction of gene-fragment library requires generation of cohesive ends on the gene-fragments, generated by partial digestion of the gene. It can be achieved by adding adapters to the ends of the gene-fragment, containing restriction sites compatible with vector for ligation. Commercially available *Xho*I adapters were used for this purpose. Due to unavailability of vector containing two *Xho*I restriction sites for gene-fragment library cloning in the lab, pComb3HSS vector was modified. The PelB leader sequence along with heavy chain stuffer sequence, containing *Xba*I site at N-terminal and *Spe*I site at C- terminal, were amplified from the vector (Figure 5.3). The selected sequence already had an *Xho*I site before the heavy chain stuffer sequence. The amplified product was purified and used as template for another PCR with reverse primer containing an additional *Xho*I sequence before the *Spe*I sequence, thus, adding an *Xho*I restriction site to the amplified product (Figure 5.4). The amplified product along with the pComb3HSS vector were digested with *Xba*I and *Spe*I and ligated.



**Figure 5.3: Amplification of *PelB* and heavy chain stuffer from *pComb3HSS*.**

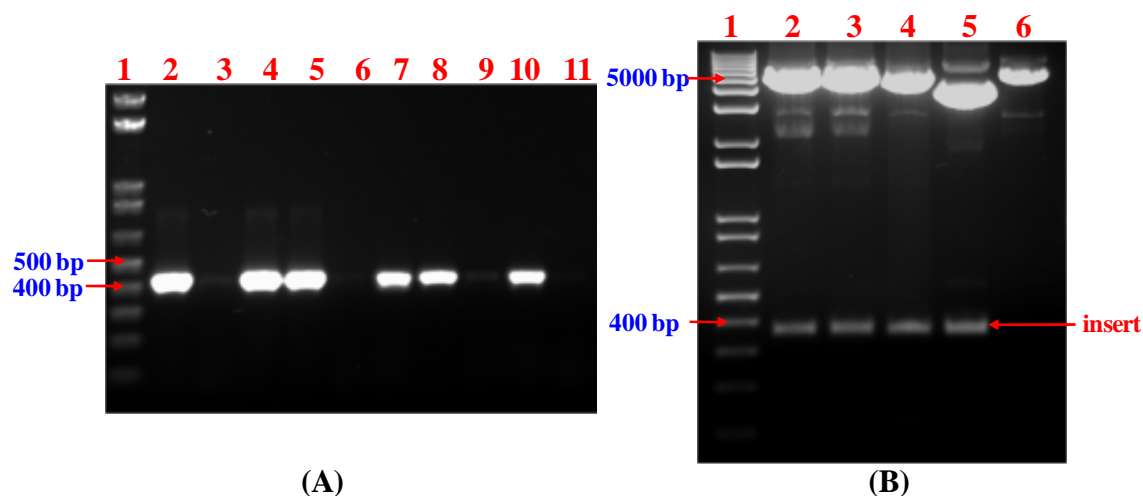
*The annealing temperature was optimised for amplifying the *PelB* leader sequence and heavy chain stuffer sequence from the *pComb3HSS* vector. No major differences in the amplification of the product were observed by varying the annealing temperature. All the amplified products were pooled, purified and cloned into the vector. Lane 1: 1 kb DNA ladder plus; lane 2: 56.0°C; lane 3: 57.2°C; lane 4: 58.0°C; lane 5: 59.1°C; lane 6: 60.0°C; lane 7: 61.2°C and lane 8: 62.5°C.*



**Figure 5.4: Modification of *pComb3HSS* vector.**

A systematic approach to introduce an *XhoI* restriction site in the vector is shown. The *XhoI* restriction site was added in the reverse primer carefully maintaining the ORF of the remaining vector for facilitating the cloning of hFABP-gene-fragments into the vector using *XhoI* adapters. Two different restriction sites (*XbaI* and *SpeI*) were selected for cloning the modified region into the vector.

The digested amplicon was cloned into pComb3HSS vector. Initially the transformants were checked by colony pick PCR (section 2.2.1.12) (Figure 5.5(A)). The positive clones from the PCR were then selected and their plasmids were digested with *Xho*I to ensure the release of inserts (Figure 5.5(B)).



**Figure 5.5: Selection of transformants with modified vector.**

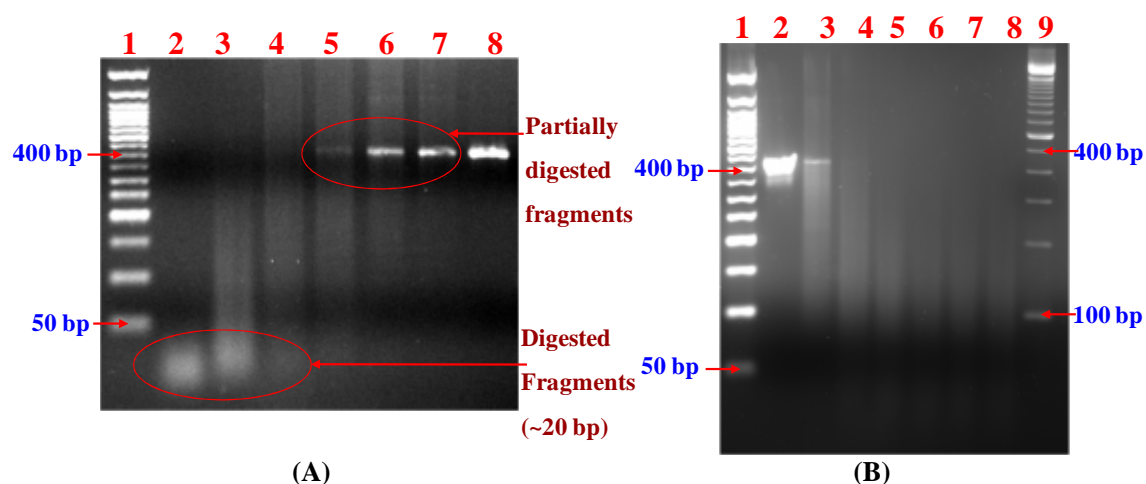
*(A) Screening of transformants by colony pick PCR. Lane 1: 1 kb DNA ladder plus; lane 2-11: colonies after transformation.*

*(B) Restriction analysis of the positive transformants from colony pick PCR. Lane 1: 1 kb DNA ladder plus; lane 2: clone 1; lane 3: clone 3; lane 4: clone 4; lane 5: clone 6 and lane 6: clone 7. Release of inserts from the digested plasmids confirmed the presence of 2 *Xho*I sites, required for the cloning of the gene-fragment library.*

### 5.3.3 Generation of gene-fragments and cloning into modified pComb3HSS vector

The gene was amplified from an hFABP-expressing clone 3 (section 4.4). The gene was digested with different concentrations of *DNase*I enzyme for different periods of time to optimise both the concentration of enzyme and duration of digestion for generation of approximately 50-100 bp fragments (Figure 5.6). Small scale reactions were carried out which were later scaled-up. The digested fragments were resolved on a 2.5% agarose gel and purified. The fragments were further polished to create blunt ends for attaching

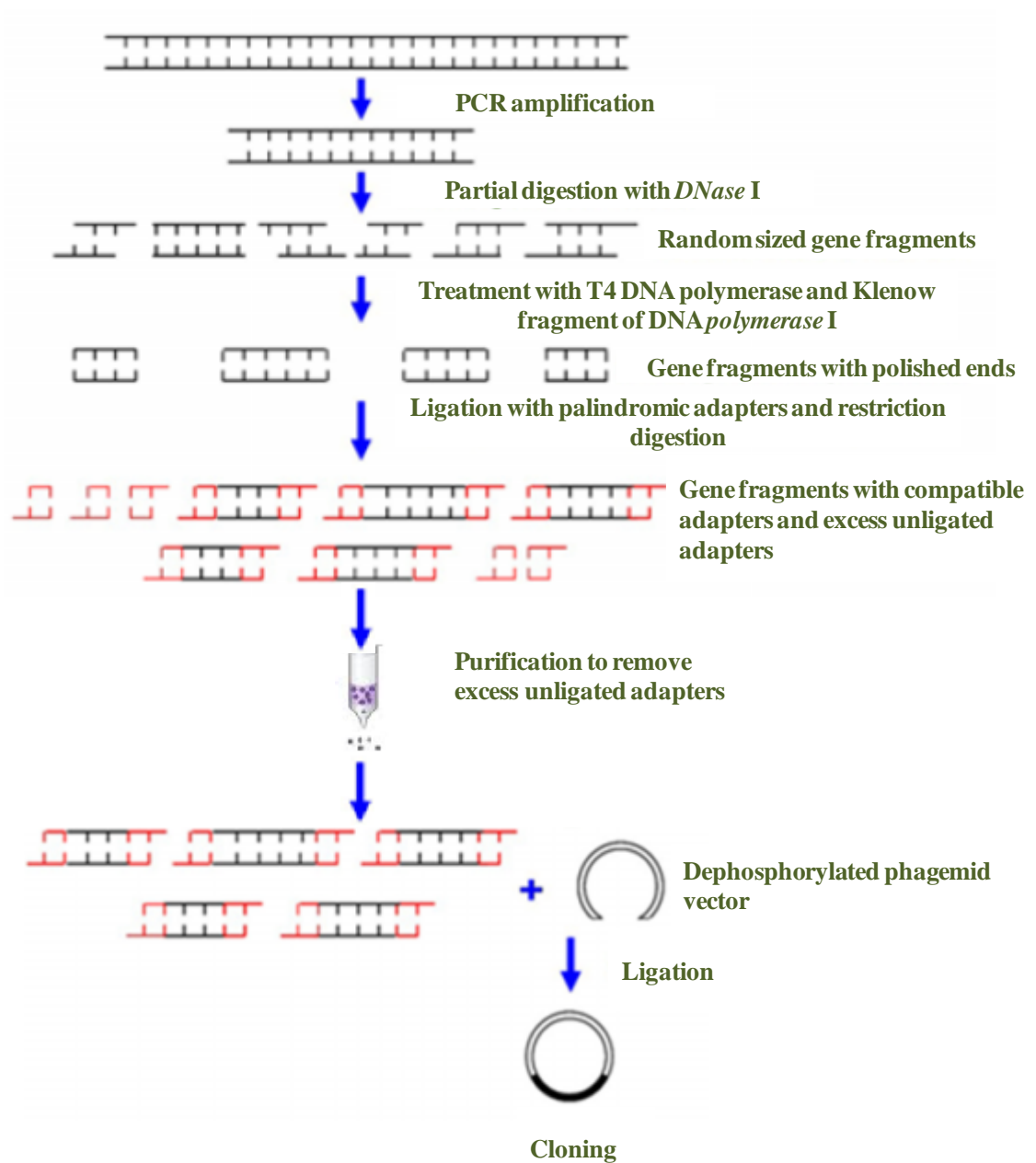
the adapters using T4 DNA polymerase and the Klenow fragment of DNA polymerase I, with robust exonuclease activity to remove 3' overhangs and filling 5' overhangs (3' recessed ends). Polished gene-fragments were ligated to an excess of *Xho*I adapters. The gene-fragments were further digested with the *Xho*I enzyme, to generate cohesive ends, and cloned into the vector (Figure 5.7).



**Figure 5.6: Optimisation of *hFABP* gene digestion by DNaseI.**

**(A)** Optimisation of DNaseI concentration. Lane 1: 50 bp DNA ladder; lane 2: 0.062 unit/μg DNA; lane 3: 0.031 unit/μg DNA; lane 4: 0.015 unit/μg DNA; lane 5: 0.007 unit/μg DNA; lane 6: 0.003 unit/μg DNA; lane 7: 0.001 unit/μg DNA and lane 8: undigested DNA. The digestion was carried out for 10 minutes. Even with very low concentrations of the enzyme 50-100 bp fragments could not be obtained efficiently. Hence, the time of the digestion was varied, using 0.010 unit/μg DNA.

**(B)** Optimisation of the duration of DNaseI digestion. Lane 1: 50 bp DNA ladder; lane 2: undigested DNA; lane 3: 2 minute digestion; lane 4: 4 minute digestion; lane 5: 6 minute digestion; lane 6: 8 minute digestion; lane 7: 10 minute digestion; lane 8: 12 minute digestion and lane 9: 100 bp DNA ladder. A four minute digestion with 0.010 unit/μg DNA yielded 50-100 bp DNA fragments efficiently which was further scaled up for the construction of the library.

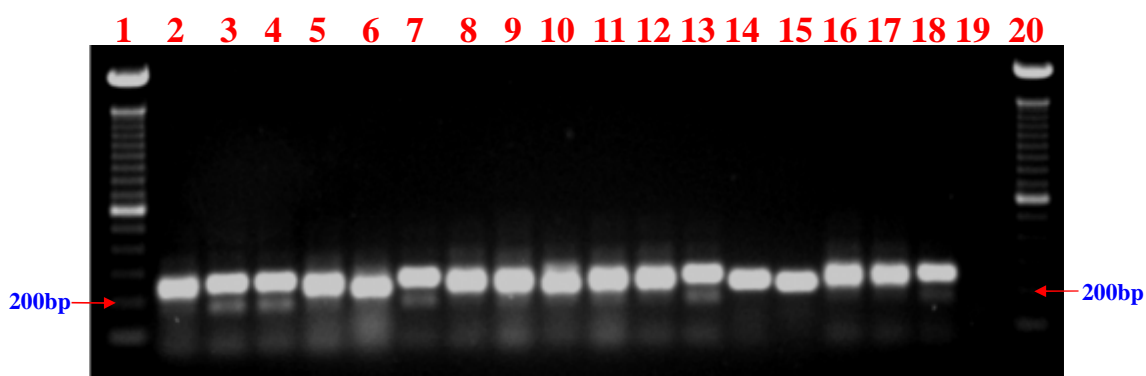


**Figure 5.7: Strategy for construction of gene-fragment library.**

Various steps used in gene-fragment library construction are diagrammatically represented. The gene was amplified and partially digested creating random sized fragments. Random fragment size is controlled by optimising the conditions of digestion.

### 5.3.4 Selection and screening of the gene-fragment library

A library of  $6.1 \times 10^5$  cfu/mL size was generated. The transformants were screened by colony pick PCR using a forward primer designed after the PelB region in the vector and the reverse primer, used in the modification of the vector, to check the inserts (Figure 5.8). The 3.G9 scFv was converted to scAb to enable stable capture of antibody using anti-chicken Fab antibody. The format for biopanning was checked to ensure that the scAb paratopes are free for binding the antigen (Figure 5.9). Three rounds of panning were carried out using similar conditions of antigen concentration and washing, for selecting and enriching scAb-specific peptides.

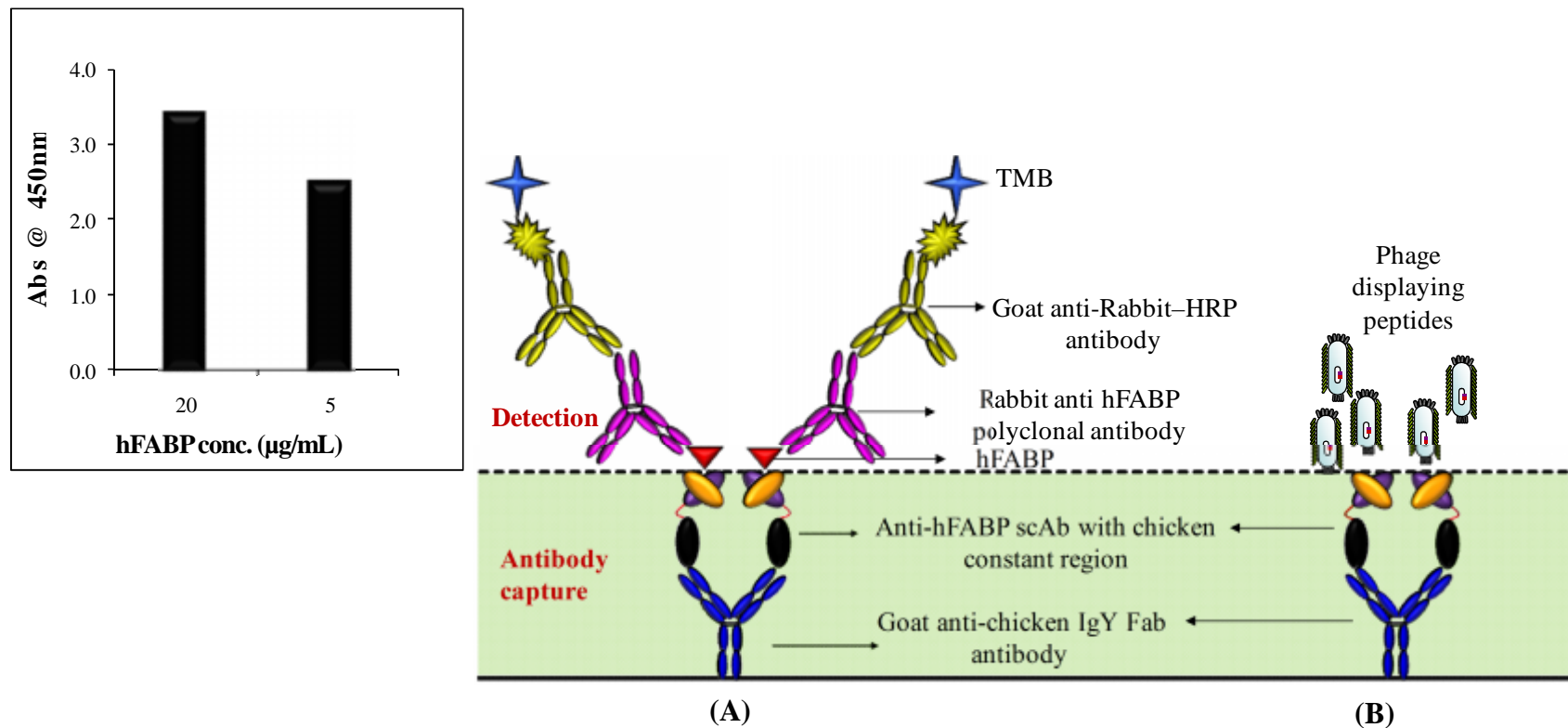


**Figure 5.8: Colony pick PCR to check the gene-fragment library.**

*Lane 1 and 20: 1 kb DNA ladder plus; lane 2-19: colonies after transformation.*

*Colony pick PCR showed the presence of inserts of variable sizes. Taking into account the amplified vector size along with the inserts, the fragments should be almost 50-100 bp in size.*





**Figure 5.9: Format selection for biopanning of hFABP-specific gene-fragment library.**

**(A)** The antibody capture format was checked for antigen binding prior to biopanning. The format was analysed by capturing the scAb (3.G9), over which the hFABP was passed. This was subsequently probed with rabbit anti-hFABP polyclonal antibody. The antigen-antibody interaction was detected using HRP-conjugated anti-rabbit antibody. This analysis was carried out using 2 different hFABP concentrations (20 µg and 5 µg), respectively. The graph on left-hand side shows that a high signal was obtained for both antigen concentrations showing that the antigenic epitope is detected in this format by the scAb.

**(B)** Diagrammatic representation of the format used for the biopanning of the gene-fragment library.

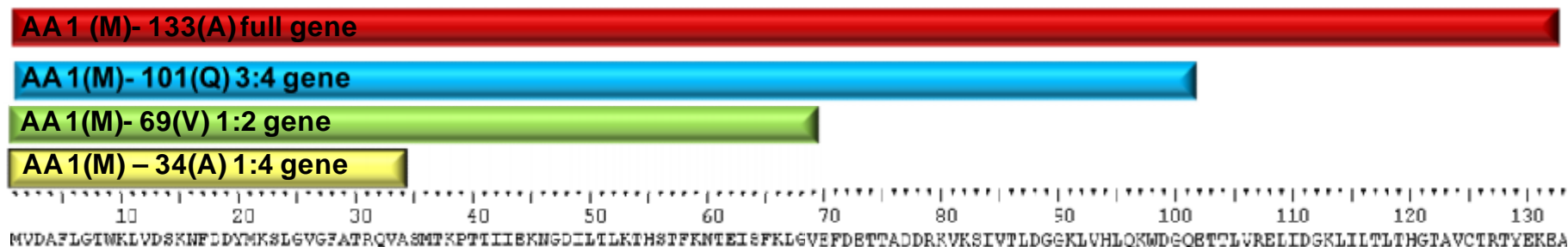
A low titre of the phage was obtained after each round of panning, indicating a low carryover of the 3.G9 scAb specific phage. No signal was obtained when the phage outputs were analysed by polyclonal phage ELISA. Biopanning was repeated 3 times changing the conditions and antibody coating concentrations. However, specific phage populations detecting 3.G9 scAb could not be obtained.

#### **5.4 Epitope mapping by recombinant expression of gene-fragments**

As the specific gene-fragment library failed to generate any result, epitope mapping using gene walking was carried out. Gene walking is a process in which overlapping gene-fragments from the entire gene are expressed and checked against the target antibody. Primers were designed for amplification of gene-fragments which were used in conjugation with the existing forward and reverse primers used for hFABP expression (section 2.2.2.1).

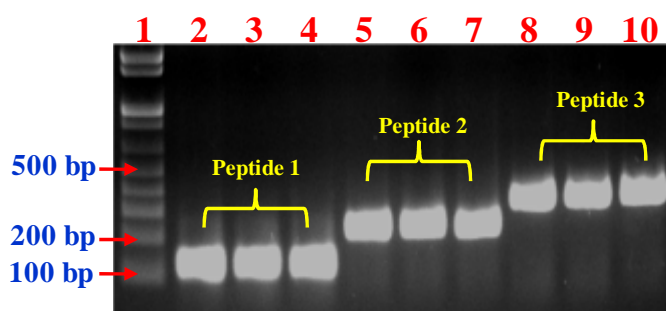
##### **5.4.1 Expression of gene-fragments by excluding regions at C-terminal of the hFABP**

Overlapping gene-fragments throughout the length of the hFABP gene were amplified. In total 7 different gene-fragments were amplified and expressed with a N-terminal fusion protein by cloning into pET-32b(+) vector. Three peptides were expressed keeping the forward primer constant and changing the reverse primer, thus, expressing approximately 1/4, 1/2, and 3/4 regions of the hFABP gene (Figure 5.10 and 5.11).



**Figure 5.10: Targeted hFABP gene-fragments for epitope mapping.**

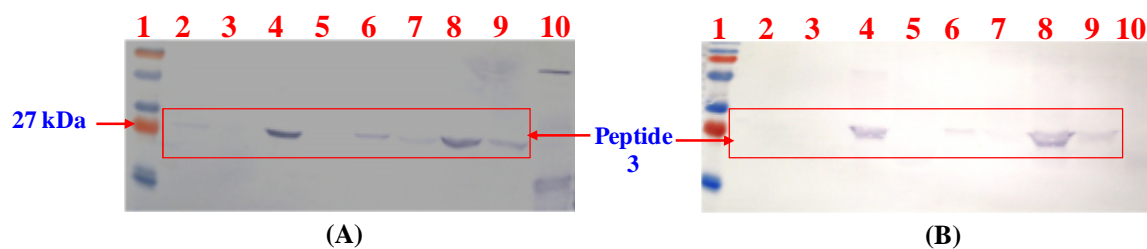
*In total 3 regions were expressed to check the epitope of 3.G9 scFv. The peptides were first expressed in 5-3' direction of the gene covering the entire sequence. Peptides covering the majority of gene sequence were checked first followed by excluding a part of the gene (C-terminal truncated) in every step. All the peptides have overlapping regions which when checked against the 3.G9 scFv will provide accurate data on its epitope.*



**Figure 5.11: Amplification of gene-fragments for peptide expression.**

The *hFABP* gene was divided into 4 regions. Three overlapping regions were amplified from the plasmid of *hFABP* expressing clone 3. Three different annealing temperatures were tried for the amplification of gene-fragments. As there was no significant influence of annealing temperature on the amplification of gene-fragments, the amplified products were pooled and purified for cloning. Lanes 1, 5 and 9: 1 kb DNA ladder plus; lanes 2, 3 and 4: peptide 1 at annealing temperatures of 60°C, 61°C and 62°C, respectively; lanes 6, 7 and 8: peptide 2 at annealing temperatures of 60°C, 61°C and 62°C, respectively and lanes 10, 11 and 12: peptide 3 at annealing temperatures of 60°C, 61°C and 62°C, respectively.

The amplified gene-fragments were cloned into the pET-32b(+) vector and transformed into BL-21 *E. coli* cells. The peptide expressing 3/4<sup>th</sup> of the gene (peptide 3) was checked by Western blot analysis using a HRP-conjugated-anti-His antibody. All the clones were simultaneously checked against the 3.G9 scFv for binding (Figure 5.12).



**Figure 5.12: Peptide 3-expression and analyses for epitope determination.**

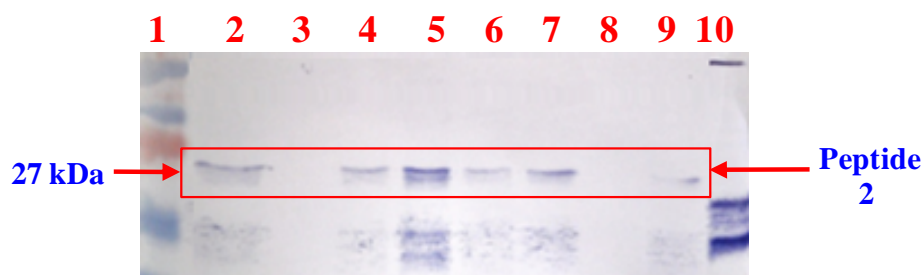
Successful expression of the peptide 3 is shown by the Western blotting analyses. The blot shows a thick band of appropriate size for peptide 3 (~24 kDa) that can be observed clearly in several clones (3, 8 and 9) which is absent in the control (10).

(A) Blot developed using HRP-conjugated-anti-His antibody.

(B) Blot developed using anti-hFABP scFv 3.G9 scFv.

In both blots, Lane 1: Fermentas prestained protein marker; lanes 2 to 9: different peptide 3 transformed clones; and lane 10: negative control (HEXA gene cloned in pET-32b(+) vector).

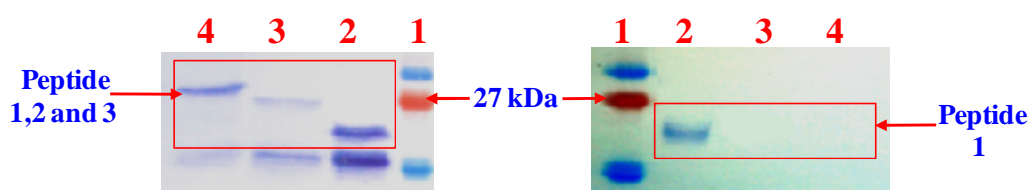
The Western blot analysis indicated the presence of the 3.G9 scFv epitope in the region represented by the peptide 3, due to the presence of the band at the appropriate size (~24 kDa) in the blot developed with the 3.G9 scFv. Peptide 3 is composed of three regions; two represented by peptide 1 and 2, along with one another region at the C-terminal (Figure 5.10). For further pin-pointing of the epitope, peptide 2-expressing clones were checked with the 3.G9 scFv. The clones obtained from peptide 2 transformation were checked by Western blot analysis with an HRP-conjugated-anti-His antibody and 3.G9 scFv. Western blot with the HRP-conjugated-anti-His antibody showed that the clones selected were expressing the peptide (Figure 5.13). However, none of the expressed peptides reacted with 3.G9 on the second blot. This analysis revealed that the region of peptide 3 containing the epitope should be in the third part of peptide (i.e. not peptide 1 and 2).



**Figure 5.13: Analyses of peptide 2 for expression for epitope mapping.**

Successful expression of the peptide 2 is shown by the Western blotting analyses with HRP-conjugated-anti-His antibody. The blot shows bands of appropriate size for peptide 2 that can be observed clearly in several clones (2,4,5,6,7 and 9), but is absent in the control (10). Another blot containing all the clones was checked using the anti-hFABP scFv 3.G9. The scFv failed to recognise the peptides. Lane 1: Fermentas prestained protein marker; lanes 2 to 9: different transformed clones of peptide 2; and lane 10: negative control (HEXA gene cloned in pET-32b(+)) vector).

A peptide (GGKLVHLQKWDG) was synthesised commercially from the region of peptide 3 (not covered by 1 and 2). This peptide was checked against 3.G9 scFv by ELISA. No positive signal was observed. Therefore, the peptide 1, 2 and 3-expressing clones were checked again by Western blotting using anti-S-tag antibody, targeting the S-tag fusion protein expressed by the pET-32b(+) vector. All the peptides were shown to be expressed. However, on reanalysing the peptides only peptide 1 reacted with the scFv (Figure 5.14).

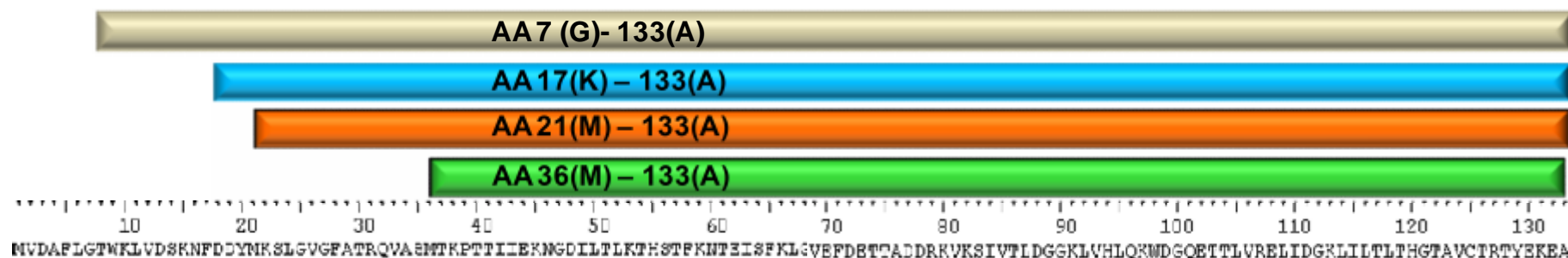


**Figure 5.14: Expression analysis of peptides and blotting with 3.G9 scFv.**

Blotting with anti-S-tag antibody showed that the peptides were being expressed. However, only peptide 1 was recognised by the 3.G9 scFv, rather than peptide 3 which was recognised before. Lane 1: Fermentas prestained protein marker; lane 2: Peptide 1 expressing clone; lane 3: Peptide 2 expressing clone and lane 4: Peptide 3 expressing clone.

#### 5.4.2 Expression of gene-fragments in by excluding regions at N-terminal of the hFABP gene

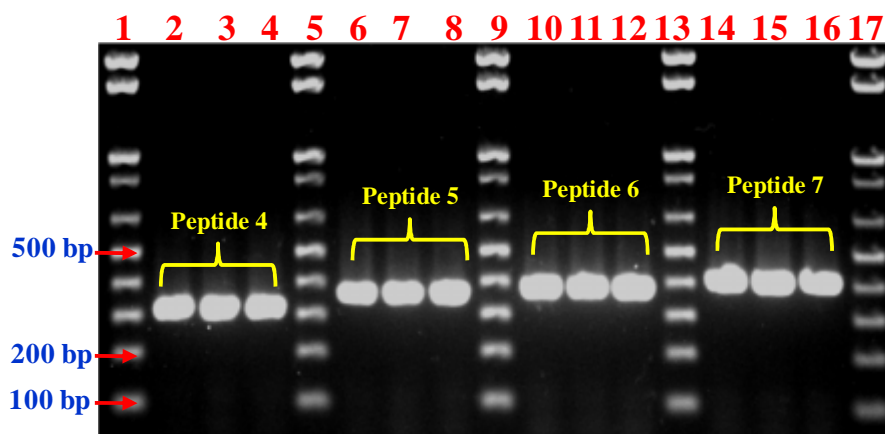
Based on the facts that the results were inconclusive and the only putative positive binding response at various stages of this experiment was found towards peptide 1. It was postulated that the epitope was located in the N-terminal region, thus, it was decided to re-express protein but with its complete C-terminal domain. It was carried out by sequentially truncating the N-terminal of the protein hoping that this would allow a precise determination of the exact epitope (Figure 5.15 and 5.16). The gene-fragments were amplified from the plasmid of hFABP expressing clone 3 and then cloned into pET-32b(+) vector. The N-terminal truncated hFABP clones were expressed and checked by HRP-conjugated-anti-His antibody. All the peptides were found to be expressed (Figure 5.17). However, when checked with 3.G9 scFv for epitope mapping, none of them were found to be binding the scFv.



**Figure 5.15: *HFABP* gene-fragments for epitope mapping expressing N-terminal truncated protein.**

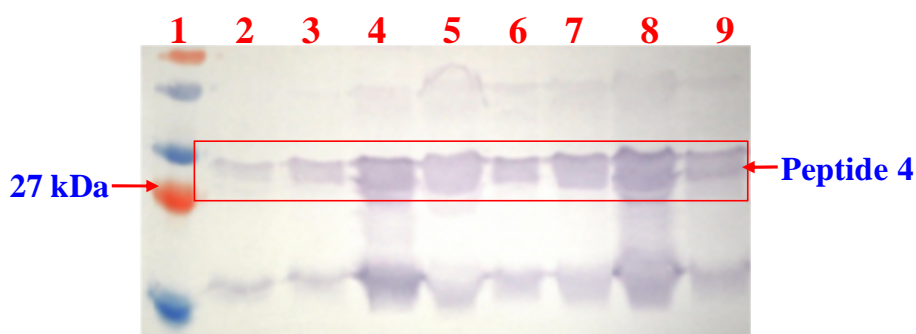
*The results obtained by truncating the hFABP gene from C-terminal were inconclusive. Therefore, in an alternative approach, truncated proteins (approximately 94-72% of the hFABP), excluding different lengths of N-terminal domain and with complete C-terminal domain, were expressed.*





**Figure 5.16: Amplification of gene-fragments for peptide expression of N-terminal truncated hFABP.**

Three different annealing temperatures were tried for the amplification of gene-fragments. As there was no significant influence of annealing temperature on the amplification of gene-fragments, the amplified products were pooled and purified for cloning into the pET-32b(+). Lanes 1, 5 9, 13 and 17: 1 kb DNA ladder plus; lanes 2, 3 and 4: peptide 4 at annealing temperatures 60°C, 61°C and 62°C; lanes 6, 7 and 8: peptide 5 at annealing temperatures 60°C, 61°C and 62°C; lanes 10, 11 and 12: peptide 6 at annealing temperatures 60°C, 61°C and 62°C and lane 14, 15 and 16: peptide 7 at annealing temperatures 60°C, 61°C and 62°C.



**Figure 5.17: Analysis of peptide 4 expression using HRP-conjugated-anti-His antibody.**

Peptide 4 was successfully expressed, as shown by the Western blotting analyses with HRP-conjugated-anti-His antibody. There was no binding observed when the same clones were checked with 3.G9 scFv. Lane 1: Fermentas prestained protein marker and lane 2-9: Peptide 4-expressing clone.

The analysis was repeated again using peptide 1 along with peptide 4, to check the results obtained earlier, where the peptide 1 was binding to 3.G9 scFv (Figure 5.14). The peptide 1 and peptide 4-expressing clones were freshly expressed and checked by Western blotting using 3.G9 scFv. Peptide 4 represented 94%, whereas, peptide 1 represented 25 % of the hFABP gene. Both the analyses were carried out in parallel as the amino acids not expressed in peptide 4 were expressed by peptide 1. There was no binding of peptides with 3.G9 scFv on western blot, showing inconsistency in the previous results obtained (section 5.4.1). The results obtained with gene walking failed to provide any conclusive result on 3.G9 epitope.

## 5.5 Discussion

The chicken 3.G9 scFv was found to be highly specific against the cardiac isoform of FABP based on cross-reactivity studies carried out against different isoforms of FABP (section 3.6). In addition to the specificity, the scFv was used to generate a high sensitivity assay for hFABP. The research described was planned in order to determine the epitope recognised by the 3.G9 scFv on hFABP which would be an interesting aspect for further characterisation of the antigen-antibody interaction. This is useful for patenting purposes, for designing the epitope synthetically for use in diagnostic assays and for immunisation of animals for generation of specific antibodies.

For epitope mapping, the starting step is usually the distinction between continuous and discontinuous epitopes by Western blotting. Previous analysis revealed that the scFv 3.G9 reacts to the antigen in its denatured form on a Western blot (section 4.4), implying that the epitope is continuous (Fack *et al.*, 1997; Morris, 2008). Phage-display technology was widely used in epitope mapping studies, to characterise antigen-antibody interactions (Fack *et al.*, 1997), owing to the advances made in optimisation of various phage display components (Irving *et al.*, 2001) and the ease of assaying a large repertoire of targets.

Initially, a commercial random peptide display library displaying 12-mer peptides was used for screening of the hFABP epitope recognised by the 3.G9 scFv. Random peptide libraries offer the advantages of being straightforward to use, simple in methodology and inexpensive to set up. Also, no prior knowledge of the target protein is required since a known receptor or antibody can be used to capture the desired ligands from random peptide libraries displayed by phage (Smith and Scott, 1993). One peptide library can also be used for many antibodies that have a variety of different specificities and affinities (Pinilla *et al.*, 1999). Peptide libraries also have the ability to select mimotopes to antigenic epitopes which are useful in certain cases (Geysen *et al.*, 1987; Felici *et al.*, 1993; Cortese *et al.*, 1995; Stephen *et al.*, 1995). A random peptide library was panned 3 times, alternatively against the scFv captured on anti-HA and on anti-His beads, to specifically enrich the peptides binding to the scFv paratope. Few peptides, from the panned output, were selected randomly by blue/white screening (<http://www.neb.com/nebecomm/manualfiles/manuale8101.pdf>) and sequenced for epitope determination. The majority of peptides were specific to the capture antibody immobilised on the magnetic beads for selective capture/orientation of 3.G9 scFv, even after a negative selection step in panning. However, 3 clones were identified that generated peptides that had the same sequence as hFABP. It was found that all the 3 peptide sequences were same and rich in histidines, making it difficult to understand whether the selection was specific to an scFv paratope or to the anti-His antibody on the magnetic capture beads. One more round of panning was carried out in the absence of any antibody capture beads to clarify the results. The peptide failed to enrich in the consecutive panning round, showing only 1 suspected hFABP-specific sequence (identified previously) among the 10 peptides from pan 4 output. These findings made it difficult to derive any conclusive information on the exact nature of the epitope.

It is a possibility that a random peptide library, despite a theoretical size of  $10^{12}$  peptides, may not include all possible amino acid combinations utilised by native antigenic epitope (Holzem *et al.*, 2001), as this method totally relies on the selection of peptides possessing high affinity towards the paratope which may or may not be similar to the antigenic epitope (Bottger *et al.*, 1995). However, short epitopes less than 6 amino acids can be identified using random peptide libraries. Therefore, epitope mapping was first attempted using a random peptide library (Fack *et al.*, 1997). However, due to the difficulties encountered with the random peptide library, a more specific gene-fragment library was constructed to carry out epitope mapping of 3.G9 scFv.

Gene-fragment libraries are small in complexity as compared to random peptide libraries. However, they offer the advantages of being more specific for the antibody, thus, requiring less rounds of selection (Holzem *et al.*, 2001). This feature makes it a rapid and reliable technique for epitope mapping. To further enhance the efficiency of the approach, Hyperphage<sup>TM</sup> was used instead of regular helper phage M13K07 to specifically enrich ORF's in order to reduce the number of junk clones and to promote polyvalent display of peptides (Hust *et al.*, 2006).

Moreover, the scFv was converted into a scAb by adding a chicken constant light chain. The scAb enables the use of an antibody against the chicken constant light chain to ensure proper orientation of the antibody thereby contributing to epitope accessibility. Three rounds of panning were carried out against the antigen. There was a decrease in the titre of phage outputs obtained at the end of each round of panning indicating less carryover of specific phage. This was verified when no positive signal was obtained in polyclonal ELISA. Three different strategies were tried for panning, i.e. by increasing

antigen coating concentration, by reducing washing steps and by the 'plating out' method to promote the growth of weak expressers. However, no signal could be obtained in polyclonal phage ELISA using any of these approaches.

The failure to obtain any antibody specific phage population from the gene-fragment library can be due to, (i) the disintegration of the epitope by *DNaseI*, which was used for random fragmenting of the gene for the construction of a gene-fragment library, (ii) low efficiency of the ligation of the adapters to the blunt ended fragments, and (iii) translational frame shift, which cannot be prevented in such libraries (Kawamura *et al.*, 2006).

Following a review of the results obtained by phage display methods, a more conventional method for epitope mapping was followed (Fack *et al.*, 1997). It involved the expression of overlapping gene-fragment sequences and probing them with the antibody by Western blotting. Peptide analysis was carried out by expressing overlapping peptides in both directions, i.e. N-terminal to C-terminal and *vice versa*. The peptides were expressed along with the fusion protein and were longer than those expressed by random and gene-fragment libraries. However, the results obtained from these libraries were not reproducible.

The results from Western blot analysis suggested that the epitope was linear. Therefore, the present study was designed on this basis. However, there are reports that some epitopes identified by Western blotting may still have conformational element(s) preserved (Morris, 2008). Several authors have obtained contradictory results from different studies pursued based on the reactivity of antigen, as determined on Western blots, as an indicator of continuous epitopes (Christensen *et al.*, 1996; Deregt *et al.*,

2005; Sui *et al.*, 2005; Veliceasa *et al.*, 2005). Renaturation of epitopes during or after the transfer of the protein (antigen) to the membrane might be the reason for the recognition of the continuous epitopes by antibodies on Western blotting (Zhou *et al.*, 2007). It was also suggested that the majority of B-cell epitopes may be conformational even though continuous (Bariow *et al.*, 1986). This concept provides a rationale for the results obtained suggesting the involvement of amino acids from several regions of hFABP contribute to the formation of the antigenic epitope. It is also a possibility that irreproducibility of results in expressed truncated peptide may be due to differences in the level of renaturation while transferring to membranes, which cannot be easily controlled.

The use of peptide libraries in epitope mapping is limited due to the small sizes of the fragments, which may/may not cover the complete determinants necessary for the formation of the antigenic epitope. However, in peptide walking the size of the fragments can be varied as required. Nevertheless, the results obtained throughout the epitope mapping of 3.G9 binding were inconclusive following multiple analyses. The consistent binding of 3.G9 scFv to hFABP (both commercial and recombinant forms) on Western blotting, in the current study, strongly suggested that the major part of the structure forming the epitope is protected from denaturation on SDS-PAGE. However, results from peptide walking suggested that there might be some conformational element involved in the epitope. All the epitope mapping analyses tried in this study were based on fragmentation of the antigen. Further attempts to identify the epitope can be made by using competition or antigen modification methods (Figure 5.1), which might be more efficient.

# **Chapter 6**

## **Generation of Rabbit Anti-cTnI scFv**

## **6.1 Introduction**

### **6.1.1 Rabbit as a host**

Rabbit antibodies were extensively used in research for decades owing to the outstanding feature of rabbit's immune system to generate high affinity and high specificity antibodies to human antigens (Popkov *et al.*, 2003; Mage *et al.*, 2006). The impetus for usage of rabbit antibodies in research is due to the evolutionarily distance of the animal from humans and mice making it an ideal host to generate antibodies to conserved antigens otherwise not recognised by rodents (Hofer *et al.*, 2007).

Rabbits are a member of the order Lagomorphs, with a somewhat different immune system organisation than humans and rodents. The major differences are the developmental processes involved in the generation of B-cell repertoire and the recombination of variable heavy and light genes to generate diversity.

In rabbits, B-cell development takes place after birth *via* a classical pathway in bone marrow (Knight and Winstead, 1997; Weill *et al.*, 2004). Following a short term lymphopoiesis, the B-cells seed gut-associated lymphoid tissues (GALT) such as the Peyer's patches, the sacculus rotundus and the appendix, where they expand and diversify. The primary antibody repertoire generated in rabbits is restricted to  $V_HDJ_H$  rearrangements resulting in a single heavy chain rearranged gene in 80% of B-cells (Knight and Winstead, 1997; Weill *et al.*, 2004). In contrast to the limited  $V_HDJ_H$  rearrangements,  $V_kJ_k$  rearrangements in rabbits are much more diverse due to the presence of two light chain isotypes K1 and K2 along with unusual germline  $V_k$ -encoded variability in the length of complementarity determining region 3 (LCDR3) (Mage *et al.*, 2006). Further diversity is generated by GALT-associated processes like gene conversion and somatic hypermutation in both heavy and light chain genes

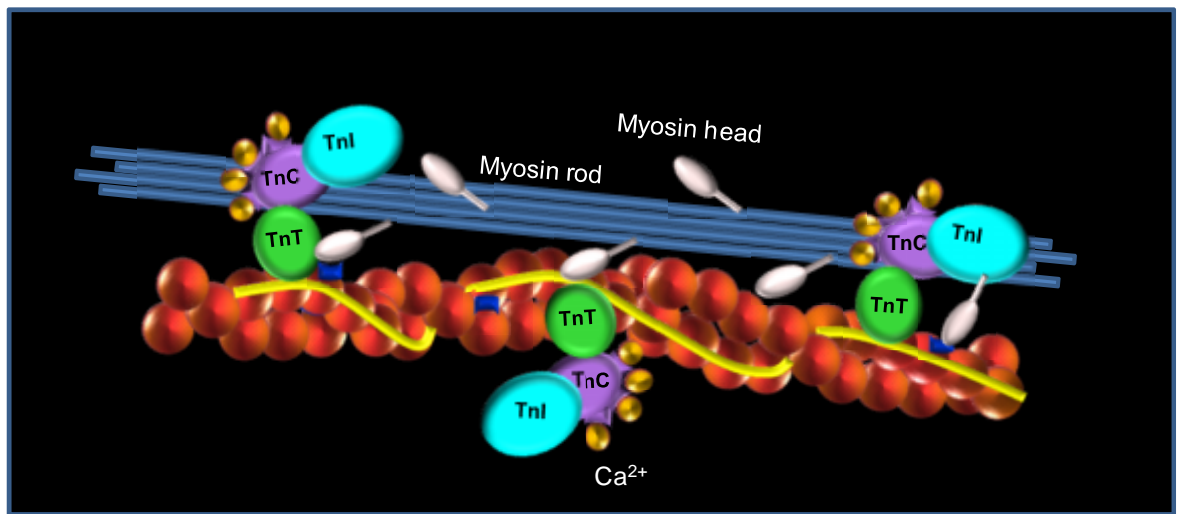


contributing to the vast array of the rabbit antibody repertoire. An interesting feature of the GALT-associated processes is the role of gut flora which acts as mitogenic superantigens for evolution of the preimmune antibody repertoire (Weill and Reynaud, 2005). After proliferation, naive B-cells move to the secondary lymphoid organs such as spleen where antigen-dependent diversification of the antibody repertoire takes place conferring to both specificity and affinity to the antibody against corresponding antigen.

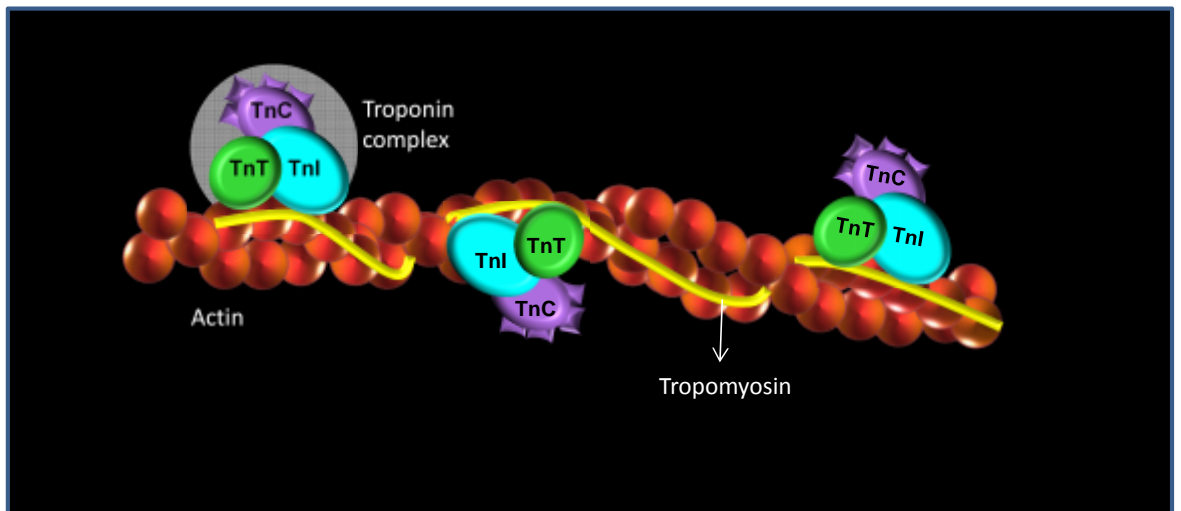
Rabbit is a major source of stable, high affinity polyclonal antibodies. This may be mostly attributed to its immune system possessing the unique characteristics of the human immune system in early stages (lymphopoiesis in bone marrow) and the that of GALT species in later stages (somatic diversification) (Jasper *et al.*, 2003). These features prompted the use of rabbit in the current research project for generating antibody to cardiac troponin I, a key marker of AMI.

### **6.1.2 Cardiac troponin I as an analyte**

The cTnI is 23 kDa a basic (pI 9.5) protein comprised of 210 amino acids, which forms a part of the regulatory complex of troponin located on the thin filament of striated muscle (Figure 6.1A) (Collinson *et al.*, 2001). TnI is an inhibitory protein that occupies a central role at the cross roads of signalling pathways between  $\text{Ca}^{2+}$  binding to TnC and cross-bridge binding to actin causing muscle contraction (Figure 6.1B). It has a high content of serine (12), threonine (8), and tyrosine (8) residues (Bovenkamp *et al.*, 2007; Zabrouskov *et al.*, 2008) making the protein prone to phosphorylation. It has 5 phosphorylation sites (Solaro and Rarick, 1998) that are highly regulated by adrenergic stimulators.



A



B

**Figure 6.1: Regulatory role of troponins in muscle physiology.**

**A:** Muscle relaxation due to inhibition of actin-myosin interaction. CTnI blocks the active site on actin; **B:** Muscle contraction, regulated by  $\text{Ca}^{2+}$  ions, renders the active site free by modifying structure of the troponin complex.

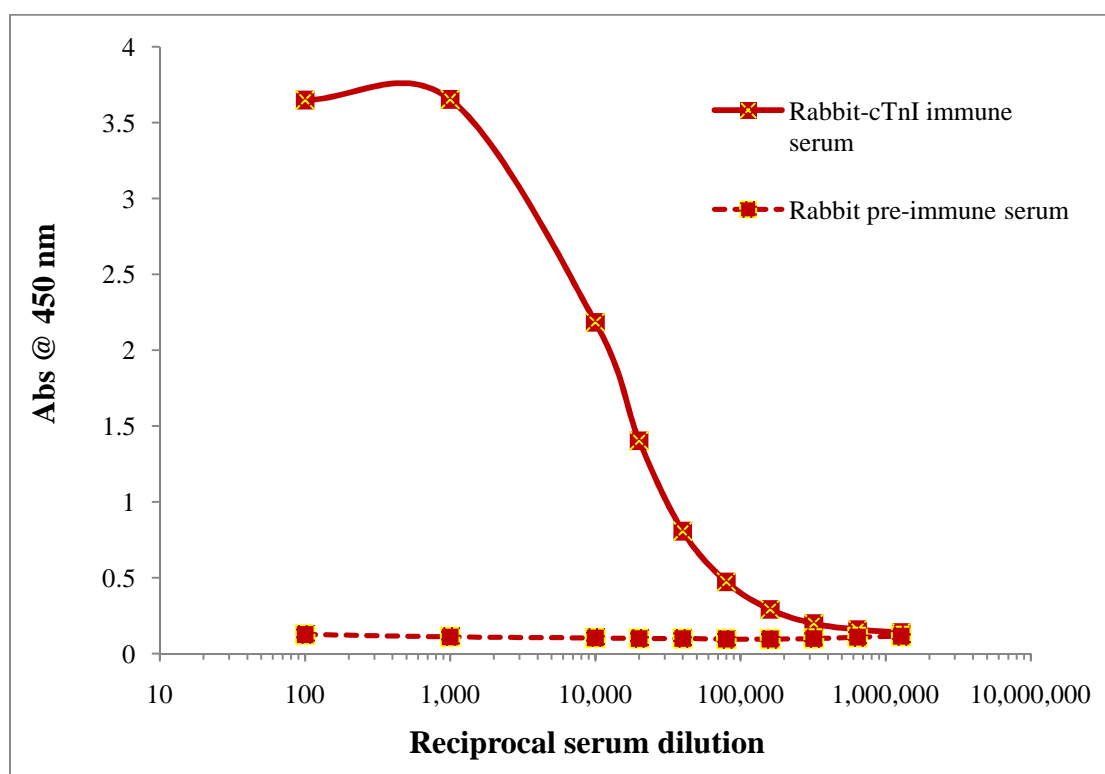
TnI has three specific isoforms, i.e. fast and slow skeletal muscles (178 amino acids, 19.8 kDa) and cardiac muscle (Cummins and Perry, 1978). CTnI is highly homologous to other isoforms with the major difference being the presence of a 32 amino acid sequence at the N-terminal of the protein, thought to be involved in regulation of heart muscle contraction and relaxation (Sadayappan *et al.*, 2008). Among the various

isoforms of TnI, cTnI becomes the sole troponin I expressed in myocardial cells after postnatal development (Lazzeri *et al.*, 2008) which makes it a highly specific biomarker for AMI.

Biologically, cTnI is found in a complex form associated with the cTnT and cTnC. The troponin complex exists in both cytosol and thin filaments of the muscle. The troponin content in cytosol is less, whereas, a majority of the troponin exists as an integral part of the thin filament of the muscle contractile apparatus. Any injury to cardiac muscle leads to the release of the protein into the blood stream initially from the cytosol, which is prolonged with the degradation of muscle thin filaments. Free cTnI is a minor proportion, whereas the majority of the cTnI released is in the form of cTnI-TnC, cTnI-TnT and TnT I-T-C covalent complexes along with small degradation products (Chang *et al.*, 2008). Following release cTnI undergoes several modifications due to proteolysis, oxidation and phosphorylation resulting in modified cTnI products (McDonough *et al.*, 2001; Wu, 2001). The aim of this project was to generate well characterised anti-cTnI-specific antibodies to the stable part of the cTnI, comprising of amino acid residues from position 30 to 110 (Katrukha *et al.*, 1998).

## **6.2 Rabbit Immunisation and cTnI Serum Titres**

A female New Zealand white rabbit was immunised with cTnI and boosted until an adequate titre was achieved. An antibody titre is a measure of animal's response towards the antigen. The higher the immune response, the better the chance of getting antibodies against the target antigen. The rabbit immune serum showed a very high antibody titre of 1:320,000 (Figure 6.2) against cTnI, making the animal a good candidate for antibody-library construction.



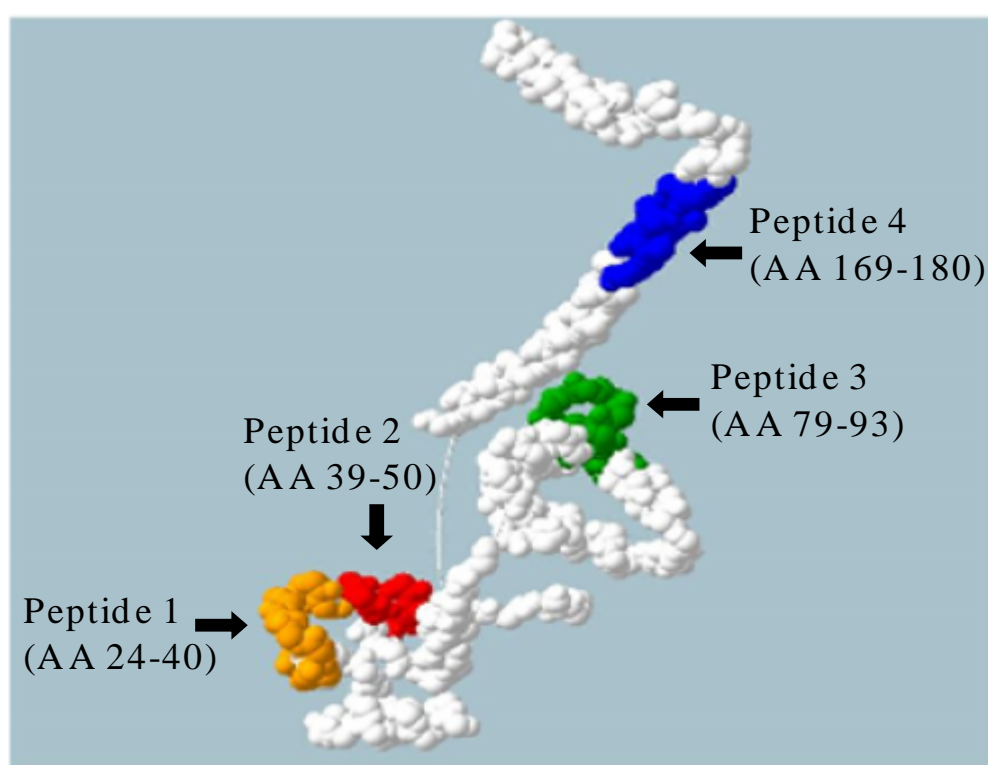
**Figure 6.2: Rabbit serum titre against cTnI.**

*The solid line represents the cTnI-specific antibody response following immunisation and the dotted line is the control sera obtained from the animal before immunisation. A high antibody titre of 1:320,000 against cTnI was obtained.*

CTnI is highly homologous to sTnI. Hence, it is necessary to ensure that anti-cTnI specific antibodies are in fact generated and isolated for specific immunoassay development. The cTnI molecule was modelled by Dr. Stephen Hearty and Dr. Niamh Gilmartin (Applied Biochemistry Group, School of Biotechnology, Dublin City University, Ireland) to identify the location of 4 different regions useful for isolation of antibodies to the cardio-specific N-terminal region of the molecule (Figure 6.3). These peptides were selected by aligning the sequences of both cTnI and sTnI and highlighting the non-homologous sequences.

The peptides were synthesized for use in the development of peptide-specific recombinant antibodies for cardiac assay development. The peptides were synthesized

with 2 different types of carrier molecules (biotin and KLH) for immunisation and screening purposes. Due to the availability of a previously immunised rabbit with cTnI, the serum antibodies were checked against cTnI-specific peptides to characterize the polyclonal antibodies based on epitopes. Due to the availability of ‘in-house’ developed anti-peptide 3 and highly hydrophobic nature of Peptide 4 in comparison with other peptides (as identified by Hopp-Woods scale) only peptide 1 and 2 (conjugated to different carrier proteins (Table 6.1) at N and C-terminals), were used for the characterisation of rabbit cTnI antisera.



**Figure 6.3: Regions of cTnI targeted for cardio-specific antibody development.**

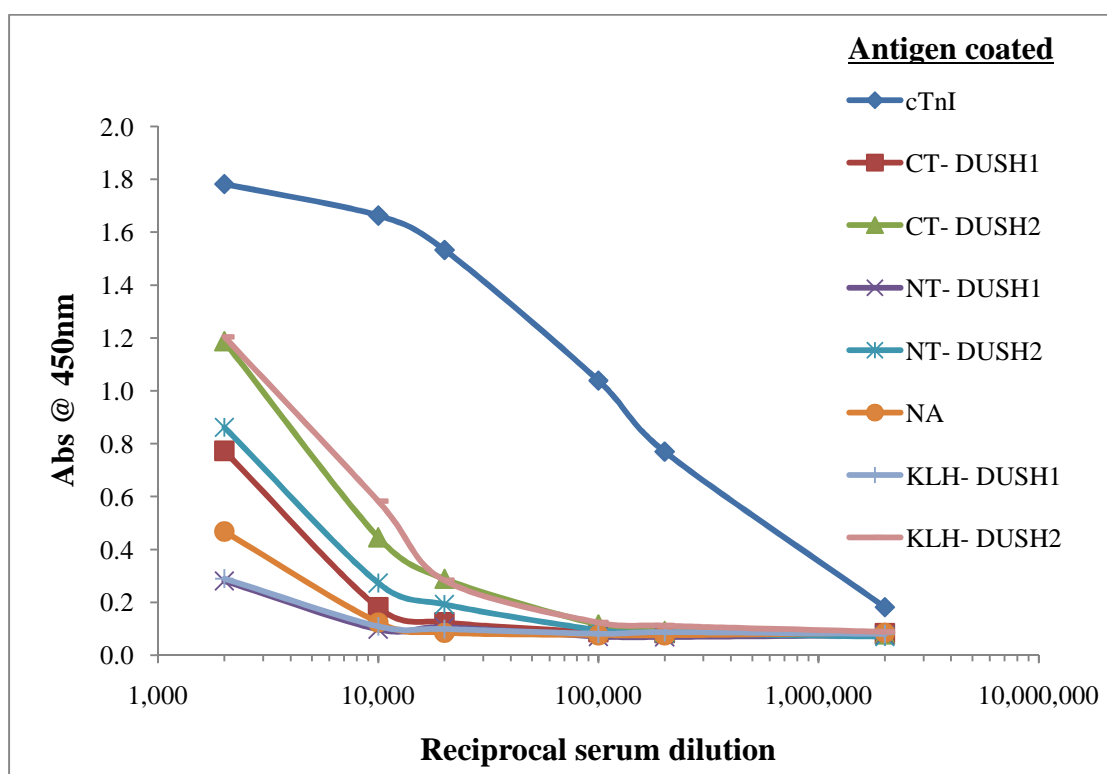
*Different peptide epitopes normally selected from the N-terminal region of the cTnI for peptide-specific antibody generation.*

An ELISA was carried out by assaying different dilutions of the rabbit polyclonal antisera (section 2.2.4.2) against cTnI-specific peptides for titre determination. The assay revealed that peptides conjugated at the C-terminal, to the carrier molecule,

showed a better response with the immune serum compared to the N-terminal conjugated peptides. However, the titre against the peptides was lower than the titre obtained against the cTnI whole molecule (Figure 6.4). This may be associated with the fact that the serum contains antibodies to regions other than those represented by the peptides. It might also be a possibility that the antibodies produced recognise discontinuous epitopes on the peptide region of the whole molecule which are not recognised in synthesized linear peptides.

**Table 6.1: *CTnI* peptides designed for antibody development.**

Peptide name	Amino acid sequence	Carrier molecule	Conjugated terminal
<b>CT-DUSH1</b>	KISASRKLQLKT	Biotin	C-terminal
<b>CT-DUSH2</b>	NYRAYATEPHAKKKSK	Biotin	C-terminal
<b>NT-DUSH1</b>	KISASRKLQLKT	Biotin	N-terminal
<b>NT-DUSH2</b>	NYRAYATEPHAKKKSK	Biotin	N-terminal
<b>KLH-DUSH1</b>	KISASRKLQLKT	KLH	C-terminal
<b>KLH-DUSH2</b>	NYRAYATEPHAKKKSK	KLH	C-terminal



**Figure 6.4: Rabbit serum titre against cTnI-specific peptides.**

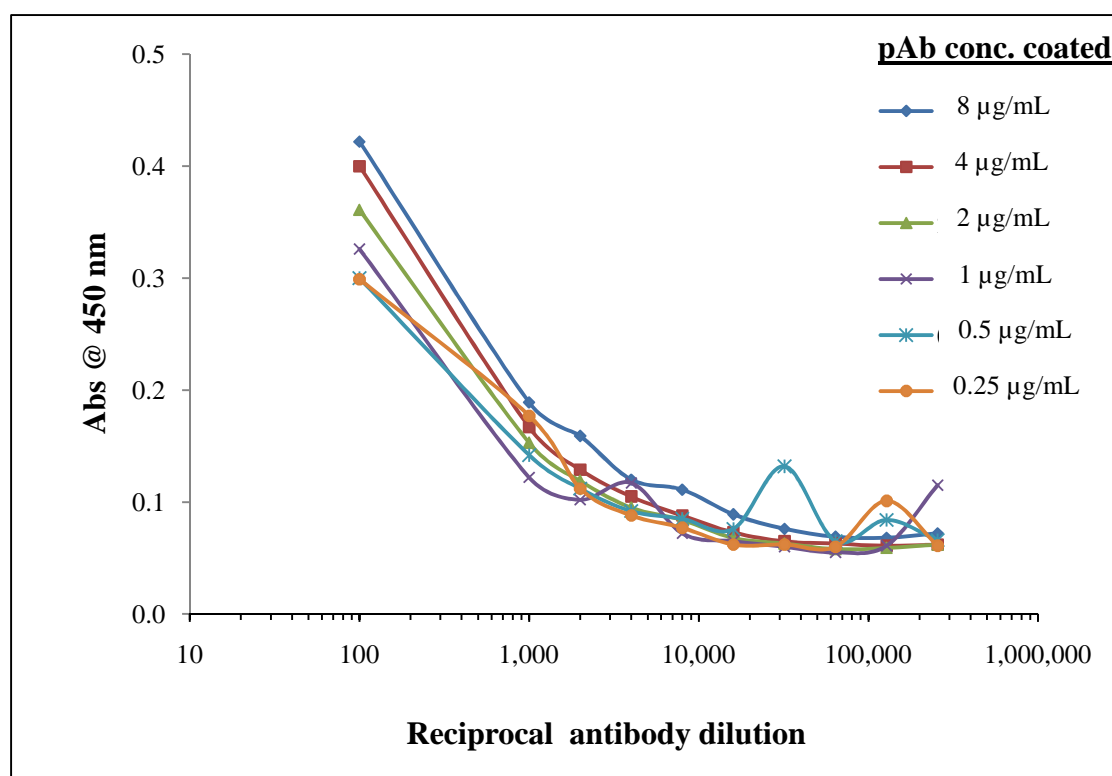
The immune serum was checked against whole cTnI and 2 peptides conjugated to two different carrier molecules (biotin and KLH). Two different types of biotinylated peptides were tested, one conjugated at the N-terminal and the other at the C-terminal. The response against cTnI (whole molecule) was higher than any other peptides favouring the isolation of antibodies to the epitopes other than those represented by the peptides on the cTnI molecule.

### 6.3 Purification of Rabbit Polyclonal Antibodies and their Analysis by Checkerboard ELISA

Protein-A purification was carried out on the rabbit polyclonal antiserum. A purified antibody fraction was analysed on SDS-PAGE gel to determine its purity. Two bands were obtained at ~55kDa and ~25 kDa, for both the heavy and light chain of antibodies, due to reduction of the disulphide bridges.

An anti-cTnI pAb was generated for use in conjunction with peptide-specific antibodies for the development of a cardio-specific immunoassay. The generated pAb was assayed

along with anti-peptide 3 chicken scFv in a checkerboard sandwich ELISA format to optimise the antibody concentrations for further assay development. In this assay the concentrations of both the pAb and the scFv were varied, keeping the antigen concentration constant at 1 µg/mL. Detection was carried out with 1:2,000 dilution of anti-chicken-HRP-labelled antibody. Very moderate levels of cTnI were captured on the pAb along with reduced functionality at high dilutions (Figure 6.5).



**Figure 6.5: Checkerboard ELISA of cTnI using rabbit anti-cTnI polyclonal antibodies.**

*Different dilutions of the purified pAbs were coated on the plate to capture different levels of cTnI which can be detected by the scFvs. As evident from the graph, the capture levels of the cTnI in this format is not optimum for development of an effective assay.*

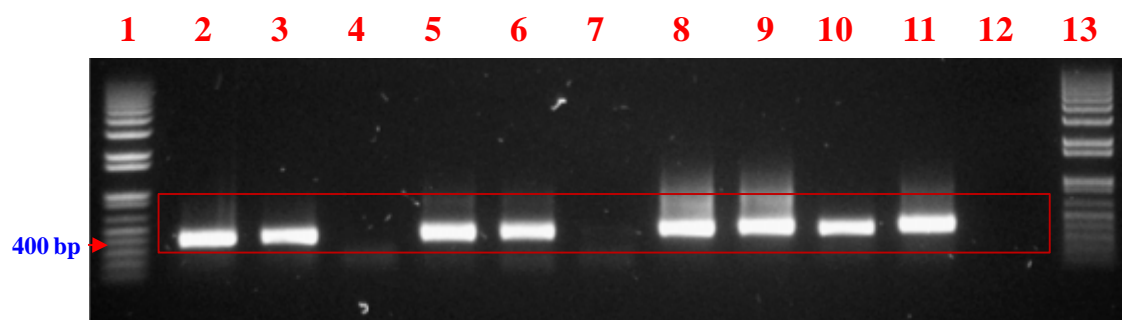
The assay was repeated using the scFv as capture antibody and the pAb as tracer antibody for detection of the cTnI. The results obtained were not satisfactory indicating the unsuitability of pAbs in cTnI assay development. However, the titre of rabbit



antisera against cTnI was promising. This led to the idea of progressing the project by developing recombinant antibodies against the antigen which can be used in assays. Besides generating the antibodies recombinantly for assay development, antibodies from New Zealand white rabbits are reported to contain an unnatural disulphide bridge between Cys 80 in variable light chain and Cys 171 of constant chain (McCartney-Francis *et al.*, 1984). Keeping this in mind, a study was designed to isolate such antibodies, characterise them and study the effect of such unnatural cysteines on antibody characteristics.

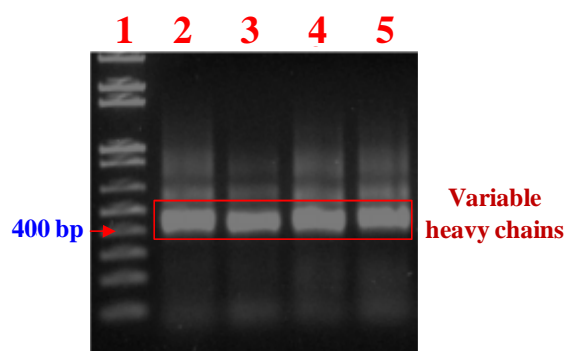
#### **6.4 Optimisation of Variable Gene Amplifications from Rabbit cDNA**

The RNA extracted from the bone marrow and spleen of rabbit followed by cDNA preparation which was further used for variable gene amplifications. The variable genes for both bone marrow and spleen were amplified separately using different combinations of light chain and heavy chain primers in the PCR reaction (section 2.2.4.7) (Figure 6.6 and 6.7). The PCR was optimised using different annealing temperatures and magnesium concentrations. In variable light chain amplifications, two combinations of primer required further optimisation for the product amplification which was achieved by substituting regular *Taq* with Phusion *Taq* (Figure 6.8). The amplified products were scaled up and purified by agarose gel electrophoresis.



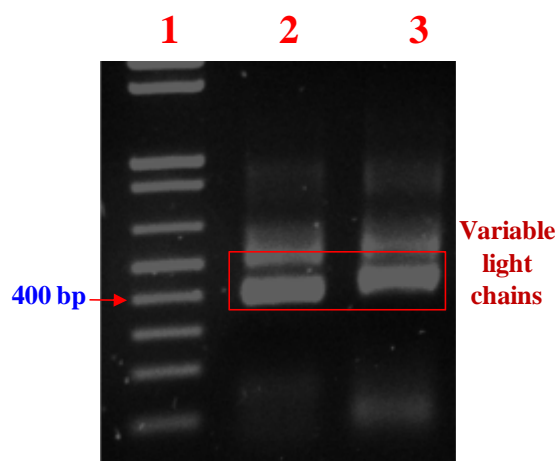
**Figure 6.6: Variable light chain amplifications from bone marrow for rabbit anti-*cTnI* scFv library.**

Nine different primer combinations were used for kappa light chain and one was used for lambda light chain amplification. Using optimised conditions for rabbit library construction, seven out of nine of the kappa light chain and lamda chain were amplified at 2 mM magnesium concentration and an annealing temperature of 56°C. Lane 1 and 13: 1 kb DNA ladder plus; lanes 2-10: different rabbit kappa chain amplifications (VK1-VK9); lane 11: rabbit lambda chain amplification (VL ) and lane 12: negative control.



**Figure 6.7: Variable heavy chain amplifications from bone marrow for rabbit anti-*cTnI* scFv library.**

Lane 1: 1 kb DNA ladder plus; lanes 2-5: four different combinations of heavy chain amplifications (VH1-VH4). Heavy chains were amplified using four different combinations of heavy chain forward and reverse primers, at 2 mM magnesium concentration and an annealing temperature of 56°C, which was further used for large scale PCR amplifications.

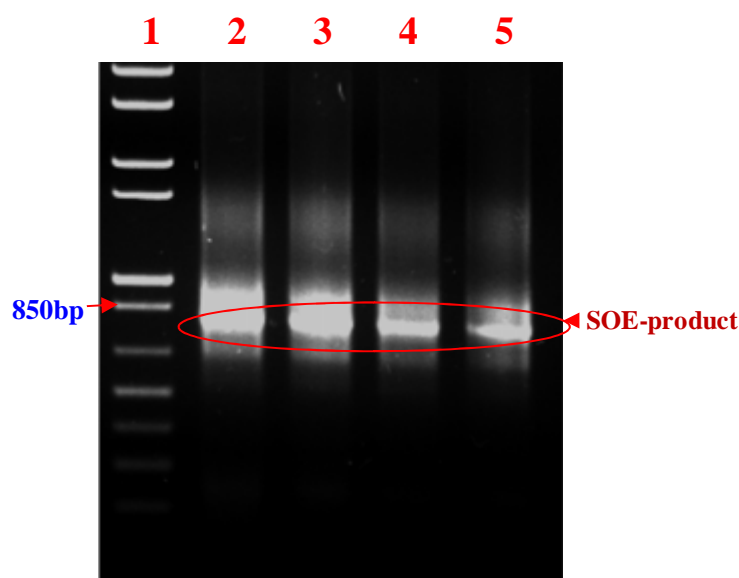


**Figure 6.8:** *Variable kappa chain amplifications from bone marrow for rabbit anti-*cTnI* scFv library.*

*This figure represents the amplification of the kappa light chain by Phusion Taq polymerase. Lane 1: 1 kb DNA ladder plus; lane 2: kappa chain amplification for VK3 bone marrow and lane 3: kappa chain amplification for VK6 bone marrow. Sufficient amplification of both the primer combinations were obtained resulting in use of Phusion Taq polymerase for large scale amplification of these light chain antibody regions.*

### **6.5 Rabbit SOE-PCR for Assembling Variable Heavy and Light Chain**

Purified heavy chains and the light chains were mixed in equimolar ratios and 100 ng of each of the variable chains were used in PCR reaction to produce SOE product. The SOE product was amplified by fusing the heavy and the light chains of the antibodies with the help of Gly-Ser (G<sub>4</sub>S<sub>4</sub>) linker (Figure 6.9). The SOE-PCR for kappa and lambda light chains was carried out separately for both bone marrow and spleen by using the same heavy chain gene product (section 2.2.4.8). The annealing temperature of 56°C and a magnesium concentration of 2 mM were found to be optimum for the PCR using the Platinum high-fidelity *Taq* polymerase.

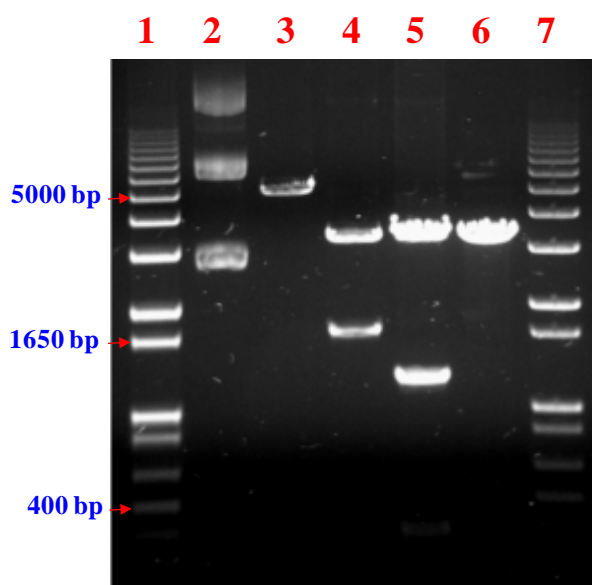


**Figure 6.9: Rabbit SOE-PCR.**

*Lane 1: 1 kb DNA ladder plus; lane 2: SOE-PCR for bone marrow using kappa light chain; lane 3: SOE-PCR for spleen using kappa light chain; lane 4: SOE-PCR for bone marrow using lambda light chain and lane 5: SOE-PCR for spleen using lambda light chain.*

## 6.6 Library Construction and Biopanning

The library was constructed as outlined in section 2.2.4. In brief, the SOE-PCR product for both bone marrow and spleen was cloned into the pComb3XSS vector after digesting with *Sfi*I. The stuffer fragment separated from the vector was further digested with *Xho*I and *Xba*I, to ensure that it does not interfere with the cloning step. Figure 6.10 shows complete digestion of vector and the stuffer fragment carried out before cloning. The cloned product obtained after ligating both the bone marrow and spleen SOE-PCR product was electroporated into electro-competent XL1-Blue cells. To obtain a potentially diverse and efficient library size, electroporation is the best method for transformation (Lin and Cornish, 2002). The transformants for both the libraries were titred to calculate the efficiency of transformation.



**Figure 6.10: Vector digestion for cloning the library.**

*The vector was digested using SfiI enzyme. The stuffer was further digested with XhoI and XbaI enzymes and the purity of vector was checked by agarose gel electrophoresis. Lane 1 and 7: 1 kb DNA ladder plus; lane 2: uncut vector showing three forms; lane 3: linearised vector using SacI enzyme; lane 3: vector digested with SfiI enzyme; lane 5: vector digested with SfiI, XhoI and XbaI enzymes and lane 6: purified vector.*

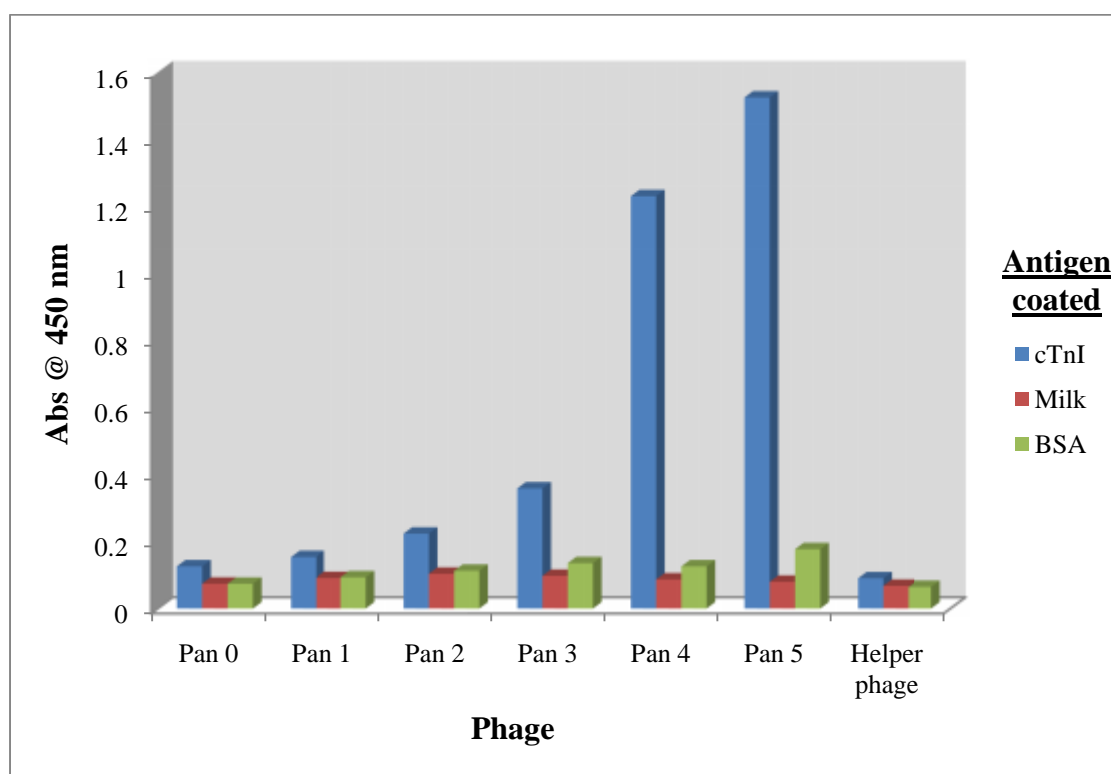
A library size of  $2.23 \times 10^7$  cfu/mL, for bone marrow and  $3.1 \times 10^6$  cfu/mL, for spleen was obtained from the transformation. The library size is limited by the concentration of DNA and efficiency the electro-competent cells used (Lin and Cornish, 2002). Both the libraries were pooled before panning and the specific scFvs against cTnI were selected and enriched *via* biopanning by immobilising the cTnI on solid surface. Five rounds of panning was carried out for the rabbit anti-cTnI scFv library, decreasing the antigen concentration and increasing the stringency in washing step-wise to reduce non-specific and low affinity binders. There was a subsequent increase in the titre of the phage obtained at each round due to enrichment of binding clones (Table 6.2).

**Table 6.2:** *Phage output and input titre obtained at each round of panning.*

Panning round	Input titre	Output titre
Pan 1	$4.25 \times 10^{10}$	$2.4 \times 10^5$
Pan 2	$2.25 \times 10^{13}$	$5.5 \times 10^6$
Pan 3	$2.75 \times 10^{13}$	$1.8 \times 10^6$
Pan 4	$7.95 \times 10^{13}$	$6.3 \times 10^6$
Pan 5	$3.52 \times 10^{13}$	$1.3 \times 10^7$

### **6.7 Analysis of Biopanning Outputs by Polyclonal Phage ELISA**

Polyclonal phage ELISA was carried out by assaying phage outputs, obtained after each round of panning, against the cTnI antigen (Figure 6.11). The phage output contains a mixture of antibodies with varying specificity and affinity, selectively enriched against the antigen. Polyclonal phage response is indicative of the panning efficiency as the specific response is expected to increase from round one onwards. It provides an idea about the phage output with maximum enrichment of the specific clones that can be used for expressing monoclonal scFvs, in order to obtain the maximum population of antigen binders.

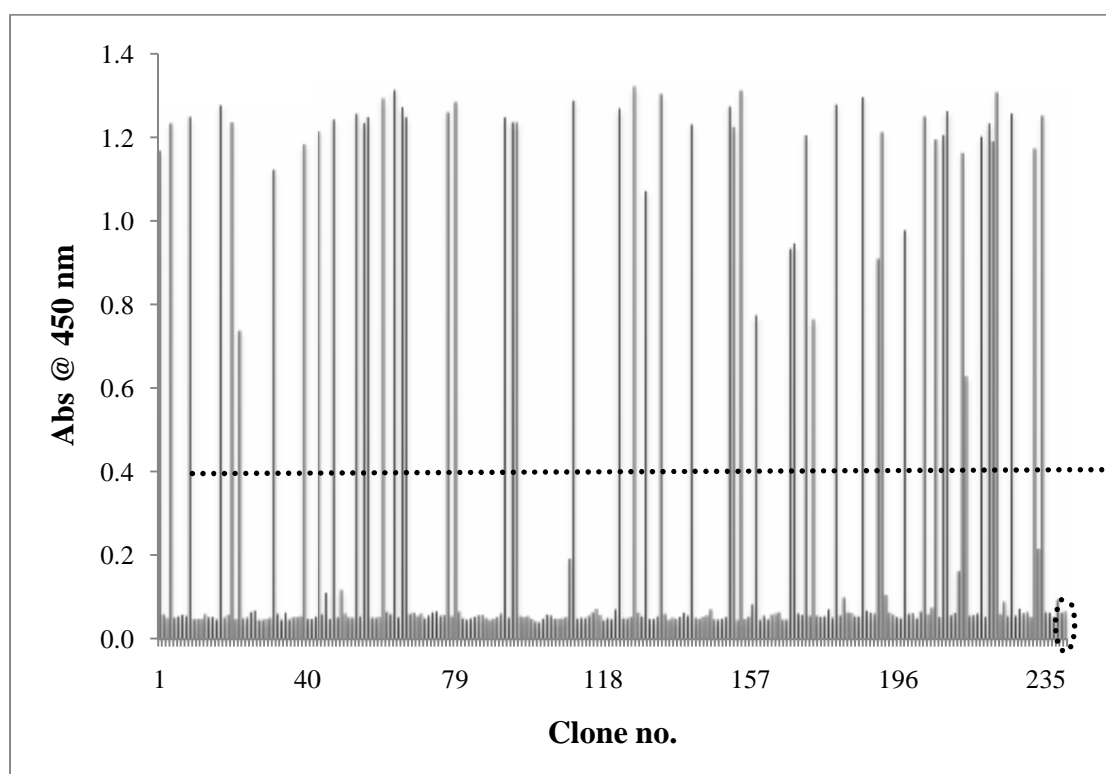


**Figure 6.11: Polyclonal phage ELISA**

A gradual increase in the detection signal with each round of panning peaking in round 5 is illustrated. This shows that round 5 outputs will be optimum for further expression of monoclonal scFvs. Blocking agents such as milk and BSA along with the helper phage, used in the panning, were also checked for non-specific phage binding. The results show that only specific-phage were amplified against the antigen with each round of panning.

## 6.8 Soluble Expression and Indirect ELISA for Screening cTnI Clones

The pan 5 phage output was infected into TOP10F cells for soluble expression and the individual clones were picked for analysing monoclonal scFvs. The clones were subsequently subcultured and the scFvs were expressed overnight using 1 mM IPTG. These clones were analysed for specific binders to cTnI by performing an indirect ELISA on the culture lysates obtained after three 'freeze-thaw' cycles. A total of 240 clones were selected from which approximately 23% of the clones were found to show binding to cTnI (Figure 6.12).



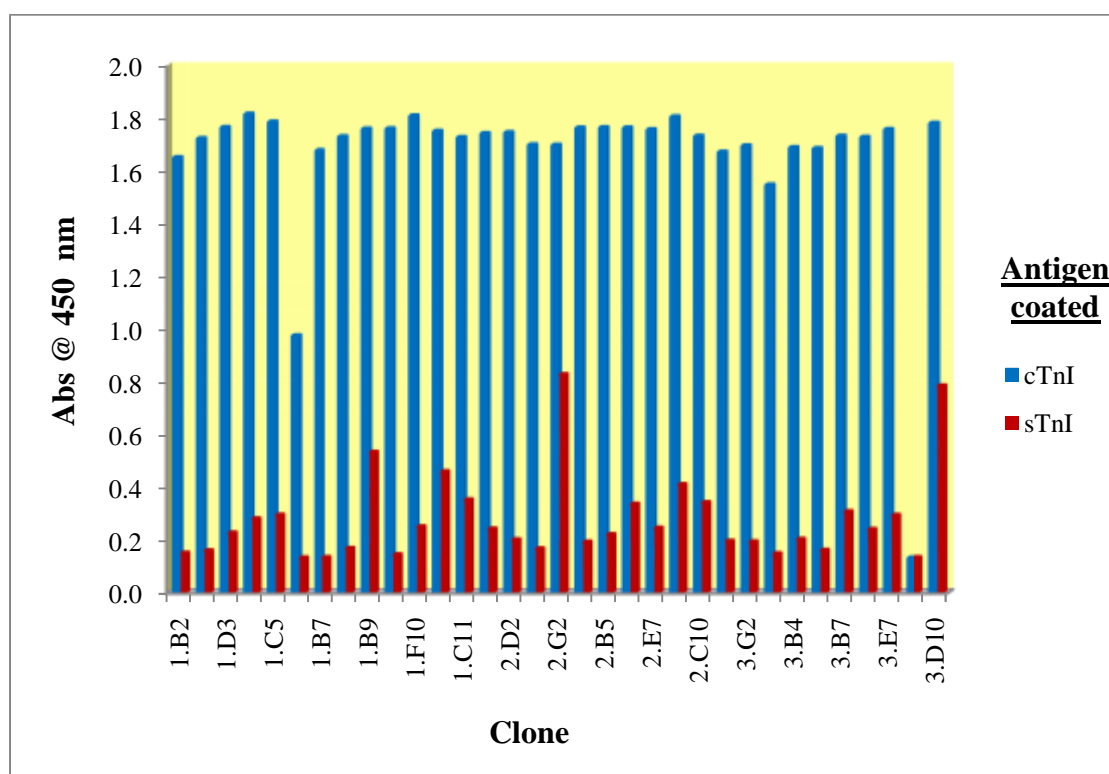
**Figure 6.12: Monoclonal ELISA of solubly expressed scFvs against cTnI.**

Twenty three percent of the clones screened were found to bind cTnI. The broken line indicates the reference cut-off limit above which the clones were selected for further analysis and the broken circle represents reagent control.

## 6.9 Cross-Reactivity Analysis of Positive Clones with sTnI

The rabbit anti-cTnI scFvs detected in section 6.8 were further analysed for cross-reactivity against skeletal troponin I (sTnI) by an indirect ELISA. The cardiac isoform, cTnI shares a high percentage of similarity with sTnI except for only a stretch of 32 amino acids at the N-terminus being different from the skeletal isoform (Le Moal *et al.*, 2007). It was necessary to analyse the reactivity of the scFvs against sTnI to confirm the specificity of the antibodies. The scFvs were found to be highly specific against cTnI (Figure 6.13).





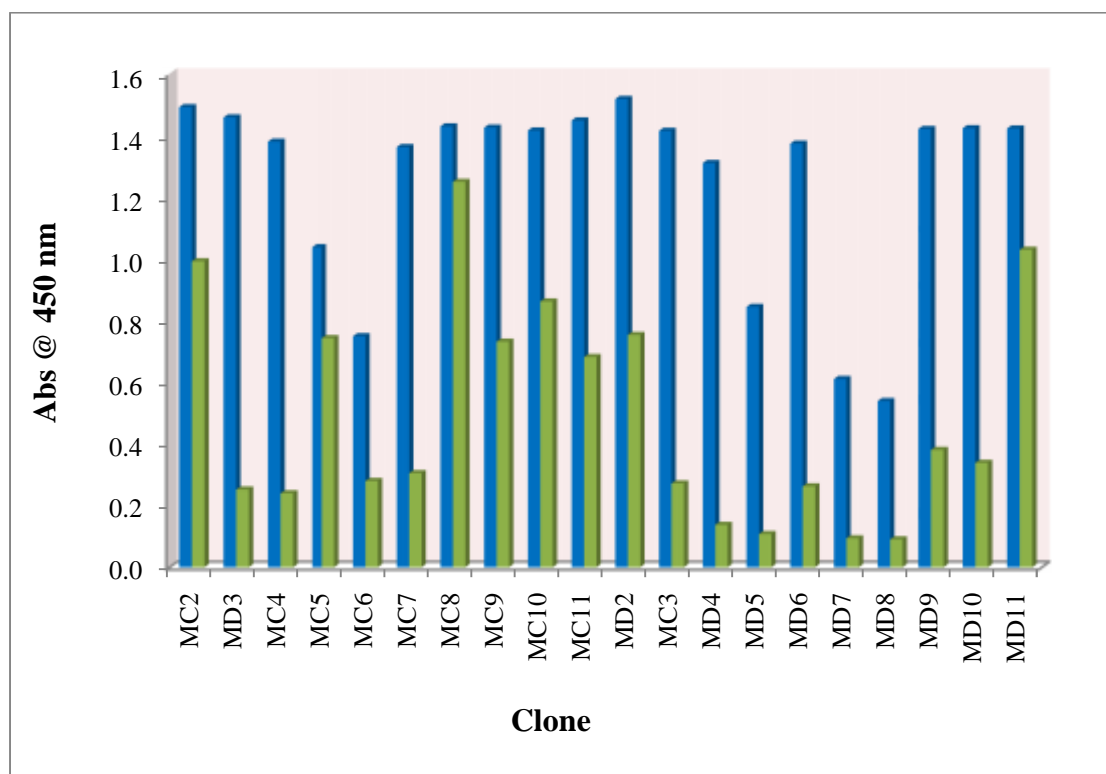
**Figure 6.13: Analysis of anti-cTnI scFvs for cross-reactivity against sTnI.**

*The majority of scFvs (48 out of 50) selected were found to be specific against cTnI although two of them showed to be moderately binding to sTnI.*

## 6.10 Solution Phase Inhibition ELISA for Selection of scFvs Capable of Use in a Competitive Immunoassay

The scFvs specific to cTnI were selected and analysed for inhibition by incubating with free cTnI and assaying them on immobilised cTnI. In total 50 scFvs were analysed for inhibition, with 5 µg/mL of free cTnI in solution against 1 µg/mL of cTnI coated on the plate. The ability of the scFvs to bind the antigen in solution, as compared to the immobilised antigen on plate, was determined by analysing the same clone along side without any inhibition by the free antigen. This analysis showed that most of the scFvs selected were inhibited from binding the immobilised antigen, depending on the given concentration of the free antigen in solution (Figure 6.14). This characteristic is of significance in relation to characterisation and assay development which relies on the

ability of antibody to detect the antigen in patients' blood. These scFvs were further selected for ranking analysis for affinity-based characterisation.



**Figure 6.14: Inhibition analysis of anti-cTnI scFvs by free cTnI in solution.**

Most of the scFvs selected were found to be competitive against cTnI when incubated with 5  $\mu$ g of antigen before assaying in solution. The blue columns indicate the  $A_0$  values, i.e. scFv without inhibition, and the yellow columns indicate the inhibition in binding of scFvs to immobilised antigen in the presence of free antigen.

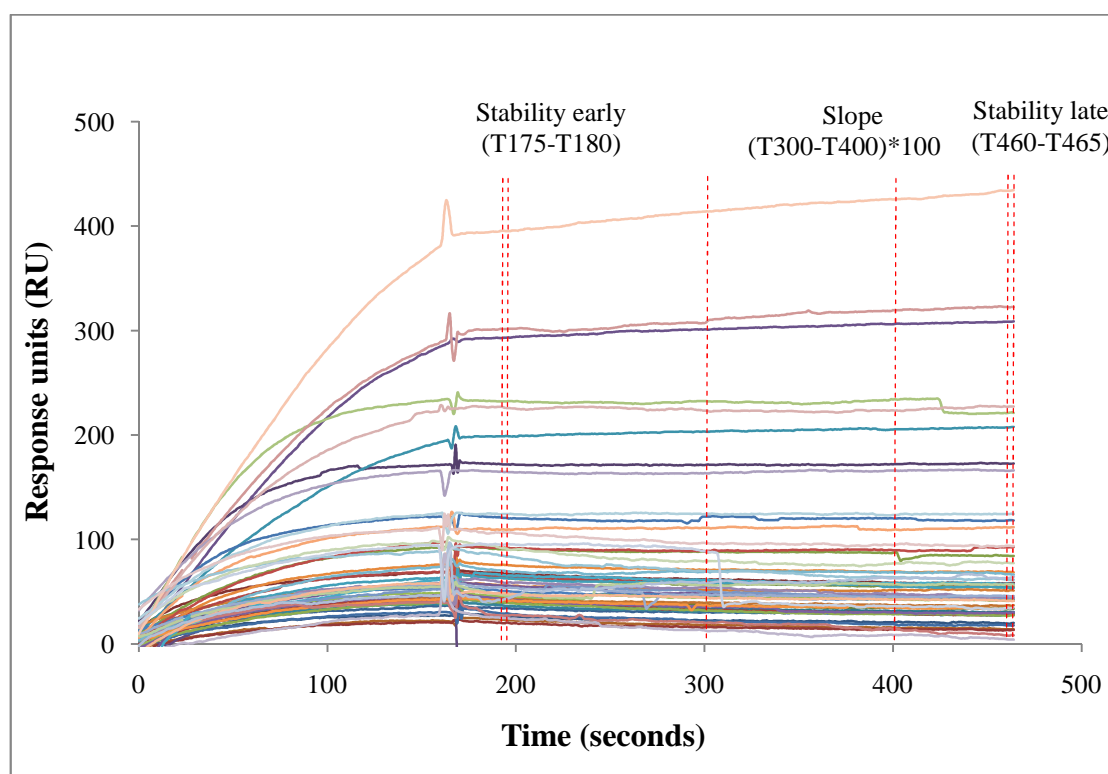
### 6.11 Ranking of Anti-cTnI ScFvs

The scFvs were ranked based on 'off-rate' kinetics analysed on Biacore 3000. A method file was created to sequentially assess the clones by passing the lysates over an anti-HA immobilised chip (prepared as in section 2.2.1.17). Each scFv was studied for its binding parameters by passing 30 nM of cTnI over the captured scFv and double referencing the values by subtracting the buffer response (zero analyte concentration) and the reference cell response for each antibody analysed. The sensorgrams were

analysed on BIAevaluation 4.1 software and the data was exported to excel for evaluation. The evaluation was carried out by calculating stability early (calculated from the average response from 175 to 180 seconds), and stability late (calculated from the average response from 460 to 465 seconds) for each individual clone. Antibody/antigen complex stability (% left) was calculated by expressing the 'stability late' as a percentage of 'stability early'. A slight increase in the RU was obtained due to the stickiness of cTnI antigen over the chip surface even after capping the surface with ethanolamine. The results are displayed in Figure 6.15 showed a slight increase in the apparent slope at the dissociation phase resulting in high stability late values. These can be attributed to aggregation or other such complications leading to a rebinding effect. Consequently these values were excluded, as this method involves preliminary shortlisting of clones based on their 'off-rates' (Table 6.3) which can be further characterised using end-point assays or by optimising antigen or antibody concentrations or by modifying the assay format. Ten antibodies with maximum % left values were selected for further analysis.

**Table 6.3: *Percentage stability calculated for each clone.***

Clone	% Retained	Clone	% Retained
<b>B2</b>	69	<b>MD2</b>	100
<b>MB3</b>	84	<b>MC2</b>	88
<b>MB5</b>	74	<b>MC9</b>	65
<b>MB6</b>	100	<b>MD8</b>	88
<b>MB7</b>	73	<b>MD4</b>	78
<b>MB8</b>	57	<b>MF2</b>	85
<b>MB4</b>	81	<b>MF3</b>	24
<b>MB9</b>	61	<b>MF4</b>	95
<b>MC3</b>	83	<b>MF5</b>	73
<b>MC4</b>	118	<b>MF6</b>	91
<b>MC5</b>	71	<b>ME2</b>	102
<b>MC6</b>	81	<b>ME3</b>	83
<b>MC7</b>	54	<b>ME4</b>	107
<b>MC10</b>	66	<b>ME5</b>	109
<b>MC11</b>	91	<b>MF7</b>	100
<b>MC8</b>	106	<b>MF8</b>	70
<b>MD3</b>	105	<b>MF9</b>	83
<b>MD5</b>	88	<b>MF10</b>	139
<b>MB10</b>	96	<b>MF11</b>	100
<b>MD6</b>	86	<b>MG2</b>	80
<b>MD7</b>	65	<b>MG3</b>	16
<b>MD9</b>	74	<b>MG4</b>	100
<b>MD10</b>	83	<b>MG5</b>	110
<b>MD11</b>	89	<b>MG6</b>	36
<b>MB11</b>	79	<b>MG7</b>	87

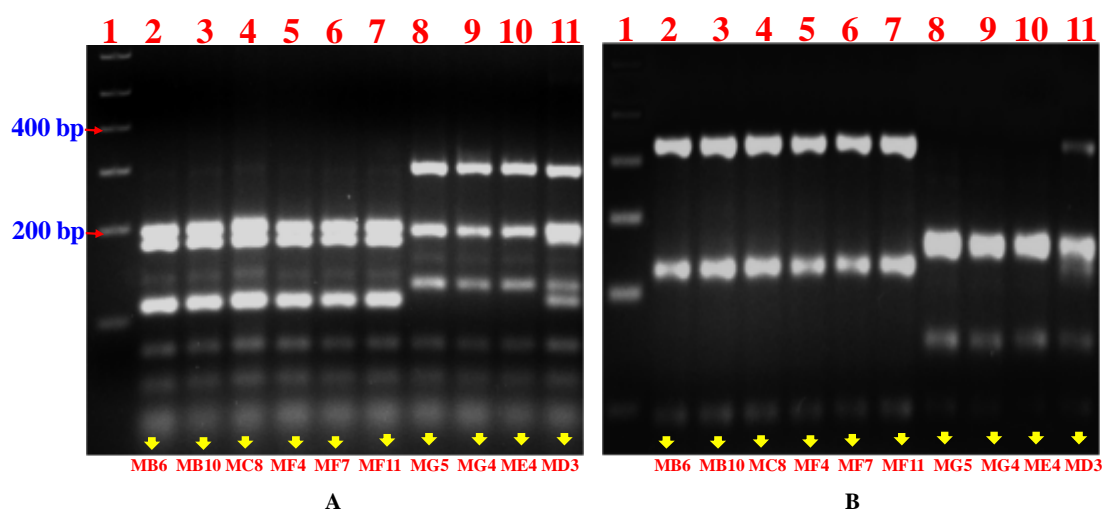


**Figure 6.15: Ranking of anti-cTnI scFvs.**

Sensorgrams of 50 clones overlayed in BIAevaluation 4.1 software. The red dotted line indicates various parameters (slope, stability early and stability late) considered for ranking the clones for choosing best performing clones for further analysis.

## 6.12 Fingerprinting Analysis of Anti-cTnI scFvs

The scFv coding sequences were digested with *AluI* and *BstMI* restriction enzymes for characterising the antibodies based on restriction profiles (Figure 6.16). Basically, three different RE profiles were obtained based on the restriction sites identified by the enzymes, i.e. CC/WGG by *BstMI* and AG/CT by *AluI*. The values obtained for different ‘off-rate’ parameters by Biacore were arranged with the finger-printing profiles to select representative clones from each group based on their affinity. The clones were processed for plasmid extraction and the sequence of the respective scFv gene was determined.



**Figure 6.16: Characterising anti-cTnI scFvs based on different RE profiles.**

**A:** Lane 1: 1 kb DNA ladder plus; lane 2-11: AluI digested scFv genes.

**B:** Lane 2: 1 kb DNA ladder plus; lane 2-11: BstNI digested scFv genes.

ScFvs were characterised into three different profiles based on the pattern obtained from AluI and BstNI enzyme digestion. This analysis also shows that either of the enzymes is capable of RE profiling the rabbit antibodies independently.

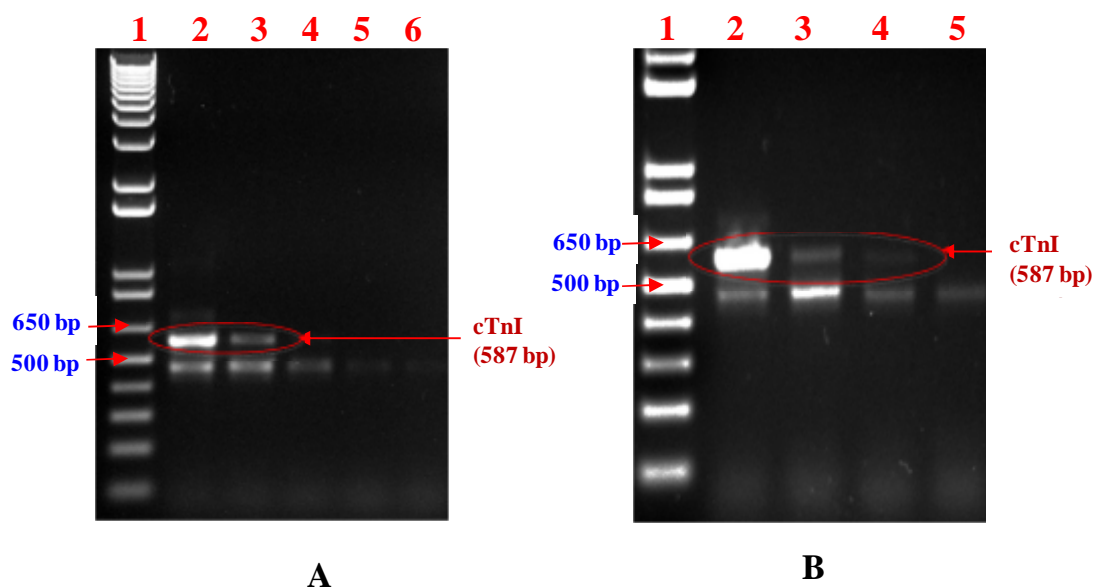
### 6.13 Expression of Recombinant cTnI

Expression of recombinant cTnI was considered necessary to maintain a constant supply of reproducible antigen for further characterisation of the antibodies. This became a necessity as batch-to-batch variations were observed in the commercially purchased antigen (such as in the ‘off-rate’ analysis of anti-cTnI scFvs the commercial antigen was found to be binding the CM dextran surface of the chip, at different rates, which is an issue when analysing the binding kinetics of the antibodies).

#### 6.13.1 Amplification of the cTnI Gene for Expression

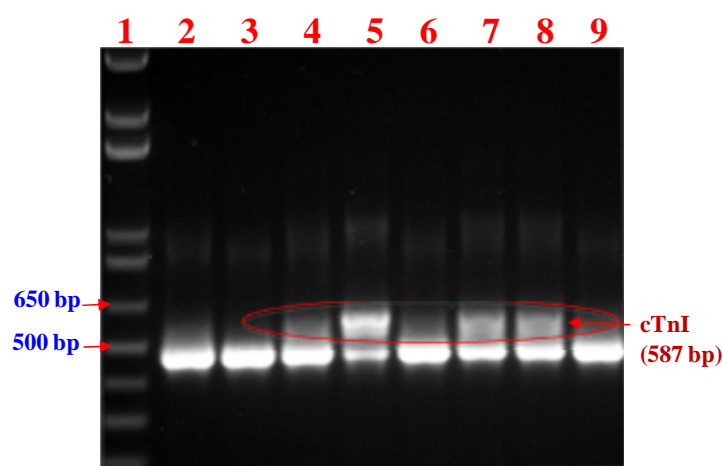
An attempt was made to amplify the cTnI gene from human heart cDNA using specific primers. The gene amplification was optimised by using different magnesium concentrations and annealing temperatures. The PCR results varied every time the gene was amplified, resulting in a strong non-specific band of low molecular weight being

amplified (Figure 6.17). Different approaches were tried for consistent amplification of the specific cTnI gene. One of the approaches involved purifying the amplified gene from the PCR-amplified product and using it as template for PCR in order to scale-up the gene for cloning. This approach failed due to non-specific amplifications which can be seen as two bands close to the cTnI gene amplified (Figure 6.18).



**Figure 6.17: Optimising cTnI amplification by PCR.**

**A:** PCR results for annealing temperature optimisation. Lane 1: 1 kb DNA ladder plus; lane 2: 59.6°C; lane 3: 61.4°C; lane 4: 63.3°C; lane 5: 65°C and lane 6: 66°C. A temperature of 59.6°C was found to be optimum for cTnI amplification. **B:** PCR results for magnesium concentration optimisation. Lane 1: 1 kb DNA ladder plus; lane 2: 1 mM MgCl<sub>2</sub>; lane 3: 2 mM MgCl<sub>2</sub>; lane 4: 3 mM MgCl<sub>2</sub> and lane 5: 4 mM MgCl<sub>2</sub>. 1 mM concentration of MgCl<sub>2</sub> was used for the amplification of cTnI gene.



**Figure 6.18:** *Optimising cTnI amplification from PCR-amplified cTnI product as template at different annealing temperatures.*

*Lane 1: 1 kb DNA ladder plus; lane 2: 59.0°C; lane 3: 59.6°C; lane 4: 60.5°C; lane 5: 61.5°C; lane 6: 62.5°C; lane 7: 63.3°C; lane 8: 64.4 °C and lane 9: 65°C. The amplification of the gene is shown to be random at different temperatures with non-specific product being amplified consistently.*

### 6.13.2 Gene synthesis, cloning and transformation into *E. coli* cells

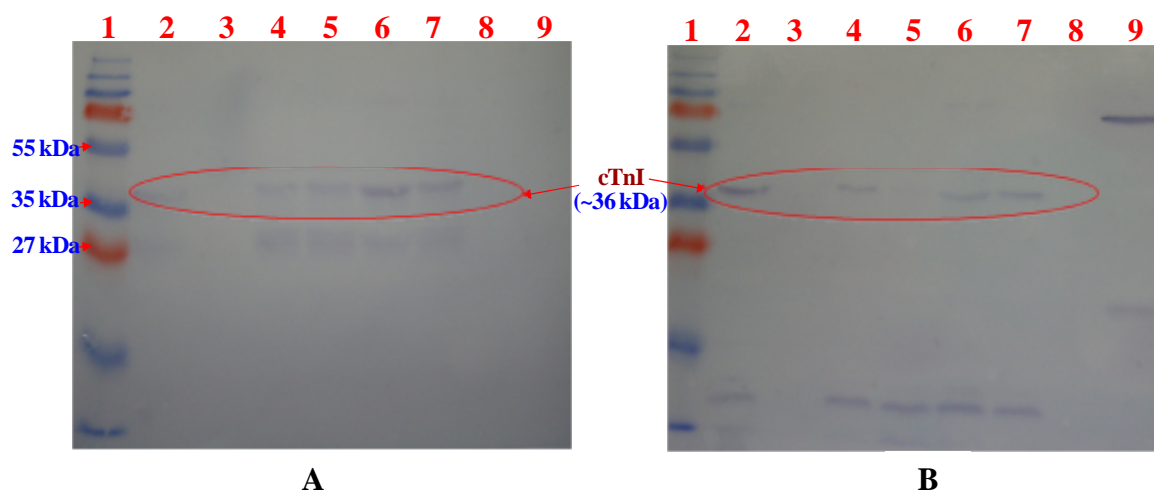
Due to the problems with amplifying cTnI gene from human heart cDNA, the gene was synthesised commercially (Genscript Inc., USA) containing *Nco*I and *Hind* III restriction sites. The gene was digested and re-cloned into an expression vector, pET-32b(+), containing two 6x His tags for purification. The cloned gene was transformed into BL-21 (DE3) RIPL cells. The transformants, containing the recombinant gene, were selected by plating them on SB-carbenicillin plates.

### 6.13.3 Analysis of cTnI expression by the transformants

The transformants were picked and grown O/N in SB-carbenicillin media. The O/N grown cultures were subcultured in SB-carbenicillin media supplemented with 505 media and grown at 37°C until the O.D. at 600 nm reached ~0.6. The mid-logarithmic phase culture was then supplemented with 1 mM IPTG to induce the lac operon-controlled expression of the cloned gene. The recombinant protein was allowed to



express O/N at 30°C. The cells were pelleted down by centrifugation and the protein was extracted from the periplasm by sonication. The expression of protein was analysed by blotting the proteins with HRP-conjugated-anti-His and cTnI-specific antibody (Figure 6.19).

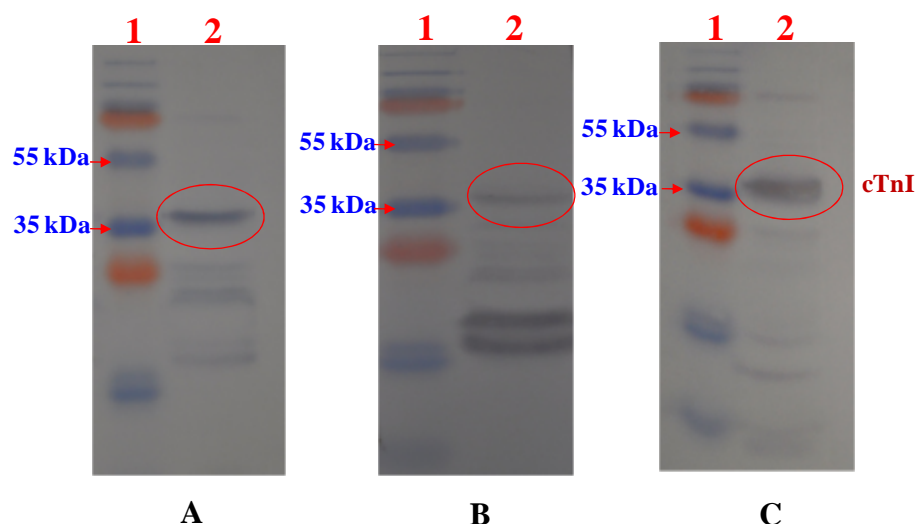


**Figure 6.19: Analysis of cTnI expression by blotting.**

**A:** Blot developed using anti-cTnI peptide 3 antibody. Lane 1: Fermentas prestained protein marker; lanes 2 to 8: different cTnI transformed clones and lane 9: HEXA protein (negative control). The blot shows that a clear band of appropriate size, of ~37 kDa, is developed for some clones but is absent in the control, confirming the expression of the gene. The actual size of the gene is 23 kDa which is increased due to the expression of an additional fusion protein along with His-tags.

**B:** Blot developed using HRP-conjugated-anti-His antibody. Lane 1: Fermentas prestained protein marker; lanes 2 to 8: different cTnI transformed clones and lane 9: HEXA protein (negative control). HEXA is used in this study as control due to the cloning of gene in similar vector (pET-32b(+)) and expression in BL-21 strain of *E. coli* as cTnI.

The blotting results also demonstrated that the recombinant protein had the relevant cardiac-specific peptide epitopes in the correct conformation to allow specific antibody binding (Figure 6.20).



**Figure 6.20: Analysis of the cTnI expressing clone by blotting with different anti-peptide antibodies.**

*A: blot developed with anti-peptide 1 antibody; B: blot developed with anti-peptide 2 antibody; C: blot developed with anti-peptide 4 antibody.*

*Lane 1: Fermentas prestained protein marker; lanes 2: cTnI expressing clone*

*There are 4 cardiac-specific peptide epitopes for cTnI. Peptide 3 was used in initial screening of clones. One clone was selected for further analysis by antibodies against 3 other peptides to check the functionality of the expressed recombinant protein. The results demonstrate that epitope peptide regions 1, 2 and 4 are functional. A blot with anti-peptide 2 antibody shows some strong bands of low molecular weight along with the specific band, which may be due to degradation of lysate during storage.*

## 6.14 Discussion

The present study was focussed on generating polyclonal antibodies to cTnI, the gold standard marker for diagnosis of cardiovascular diseases (Thygesen *et al.*, 2007). CTnI is regulatory protein in the myocardial contractile apparatus, contained in both cytosol and structurally bound to the muscle thin filament (Gowda *et al.*, 2009). Myocardial necrosis results in the release of this particular protein in various forms, including free and complex forms, with or without modifications due to oxidation and degradation (Abramov *et al.*, 2006). Detection of different epitopes is an essential paradigm for the generation of an effective immunoassay for diagnosing myocardial infarction.

The development of pAb was intended to be used in conjugation with the existing panel of avian cardio-specific epitope-based recombinant antibodies. Anecdotal references in lab found recombinant antibodies pose problems while conjugations for various assay formats like loss of functionality or sensitivity. Use of pAb can circumvent this problem due to ease of conjugation and stability. As one of the major concerns in cTnI detection in blood is the various forms released during cardiac injury, pAb is highly desirable as a tracer in assays due to their ability to recognise multiple epitopes and toleration to minor changes in antigen such as due to polymorphism, heterogeneity of glycosylation, or slight denaturation (Lipman *et al.*, 2005).

Rabbits are major source for provision of stable, high affinity pAb for research (Rader, 2009). High serum titre against cTnI was achieved from the immunised animal favouring the isolation of high affinity anti-cTnI pAb for assay development. However, the performance of pAb in assay was unsatisfactory in initial studies. High titre rabbit anti-sera warranted the generation of high affinity antibodies, so the research was continued to see if cTnI-specific recombinant antibodies could be isolated.

Recombinant antibody libraries were prepared separately for rabbit spleen and bone marrow to get maximum diversity of the genes by pooling both naïve and immune antibody repertoire of the animal. This procedure allows the shuffling of gene segments that might provide stable variable gene combinations and, in certain cases, antibody with improved affinity (Ewert *et al.*, 2003). The library was panned using stringent conditions to select high affinity clones during the process. Common problems associated with rabbit scFvs and Fabs are weak expression which may or may not be due to the presence of an aberrant Cys 80 in the sequence of antibody produced by certain allotypes of rabbits (Schmiedl *et al.*, 2000). This fact led to the use of a panning

strategy which allowed unbiased selection of weak expressers. After each phage selection round in the biopanning process, the infected *E. coli* cells were plated out in order to provide sufficient time to the weak expressor cells to grow and be amplified for further rounds.

A large panel of recombinant antibodies were successfully generated which was characterised to be highly specific for the cardiac isoform of the TnI in addition to the polyclonal serum. Along with the development of antibodies, recombinant cTnI was expressed to meet the increasing demands of antigen for further antibody characterisation and assay development. The expressed protein retained its immunological activity in the absence of post-translational modification which was verified using various N-terminal specific anti-peptide specific antibodies. It is anticipated that these reagents will be useful in the development of novel, proprietary immunoassays for cardiac disease diagnosis.

# **Chapter 7**

## **Formatting Rabbit Anti-cTnI Antibodies for Optimal Expression**

## 7.1 Introduction

Antibody libraries were developed to mimic the diversity of the natural immune system and have been shown to have many potential applications. A thorough understanding of the underlying principles of antibody generation coupled to the knowledge of various genetic engineering tools have facilitated the exploitation of this natural process *in vitro* (Dinnis and James, 2005; Fellouse *et al.*, 2007).

The realisation that only a small proportion of the entire antibody structure is required for binding the antigen in conjugation with development of advanced molecular biology tools, led to engineering of antibodies into various formats. The scFv format is one of the most commonly used in both diagnostics and therapeutics, followed by the Fab format (Benhar, 2007). However, recent understanding in the immune system of various species has led to further miniaturisation of antibodies to single domain antibody fragments e.g.  $V_H$ ,  $V_{H_H}$ , etc. (da Silva *et al.*, 2008). ScFvs can be made in either domain orientation  $V_L$  (NH<sub>2</sub>)-linker- $V_H$  (COOH) or  $V_H$  (NH<sub>2</sub>)-linker- $V_L$  (COOH), employing linker of variable lengths and both scFv formats have been described (Dorai *et al.*, 1994; Kipriyanov *et al.*, 2003; Albrecht *et al.*, 2006).

Expression and production of scFvs in *E. coli*, is greatly influenced by the stability and folding of the antibody fragment in the *E. coli* periplasm which, in turn, depends on several factors such as disulphide bonds, salt bridges between oppositely charged amino acids amino acids and repulsions between equally charged amino acids, hydrophobic core packing, buried hydrophilic residues and exposed hydrophobic residues. Several studies focussed on studying the effect of various biophysical factors to improve stability (Knappik and Pluckthun, 1995; Forsberg *et al.*, 1997; Kipriyanov *et al.*, 1997; Nieba *et al.*, 1997; Ewert *et al.*, 2003).

The stability of an antibody can be significantly affected by any modification, required for various applications. ScFvs are regularly modified by processes such as biotinylation (Santala and Lamminmäki, 2004), PEGylation (Natarajan *et al.*, 2005; Kubetzko *et al.*, 2006) and thiolation (Albrecht *et al.*, 2004). The introduction of cysteines into antibody structure may be used for different conjugation purposes (Kipriyanov *et al.*, 1994; Segal *et al.*, 1999; Schmiedl *et al.*, 2000; Junutula *et al.*, 2008b) and specific vector constructs are commonly used for site-specific insertion of a cysteine (Gentle *et al.*, 2004; DeNardo *et al.*, 2005).

The present study harnessed the ability of the rabbit immune system to generate antibodies, containing an unnatural disulphide bridge between Cys 80 in FR3 of variable light chain and Cys 171 of constant chain (McCartney-Francis *et al.*, 1984). This feature is observed in certain allotypes of rabbits, such as B4, B5 and B6, which utilises the K1 isotype of light chain. In rabbits with the K2 isotype the disulphide bridge is found between Cys 108 and Cys 171, which generates a free thiol group in the scFv format.

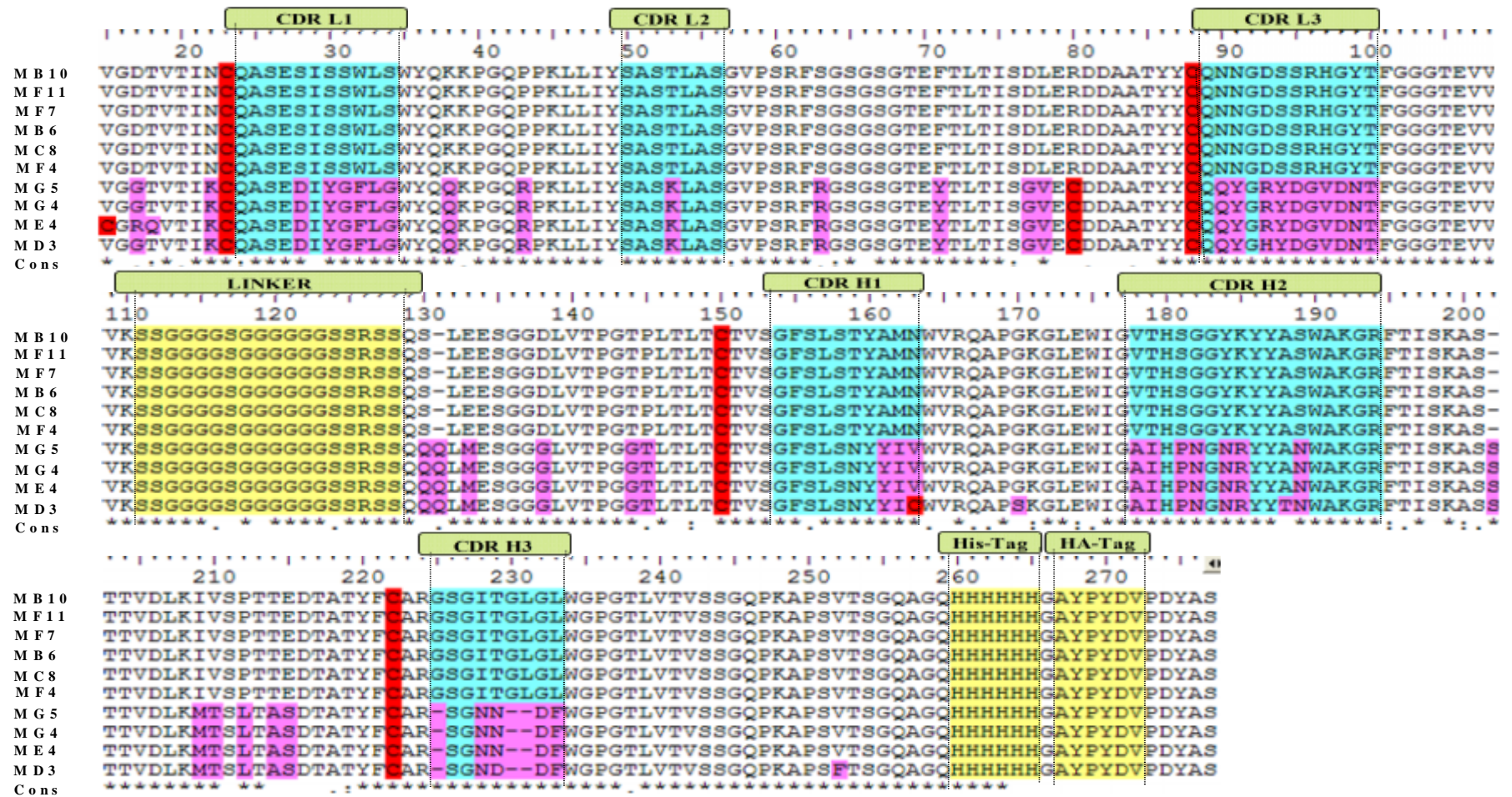
Introduction of a cysteine into an already well characterised scFv might require extensive analysis to ensure its antigen binding characteristics (Schmiedl *et al.*, 2000; Junutula *et al.*, 2008a; Del *et al.*, 2009). The objective of this study is to isolate antibodies containing naturally occurring free Cys 80 and study its application for conjugation purposes. ScFvs used in this study were selected by stringent biopanning conditions to identify only high affinity antigen-specific antibodies with a free Cys. Isolated of scFvs containing free Cys residues were further analysed to check stability, expression and binding affinity before studying their efficacy for conjugation in immunoassay platforms.

## 7.2 Sequencing of Rabbit Anti-cTnI scFvs

The rabbit scFv clones selected from ranking analysis on Biacore 3000 were sequenced using SOE-PCR primers. The sequences were aligned using ClustalX software and the CDR'S were identified. The analyses showed that basically two set of scFv sequences were obtained with major differences in CDRs in addition to some minor differences in the FRs. Exceptionally, one clone contained few substituted amino acids in the sequence, however, it was highly homologous to one set of the sequence. Sequencing data of the scFvs correlated with their restriction mapping analysis in which antibodies with three different restriction profiles were obtained.

The scFv sequences were checked for a Cys residue at position 80 (McCartney-Francis *et al.*, 1984) in the FR3 of the light chain variable domain. It was observed that there were two sets of scFvs, classified on the basis of CDRs and FR sequences that differed in their amino acid at position 80. ScFvs of one set had Cys 80 while the members of other set shared an Arg (arginine) 80. Figure 7.1 shows the sequences of the scFvs when aligned and the Cys residues highlighted with the help of Bioedit 7 software. Out of a total of 10 clones, 6 clones had Arg 80 and 4 clones had Cys 80. The clones with Arg 80 had similar sequences in the CDRs and framework regions whereas the clones with Cys 80 shared similar CDRs and FR except MD3 clone which possessed extra cysteine with few other amino acid differences.



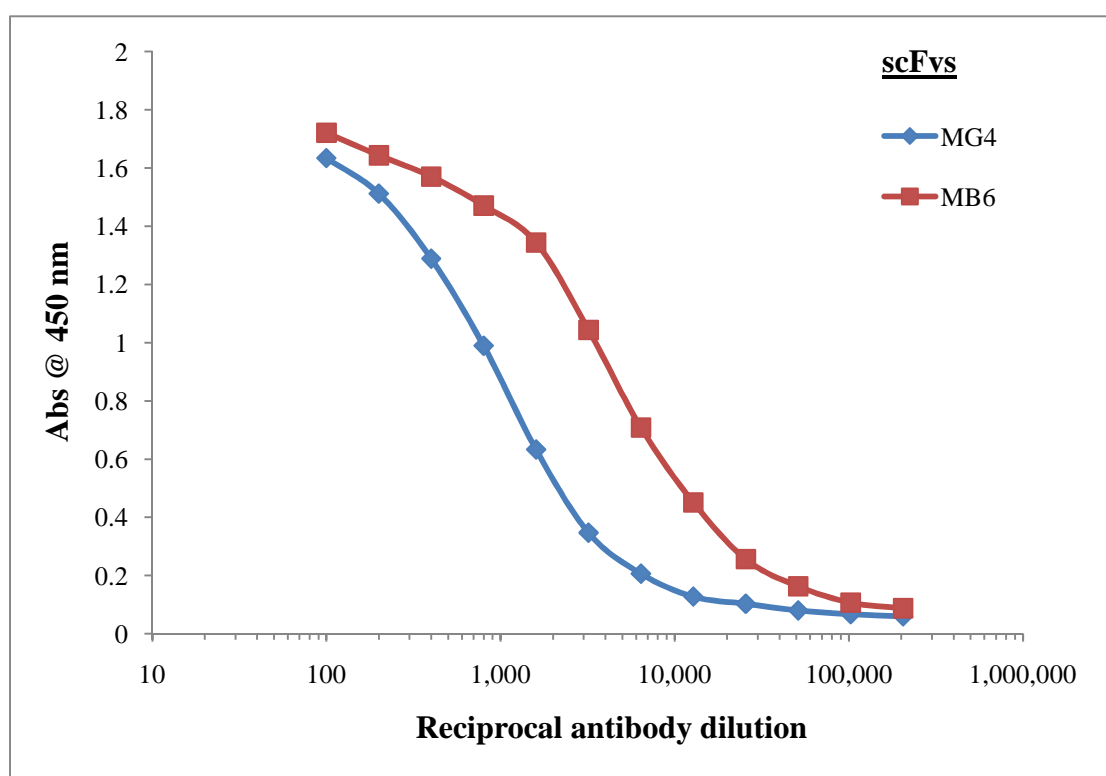


**Figure 7.1: ScFv sequence alignments for diversity analyses.**

The scFv clones were aligned against each other to determine the diversity in sequences. The diversity between two main set of scFv sequences are highlighted in pink whereas the Cys residues are highlighted in red. The CDRs shared between the same groups of scFvs are predominantly similar whereas the diversity is much higher when compared to non-related scFvs.

### 7.3 Comparative Expression Analysis of Anti-cTnI scFvs with Different Amino Acid at Position 80

Based on the ‘off-rate’ analysis, two scFvs with different amino acid sequence at position 80 were selected. The scFvs were analysed for their expression by ELISA and purification by SDS-PAGE analysis. ScFv (MG4) with Cys80 and the scFv (MB6) with Arg80 were grown in large volume cultures (500 mL) and their lysates were checked for titre by ELISA. ELISA analysis revealed better expression of MB6 (Arg80) as compared to MG4 (Cys80) (Figure 7.2).

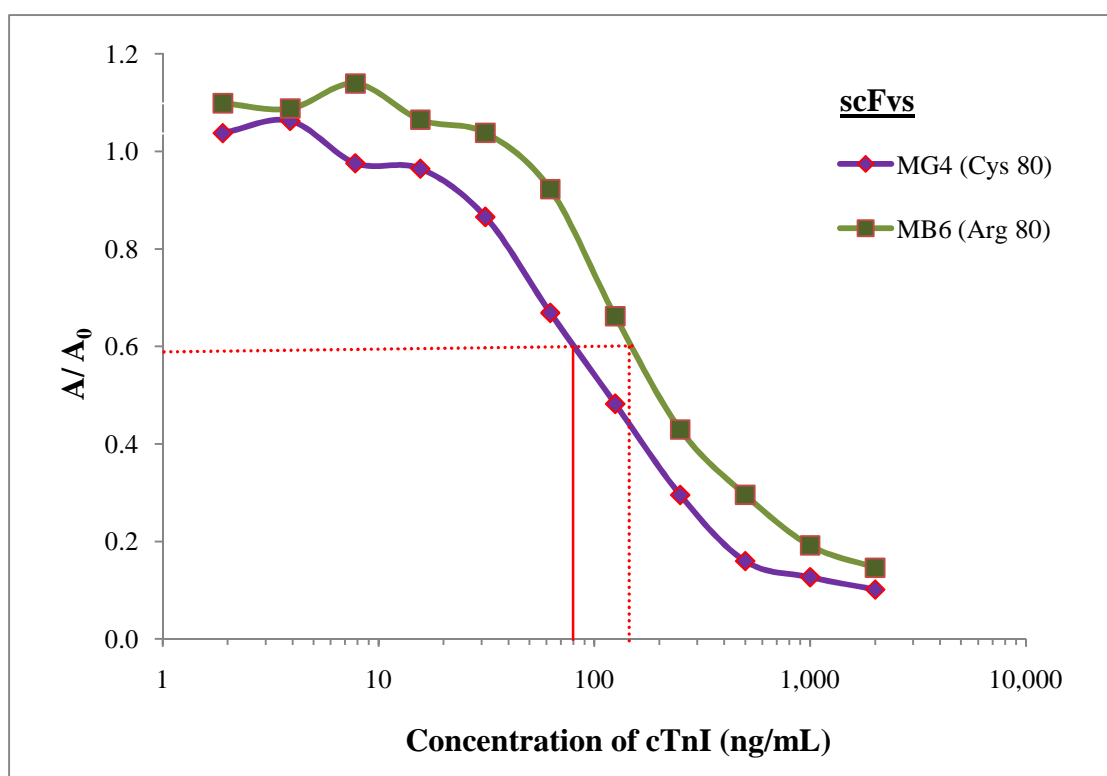


**Figure 7.2: Titre of purified rabbit anti-cTnI scFvs.**

*The purified scFvs were titred using a range of dilutions from 1:1,000 to 1:2,560,000. It is evident from the graph that a less titre of MG4 (1:6,400) is obtained when compared to MB6 (1:51,200), under similar conditions of growth, expression and purification.*

These lysates were purified using IMAC column and the purified antibody concentrate was quantified on the Nanodrop™ ND-1000 and checked on SDS-PAGE. Both the

analyses indicated that a lower protein yield of MG4 (0.5 mg/mL) was obtained, as compared to MB6 (1mg/mL) after purification. An inhibition ELISA was performed with the purified scFvs. This provided preliminary data on sensitivity of both the scFvs towards the antigen. Inhibition ELISA showed that MG4 scFv was more sensitive than that of MB6 (Figure 7.3), suggesting that Cys 80 may not have any influence on the antigen-binding sensitivity of scFv, as observed with its expression.



**Figure 7.3: Inhibition ELISA of purified anti-cTnI scFvs.**

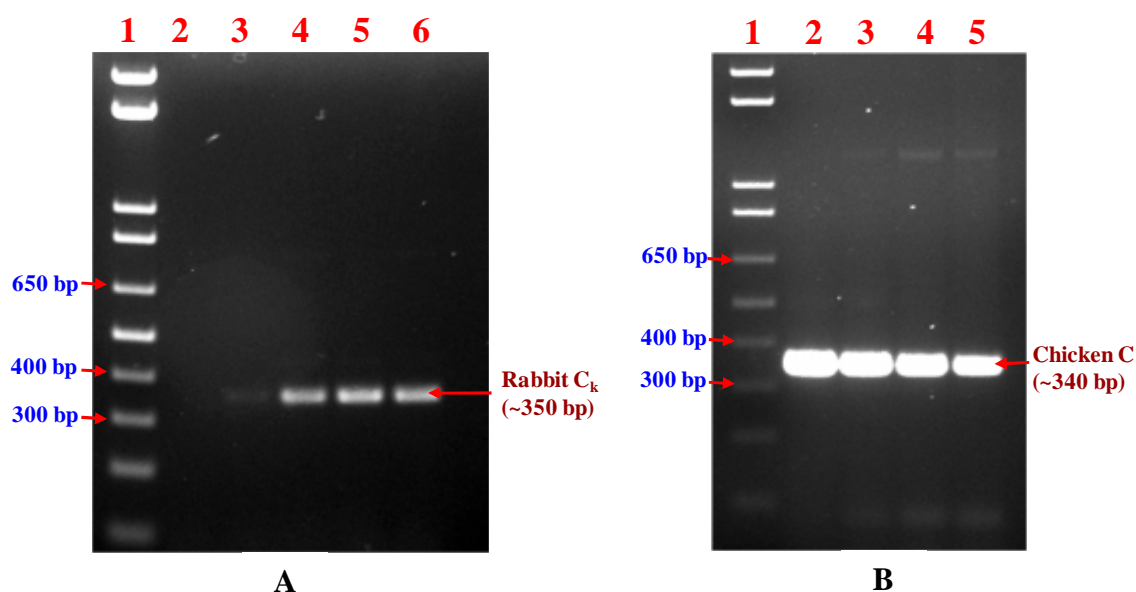
*Inhibition ELISA was carried out, in duplicate, on purified scFvs by diluting them according to their mid-point values obtained from their titre, as determined by ELISA. Different concentrations of free antigen were added to the diluted scFv ranging from 2,000 ng/mL to 1.90 ng/mL. The MG4 scFv with Cys 80 was slightly more sensitive ( $IC_{50}$ =80 ng approx) as compared to MB6 with Arg80 ( $IC_{50}$ =105 ng approx.), having a low limit of quantitation of 15.62 ng/mL*

## **7.4 Reformatting MG4 scFv**

The effect of Cys80 on low yield of scFv following expression and on purification was studied by analysing different formats for the MG4 antibody. This involved reversing the orientations of the variable domain in the scFv along with changing the scFvs, with different variable domain orientations into a scAb. The effect of the constant region on scAb expression was also analysed by expressing the scAb with constant domains of 3 different species (chicken C , rabbit C and human C ). In total, 8 different formats of a rabbit anti-cTnI antibodies were studied for expression.

### **7.4.1 Modification of pMoPac vector**

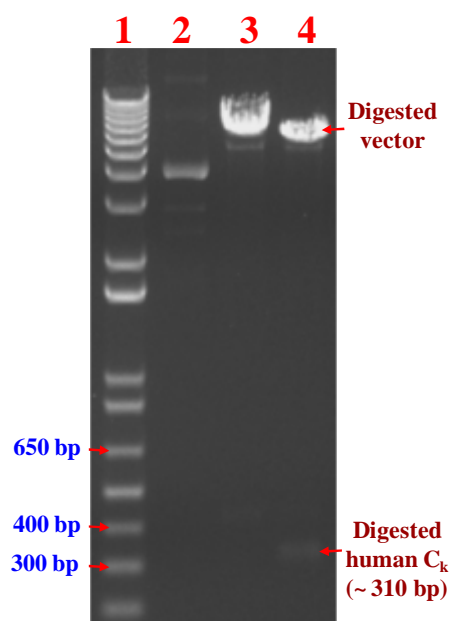
The pMoPac 16 vector was obtained from Dr. Conor Hayes with a scFv against prostate specific antigen (PSA) cloned in it. This vector was originally constructed by Dr. Andrew Hayhurst and co-workers with a human C constant chain for improving the expression and stability of scFvs (Hayhurst, 2000). The pMoPac vectors are loosely based on the pAK 400 Krebber vector for soluble expression of scFvs. The vector was modified by switching the constant region from native human C to chicken C and rabbit C . The cDNA extracted for preparation of hFABP and cTnI immune libraries were used as template for amplifying chicken and rabbit constant regions (Figure 7.4).



**Figure 7.4: Amplification of rabbit and chicken constant light chains.**

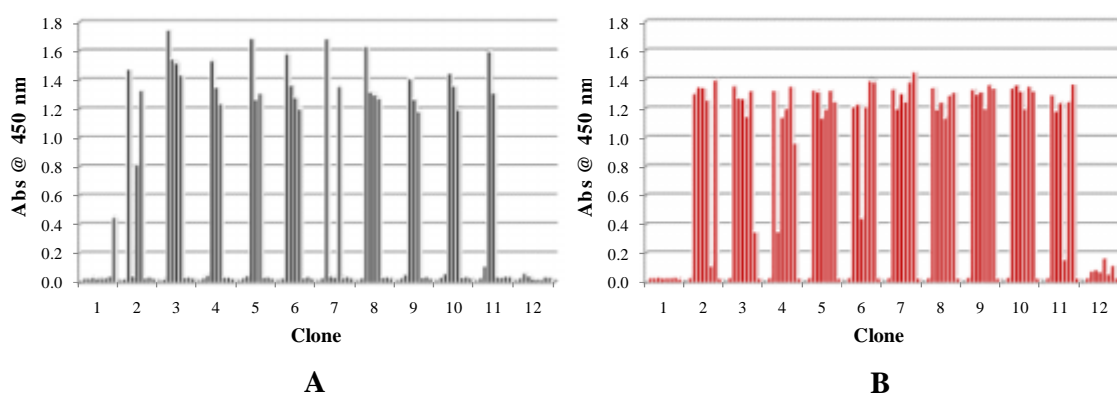
Rabbit (A) and chicken (B) constant light chains were amplified from their respective cDNA using different  $MgCl_2$  concentrations. Lane 1: 1 kb DNA ladder plus; lane 2: 1 mM  $MgCl_2$ ; lane 3: 2 mM  $MgCl_2$ ; lane 4: 3 mM  $MgCl_2$ ; lane 5: 4 mM  $MgCl_2$  and lane 6: 5 mM  $MgCl_2$ . It is shown that a  $MgCl_2$  concentration of 4 mM for rabbit C and 1 mM for chicken C gave better amplification of the product. The optimised  $MgCl_2$  concentration was then used for 'large-scale' PCR.

The amplified product and the pMoPac 16 vector (Figure 7.5) were digested with *NotI* and *SalI* enzymes. This enzyme combination cuts the human C<sub>k</sub> in the pMoPac vector, upstream to the scFv cloning site, which was substituted with the antibody constant domains of other species. Chicken and human constant regions were cloned into the vector and were transformed into TOP10F cells. The colonies were screened on a PSA-coated plate due to the presence of an anti-PSA scFv gene in the modified vector. The positive clones were detected with anti-chicken and anti-rabbit secondary antibodies thus enabling the selection of clones with substituted constant regions (Figure 7.6).



**Figure 7.5: Sequential digestion of vector with *NotI* and *SalI* enzymes.**

*Human C<sub>k</sub>* digested from the vector can be seen as a faint band at approx. 300 bp. Lane 1: 1 kb DNA ladder plus; lane 2: uncut vector; lane 3: *NotI* linearised vector and lane 4: *NotI* and *SalI* digested vector.



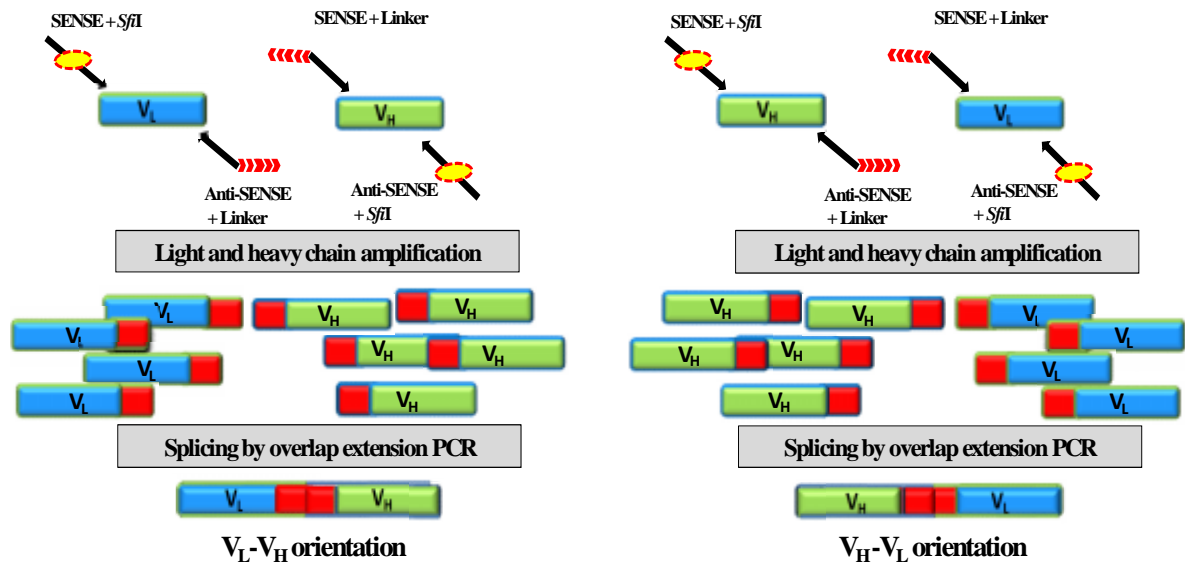
**Figure 7.6: Screening of clones with modified constant region.**

Graph A represents the clones for chicken constant region modification whereas graph B is showing clones with rabbit constant region. The pMoPac used for this purpose, had a PSA scFv gene cloned in it which facilitated the screening of clones by binding the scAb expressed to PSA-coated plate and the scAbs with modified constant region were detected using secondary antibody-specific for the constant region. This resulted in the selection of functional scAbs having two different constant light chains.



#### 7.4.2 Changing variable domain orientation

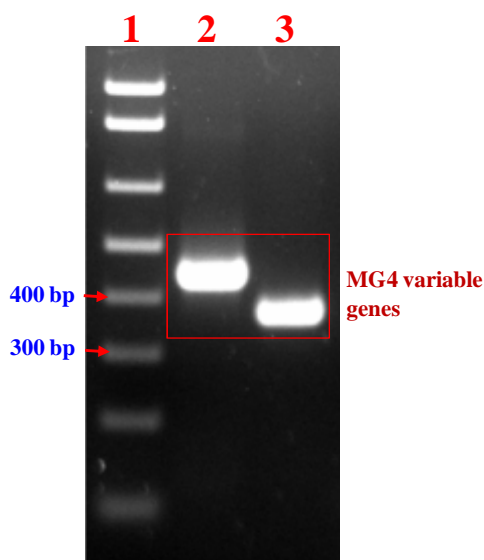
The scFv MG4 was originally cloned in pComb3XSS vector in  $V_L$ - $V_H$  orientation. To change this orientation to  $V_H$ - $V_L$ , the position of linkers and the *Sfi*I site in the primers were changed. The strategy used for designing the primer and amplifying the genes is represented in Figure 7.7.



**Figure 7.7: Strategy for changing variable domain orientation.**

The  $V_L$ - $V_H$  orientation is the original variable-domain orientation of scFv. The *Sfi*I site is present in the sense primer of light chain and in the anti-sense primer of heavy chain to enable directional cloning into pComb3XSS vector. For assembling the genes, the linker is coded by the anti-sense primer for light chain and sense primer of heavy chain. In reversing the domain orientation the reverse was carried out. This means the *Sfi*I site is present in the sense primer of heavy chain and anti-sense primer of light chain, and the linker is present in the sense primer for light chain and anti-sense primer of heavy chain.

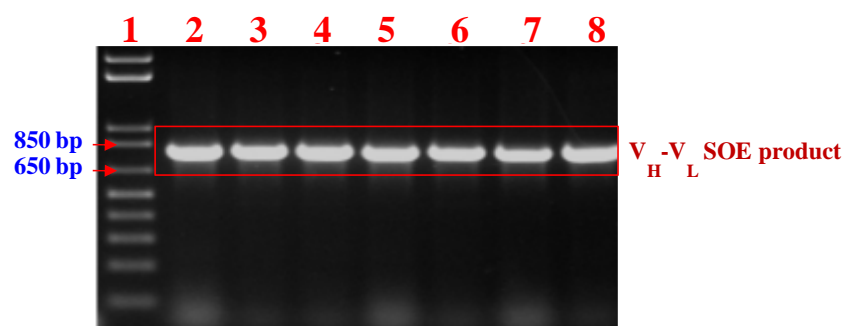
The variable genes were re-amplified from the plasmid of MG4 scFv using the new primers, which reversed the position of *Sfi*I restriction site and linker (Figure 7.8). These variable genes were assembled by SOE-PCR using primer combination containing the heavy chain coding sequence in the sense primer and light chain coding sequence in the anti-sense primer. This overlap SOE-PCR joined the antibody variable genes in V<sub>H</sub>-V<sub>L</sub> domain orientation which was later cloned into pMoPac vector constructs with human C<sub>κ</sub>, chicken C<sub>κ</sub>, and rabbit C<sub>κ</sub> light chains (Figure 7.9).



**Figure 7.8: Amplification of variable genes from MG4 scFv, with V<sub>L</sub>-V<sub>H</sub> variable domain orientation.**

*Lane 1: 1 kb DNA ladder plus; lane 2: variable heavy chain and lane 3: variable light chain. No optimisation was required as satisfactory amplification was observed at an annealing temperature of 56°C and a 1 mM MgCl<sub>2</sub> concentration.*





**Figure 7.9: Assembling variable genes from MG4 scFv using splicing by overlap (SOE) PCR.**

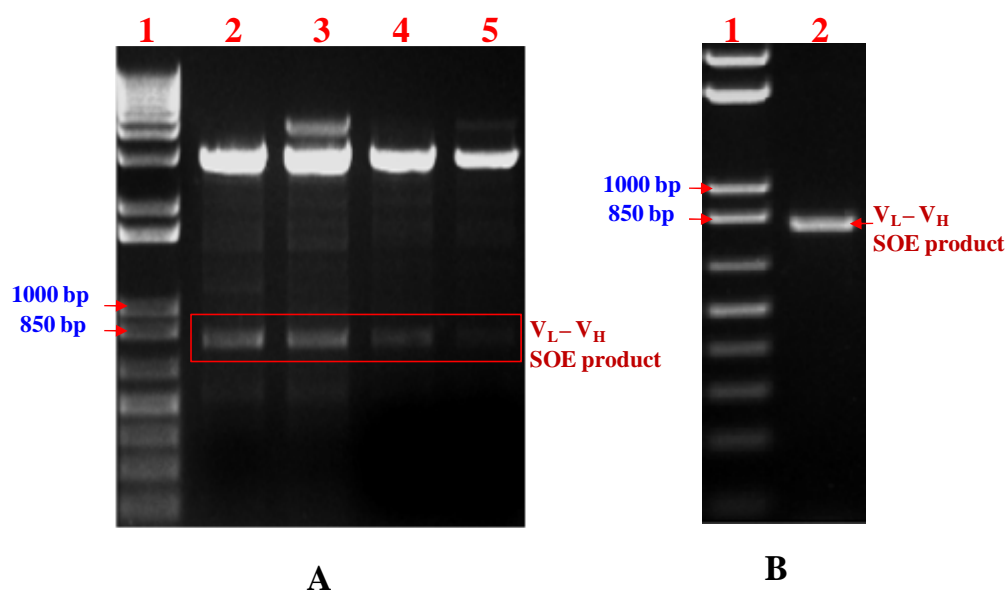
A range of annealing temperature was tried for assembling the variable chains in  $V_H$ - $V_L$  orientation. A  $MgCl_2$  concentration of 1 mM was used. There was no effect of the annealing temperature on the amplification of product. All the products were pooled and purified and used as template for cloning into the pMoPac 16 vector. Lane 1: 1 kb DNA ladder plus; lane 2: 58.0°C; lane 3: 58.6°C; lane 4: 59.5°C; lane 5: 60.0°C; lane 6: 61.5°C; lane 7: 62.5°C and lane 8: 63.4°C.

#### 7.4.3 Changing the restriction sites on the SOE-PCR product

The SOE-PCR product in both  $V_L$ - $V_H$  and  $V_H$ - $V_L$  domain orientation were reamplified with SOE-primers containing vector compatible *Sfi*I restriction sites (Figure 7.6 and 7.7). The *Sfi*I enzyme recognises a sequence coding for 5 amino acids flanked by a stretch of 4 amino acids ggcc on both sides which can be represented as “ggccnnnnnggcc”. This 5 amino acid sequence was designed in the primer to comply with vector *Sfi*I sites, for directional cloning of the amplified gene product.

The *Sfi*I restriction site in the MG4 scFv is compatible with pComb vector system whereas the *Sfi*I site in the SOE-PCR product, amplified in section 7.4.2, is compatible with the pMoPac vector. The restriction sites on the SOE primers were changed, so that SOE products compatible with pMoPac and pComb vector systems can be generated in order to construct scAbs with  $V_L$ - $V_H$  domain orientation and scFvs with  $V_H$ - $V_L$  domain orientation.

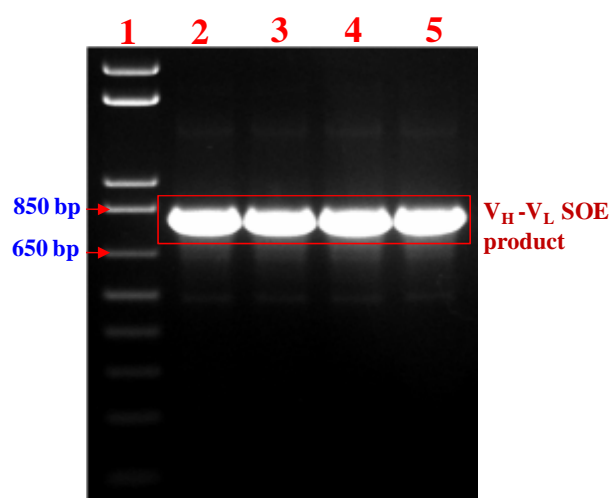
The scFv gene was amplified from MG4 scFv with  $V_L$ - $V_H$  orientation and was cloned into the pMoPac 16 vector constructs (with human  $C_k$ , chicken  $C$ , and rabbit  $C_k$  light chains). The scFv gene (from section 7.4.2) was reamplified with pComb compatible SOE-primers and cloned into the pComb vector for producing an scFv with  $V_H$ - $V_L$  orientation.



**Figure 7.10: Optimisation of SOE amplification.**

(A) Amplification of scFv gene  $V_L$ - $V_H$  from MG4 scFv. The gene was amplified with Phusion Taq as there was no amplification generated with Platinum Taq polymerase. The amplification was optimised using various annealing temperatures and DMSO concentrations. This figure represents the optimisation of annealing temperature for the amplification of  $V_L$ - $V_H$  SOE product. The PCR yielded many partially amplified products along with the SOE product. Lane 1: 1 kb DNA ladder plus; lane 2: 55.0°C; lane 3: 56°C; lane 4: 58°C and lane 5: 59°C.

(B) The  $V_L$ - $V_H$  SOE product was directly amplified from the SOE product digested and purified from the plasmid at an annealing temperature of 56°C.



**Figure 7.11: Amplification of  $V_H$ - $V_L$  gene from purified SOE-PCR product.**

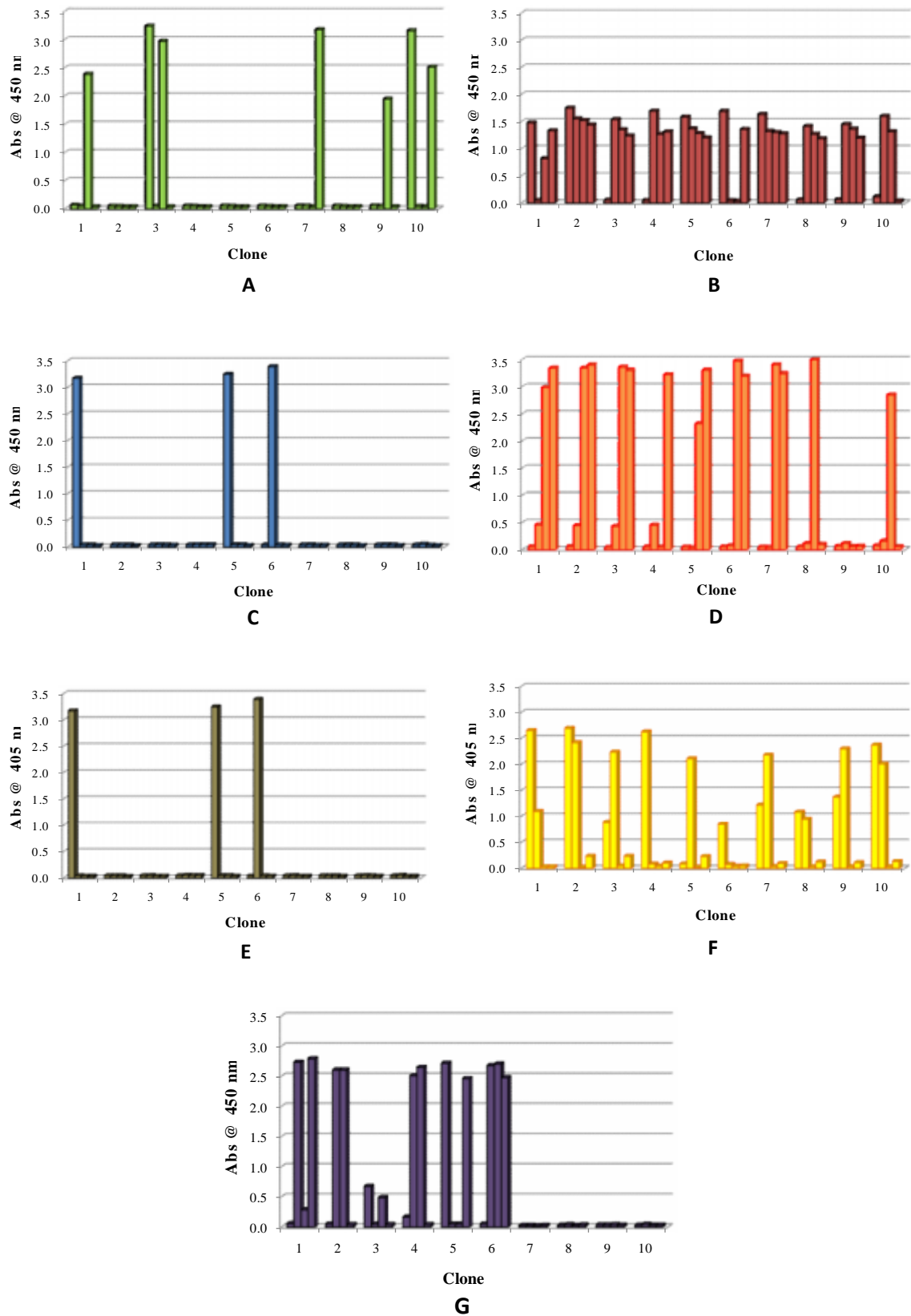
*The annealing temperature was optimised as carried out for  $V_L$ - $V_H$  gene amplification. Lane 1: 1 kb DNA ladder plus; lane 2: 55.0°C; lane 3: 56°C; lane 4: 58°C and lane 5: 59°C. There was no effect of annealing temperature on the product amplification so all the products were pooled and purified for cloning.*

#### **7.4.4 Transformation and screening of antibodies with different formats**

Different constructs prepared with both pComb3XSS and pMoPac vectors, with different constant light chains and different domain orientations of scFvs, were transformed into TOP10F cells. The colonies were screened (section 7.4.1). The clones were screened on a cTnI-coated plate by ELISA (Figure 7.12). The detecting antibody used was specific for the vector construct, i.e. for the pMoPac vector the secondary antibody was against the constant regions and for pComb3XSS, an anti-HA antibody was used.

The antibodies with different constant regions and different domain orientations were sequenced to confirm cloning of the gene into different vector constructs with desired orientations. The sequence also confirmed that the antibody gene sequence was intact with no undesirable mutations while amplification or cloning into the vector, enabling

the comparison of the antibodies based purely on different molecular formats with no bias in relation to any changes in the original antibody gene.

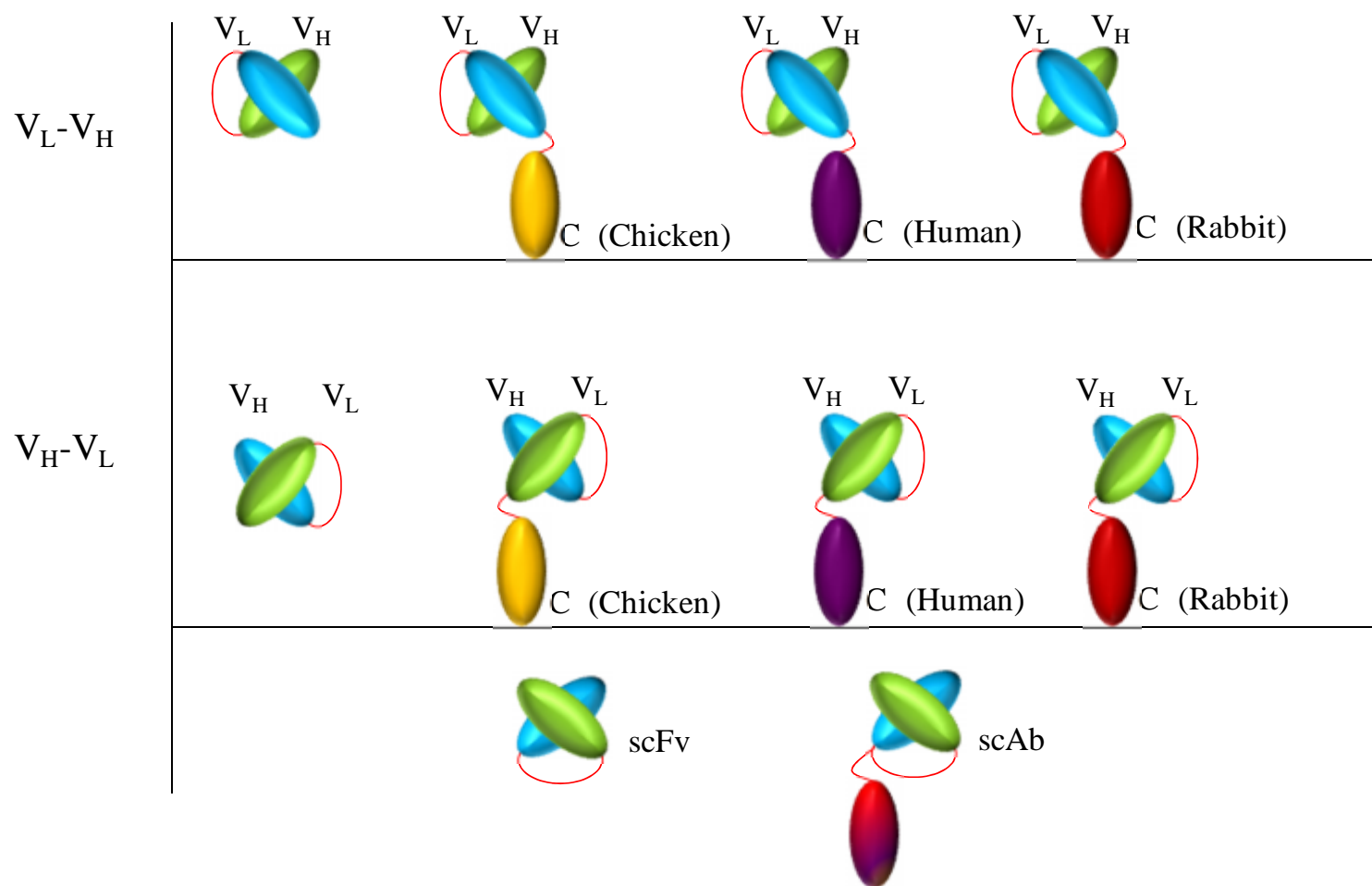


**Figure 7.12: Screening of antibodies with different formats.**

**A)** scAbs with rabbit C domain ( $V_L$ - $V_H$  domain orientation); **B)** scAbs with rabbit C domain ( $V_H$ - $V_L$  domain orientation); **C)** scAbs with chicken C domain ( $V_L$ - $V_H$  domain orientation); **D)** scAbs with chicken C domain ( $V_H$ - $V_L$  domain orientation); **E)** scAbs with human C domain ( $V_L$ - $V_H$  domain orientation); **F)** scAbs with human C domain ( $V_H$ - $V_L$  domain orientation); and **G)** scFv ( $V_H$ - $V_L$  domain orientation).

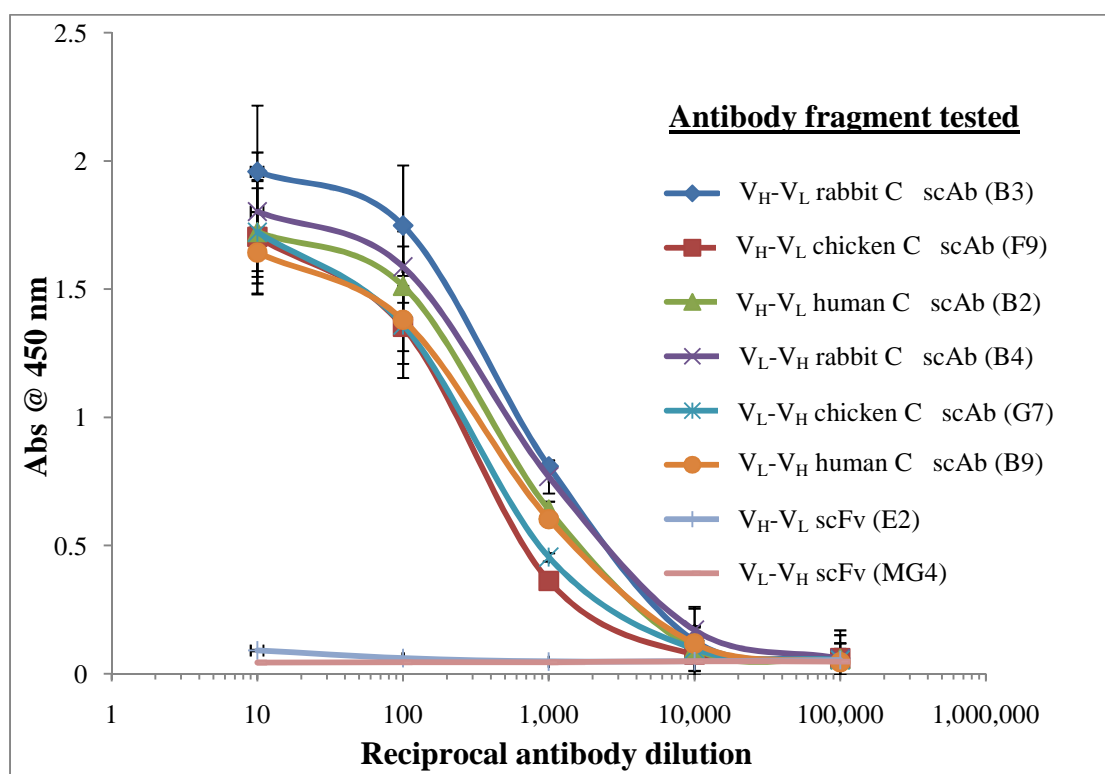
## 7.5 Expression Analysis of Different Antibody Formats

In total eight different antibody-derived formats were generated as shown in Figure 7.13. Clones were selected for the different antibody formats and analysed for expression *via* ELISA. All the clones were grown and expressed under similar conditions. The clones were processed similarly for periplasmic extraction of the expressed protein and assayed against cTnI, in triplicates, taking standard deviations of the measurement results into consideration. ScAbs showed a very high signal as compared to scFvs (Figure 7.14), with quick development of colour when TMB was added. This may be attributed to the high expression of scAb with respect to scFvs (Hayhurst, 2000). ScAbs with different constant regions or domains did not show any major difference in the expression although the scAbs with rabbit constant region had better  $IC_{50}$  values. The standard deviations obtained for each of the data point were acceptable, verifying the results obtained.



**Figure 7.13: Types of scFv and scAb formats used.**

Two types of scFvs were used one with the  $V_L-V_H$  domain and the other with a  $V_H-V_L$  domain. Six different types of scAbs were made with three different constant regions containing variable genes of both  $V_L-V_H$  and  $V_H-V_L$  domain orientation.

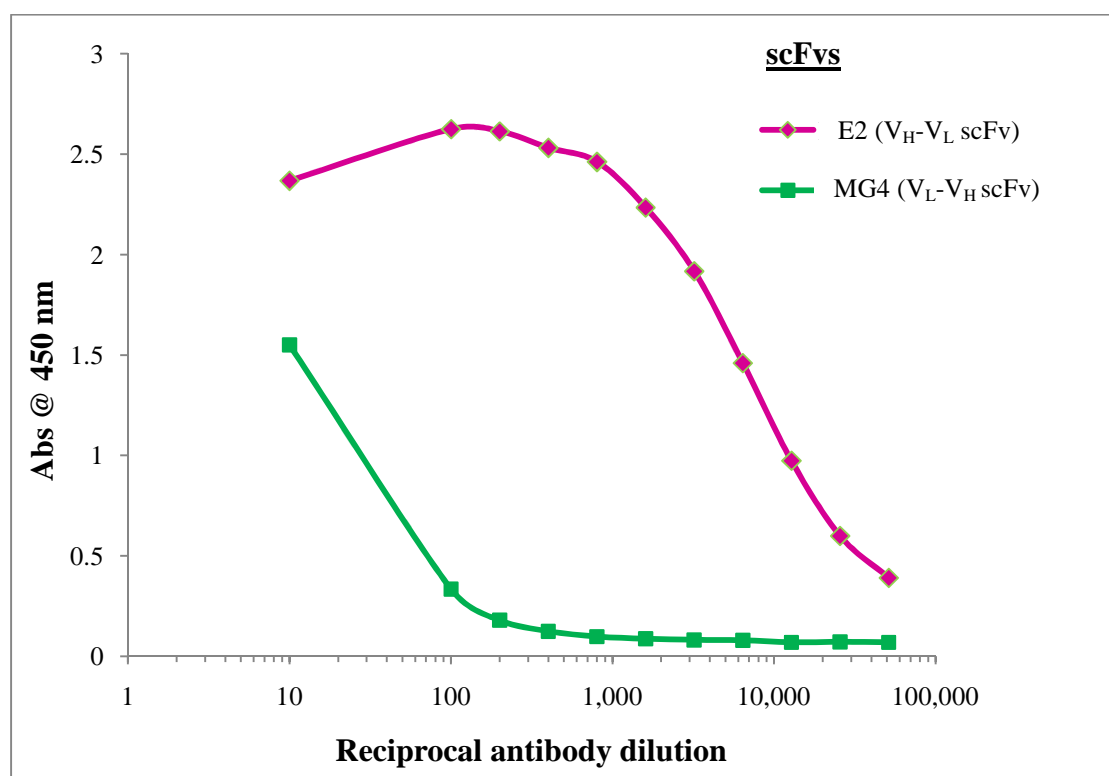


**Figure 7.14: Comparative expression analysis of different antibody formats.**

Different antibody formats were compared using five different antibody dilutions (1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000). ELISA was performed on a single plate and developed using HRP-conjugated-anti-His antibody which was common for the antibodies expressed in pComb and pMoPac vectors. ScAbs were found to be better expressers than scFvs. A slight increase in the signal of scFv with the V<sub>H</sub>-V<sub>L</sub> domain as compared to one with the V<sub>L</sub>-V<sub>H</sub> domain was observed.

The scFv with V<sub>H</sub>-V<sub>L</sub> domain orientation showed a better signal as compared to the scFv with V<sub>L</sub>-V<sub>H</sub> domain orientation (Figure 7.15). It can be assumed that the dilution range used in the above comparison may not be appropriate for scFvs while it sufficed for titration of the scAbs. Besides, while comparing different formats, the scAbs were developed relatively quickly which necessitated the reaction to be stopped. This would not provide enough time for the scFvs to generate a signal due to their low expression levels. To analyse the effect of variable domain orientation on scFv expression, both the scFvs were compared separately using a wide range of dilutions. As the scFvs are in pComb3XSS vectors the ELISA was carried out using an HRP-conjugated-anti-HA, as

secondary antibody. The scFv with the  $V_H$ - $V_L$  domain showed a 100-fold better expression than the one with  $V_L$ - $V_H$  domain orientation.



**Figure 7.15:** *Comparative expression analysis of scFvs with  $V_H$ - $V_L$  domain and  $V_L$ - $V_H$  domain.*

*ScFvs with  $V_H$ - $V_L$  and  $V_L$ - $V_H$  domains were compared using a range of dilution starting from 1:10 to 1:51,200). A very significant difference was observed in the expression levels as shown in the graph.*

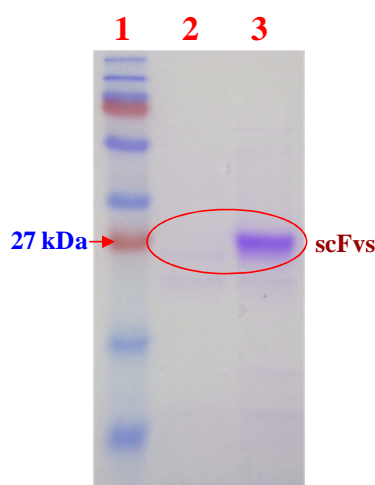
## 7.6 Comparative Purification Analysis of Rabbit Anti-cTnI Antibodies by IMAC

ScFvs with  $V_H$ - $V_L$  domain and  $V_L$ - $V_H$  domains were grown in parallel into 250 mL culture volumes and expressed O/N. Cultures were spun down and the pellet was resuspended in lysis buffer. The resuspended pellet was sonicated for periplasmic extraction of the protein followed by purification using a standard IMAC purification protocol (section 2.2.1.20). The protein concentration was quantified on Nanodrop™ ND-1000 and the purity was checked by SDS-PAGE analysis (Figure 7.16). Data from



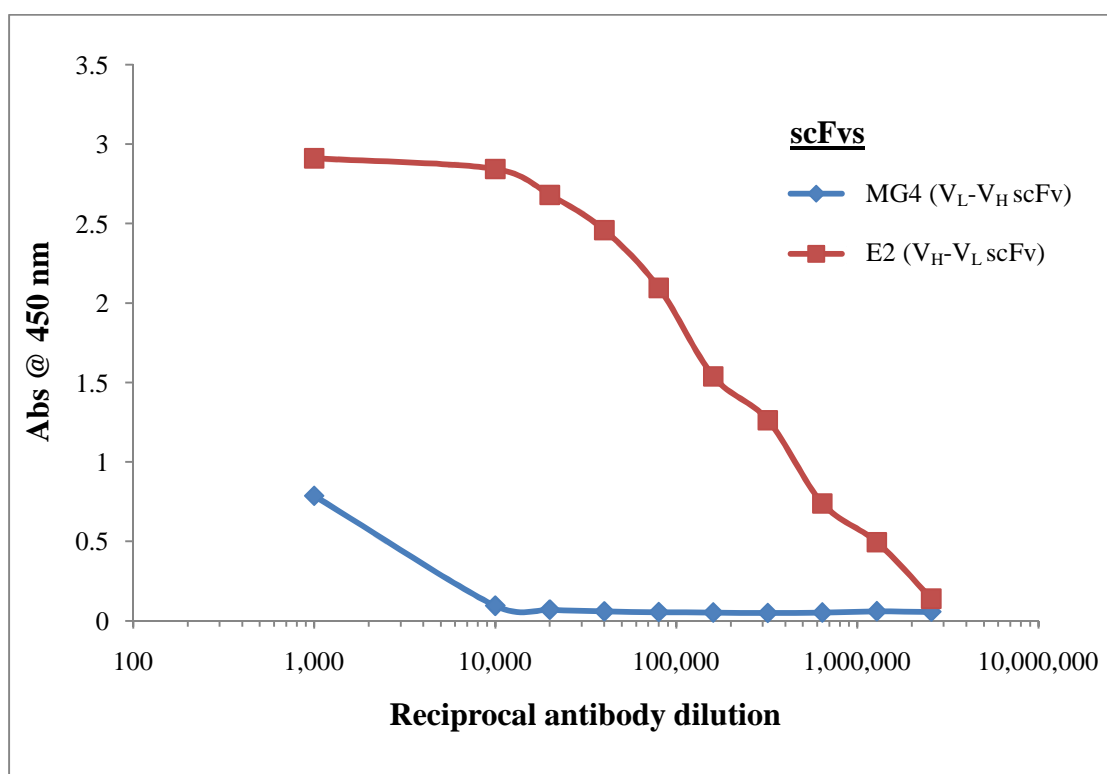
Nanodrop™ ND-1000 showed that three times higher concentration of protein was obtained for the V<sub>H</sub>-V<sub>L</sub> domain scFv as compared to V<sub>L</sub>-V<sub>H</sub> domain scFv.

SDS-PAGE analysis confirmed the Nanodrop™ ND-1000 data showing a clear band of appropriate size for the scFv with the V<sub>H</sub>-V<sub>L</sub> domain, whereas no significant band was observed for the other scFv with V<sub>L</sub>-V<sub>H</sub> domain orientation. The purified proteins were titred and analysed by ELISA (Figure 7.17). The titres of the purified scFvs showed that the MG4 scFv, with the V<sub>L</sub>-V<sub>H</sub> domain, had a low titre compared to that of ME2 scFv with the V<sub>H</sub>-V<sub>L</sub> domain.



**Figure 7.16: SDS-PAGE analysis of purification of scFvs with different domains.**

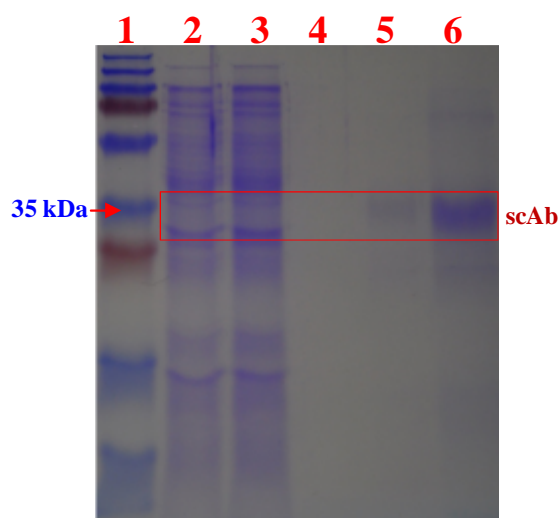
*There was no visible band on the gel for the scFv with the with V<sub>L</sub>-V<sub>H</sub> domain, whereas a very clear and sharp band was observed for the scFv with V<sub>H</sub>-V<sub>L</sub> domain. The scFv seems to be quite pure and concentrated as shown on the gel. Lane 1: Fermentas prestained protein marker; lane 2: scFv with V<sub>L</sub>-V<sub>H</sub> domain and lane 3: scFv with V<sub>H</sub>-V<sub>L</sub> domain.*



**Figure 7.17: Graph showing the titre of purified scFvs obtained.**

The ELISA verified the data from Nanodrop™ ND-1000 and SDS-PAGE gel showing significantly less yield of protein was obtained for the scFv with the  $V_L$ - $V_H$  domain. The scFv with  $V_H$ - $V_L$  domain had a titre of approximately one in a million whereas the scFv with  $V_L$ - $V_H$  domain had a titre less than one in 10,000.

The scAb with a rabbit constant domain and  $V_L$ - $V_H$  orientation was also subjected to IMAC to analyse the effects of the constant domain modifications on purification. The scAb contains an additional constant domain as compared to an scFv which adds to its size. The pMoPac vector contains six histidine tags like the pComb vector which facilitates its purification using IMAC. The isolated scAb was checked on SDS-PAGE to verify its purity. The gel showed a clear and single band at appropriate size (40 kDa) demonstrating efficient purification of the  $V_L$ - $V_H$  orientation antibody (Figure 7.18).



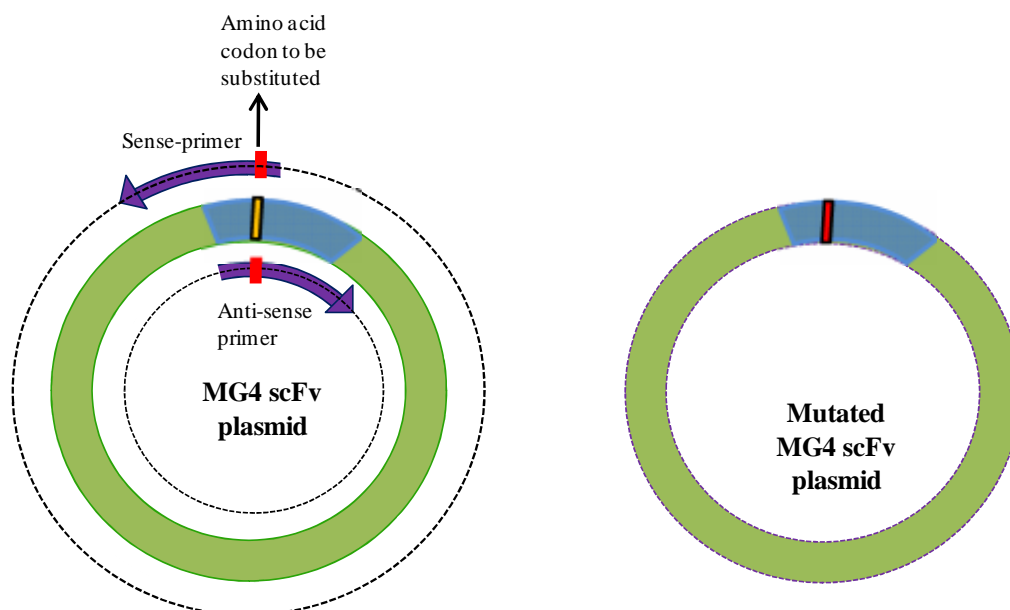
**Figure 7.18: SDS-PAGE gel analysis of rabbit anti-cTnI scAb purification.**

*Lane 1: Fermentas prestained protein marker; lane 2: scAb lysate; lane 3: flow-through from IMAC column; lane 4: wash-through from IMAC column; lane 5: 1:10 dilution of purified scAb and lane 6: neat purified scAb.*

### **7.7 Site-Directed Mutagenesis of Cys80 on MG4 scFv with V<sub>L</sub>-V<sub>H</sub> Domain**

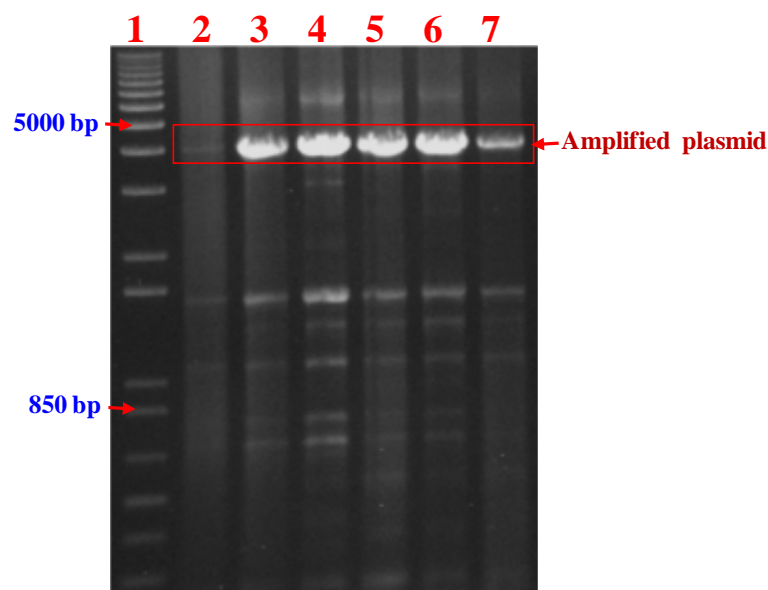
Site-directed mutagenesis is an *in vitro* technique carried out to replace/mutate an amino acid at a particular site for studying structure to function relationships. In the present study, the Cys 80, in the framework region of MG4, was replaced with another amino acid to study its influence on the expression and purification of the antibody. Basically, two types of amino acids were considered in this study, i.e. alanine (Ala) and serine (Ser), due to their small sizes. Primers were designed for site-directed mutagenesis to convert the Cys into Ala and Ser. The mutagenesis was carried out by using primers which contained the coding sequence from the antibody gene with the codon for Cys replaced by the codon for Ser or Ala. The diagrammatic representation in Figure 7.19 explains the site-directed mutagenesis process. The amplified plasmid (Figure 7.20) was gel-purified and digested with *Sfi*I enzyme to extract the mutated scFv gene from the plasmid. The gene was re-cloned into the pComb3XSS vector and transformed into

TOP10F cells. This re-cloning was performed to ensure that any mutation in the vector during amplification of the complete plasmid is not carried over in transformed scFvs.



**Figure 7.19: Site-directed mutagenesis.**

*Site-directed mutagenesis was carried out by designing the primers with the coding sequence of the scFv gene. Primers were designed by selecting the region of the gene where the Cys 80 is present. Both the forward and reverse primers were designed so as to amplify the whole plasmid starting from the region selected. The codon for Cys is replaced in the primer with the desired amino acid codon. Both sense and reverse primers contained overlapping codons thus amplifying the complete plasmid containing the scFv gene with a mutated amino acid at position 80.*

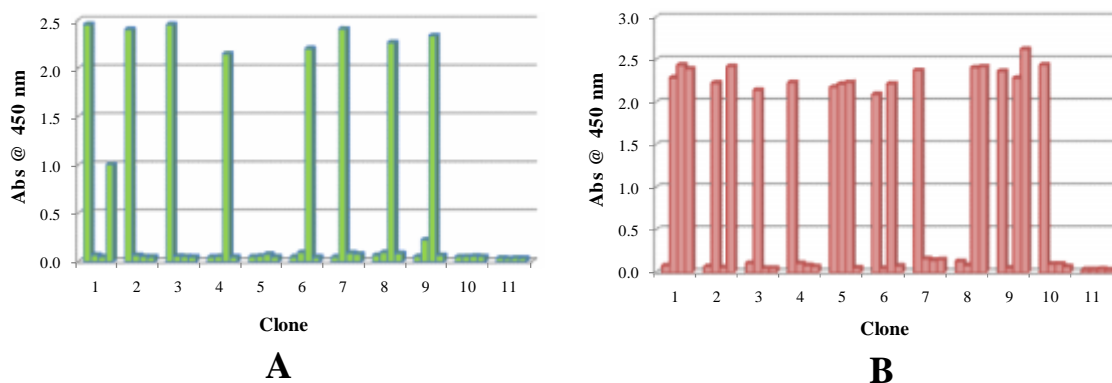


**Figure 7.20: Amplification of plasmid.**

*Optimisation of annealing temperature was carried out for plasmid amplification. Lane M: 1 kb DNA ladder plus; lane 1: 58°C; lane 2: 59.0°C; lane 3: 60°C; lane 4: 61°C; lane 5: 62°C and lane 6: 63°C. The amplification was optimal when 60°C was used as annealing temperature.*

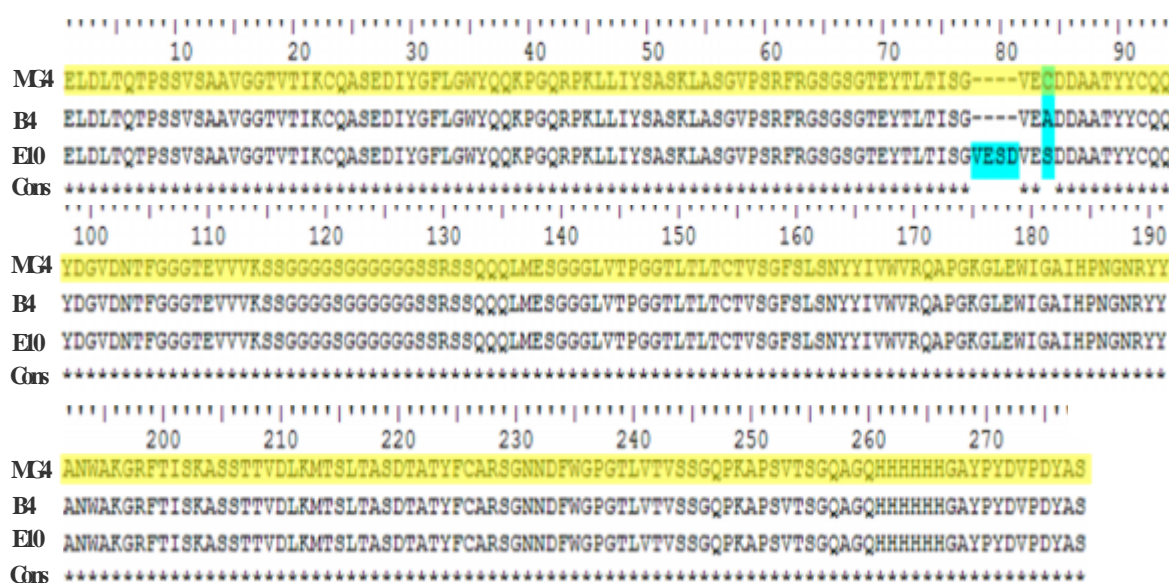
## 7.8 Screening of Mutated Clones

The colonies were screened against cTnI by indirect ELISA (Figure 7.21) and the positive clones were confirmed for mutation by sequencing with SOE-PCR primers, for V<sub>L</sub>-V<sub>H</sub> domain orientation. The sequences of the two mutated scFvs were aligned with the original scFv and the sequences were compared for mutation. The alignment showed an addition of four amino acids in the clone with Ser 80, which were repetition of the region which was targeted for primer synthesis. However, the primer sequences were re-checked to justify the cause for the addition of extra amino acids in the mutated clone. In the absence of any error in the displayed primer sequence, it may be conjectured that such a sequence may have resulted due to some error during primer synthesis or mispriming of the primer to the scFv. The other scFv was successfully mutated having Ala in place of Cys, which was verified by sequencing analyses (Figure 7.22).



**Figure 7.21: Screening of anti-cTnI scFvs after site-directed mutagenesis.**

Site-directed mutagenesis was carried out for replacing Cys 80 in the scFv gene. The codon for Cys is replaced in the primer with desire codon (**A**-alanine, **B**-serine). Screening of the clones was carried out on a cTnI-coated plate and the mutation was confirmed by sequencing.

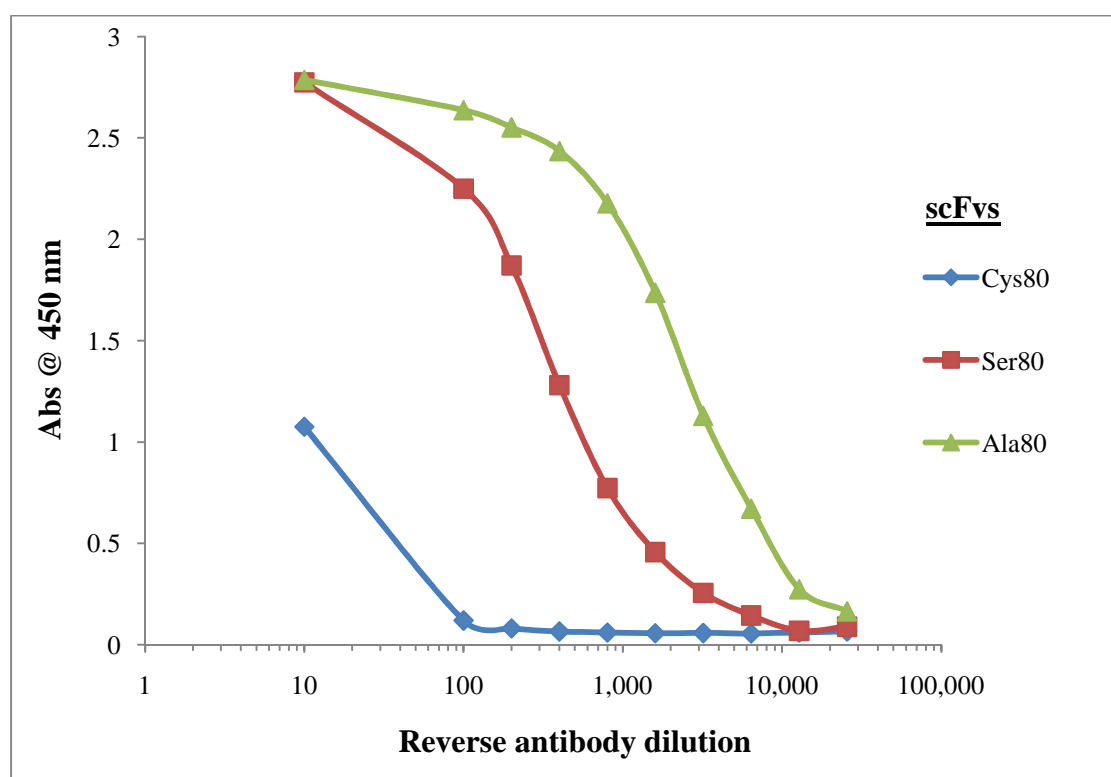


**Figure 7.22: Sequence analysis of wild-type clone with mutated clones.**

MG4 is the wild-type clone (highlighted). B4 and E10 are mutated clones with Arg 80 and Ser 80. Sequencing analysis showed that the mutagenesis was successful but a small stretch of additional amino acids was observed in the clone with the serine residue.

## 7.9 Comparative Expression Analysis of MG4 scFv with Different Amino Acids at Position 80 in the Variable Light Chain Region

The mutated scFvs, with variation in amino acid at position 80, along with original MG4 scFv (with Cys 80) were compared for expression using standard ELISA. The scFvs were grown and expressed under similar conditions followed by assaying them in triplicate using a wide range of dilutions. End-point analysis showed that the scFv with Cys 80 had the lowest titre, among the three scFvs checked, whereas the scFv with Ala 80 had the greatest titre of more than 1 in 25,600 (Figure 7.23). This analysis correlated with the results obtained in Section 6.6, where changing the position of Cys 80 *via* reversing the domain orientation, improved expression.



**Figure 7.23:** *Comparative expression analysis of scFvs with different amino acids at position 80.*

*The scFv with Cys80 had a titre of only 1:100 whereas the scFv with Ser 80 was 1:6,400 while the scFv with Ala 80 was the highest expresser with a titre of 1:25,600.*

## 7.10 Discussion

Antibody framework is based on several interactions providing a unique architecture to the protein. Among several interactions, disulphide bridges play an important role by providing both stability and folding for the immunoglobulin architecture (Glockshuber *et al.*, 1992). Particularly in antibody variable domains,  $\beta$ -sheets are connected by disulphide bridges contributed by Cys residues at L23 and L88 in  $V_L$ , and H22 and H92 in  $V_H$  (Kabat *et al.*, 1991; Proba *et al.*, 1998). Studies were carried out to analyse the precise role of the disulphide bonds on the thermodynamics of the antibody molecule (Glockshuber *et al.*, 1992; Wörn *et al.*, 2000).

Antibodies were generated using New Zealand white rabbit strain (b4 allotypes), known to possess an intrinsic disulphide bond between Cys 80 of FR3  $V_L$  region and Cys 171 of the  $C_L$  domain of the antibody. This study involved the generation of a recombinant scFv library primarily using  $V_L$  (NH<sub>2</sub>)-linker- $V_H$  (COOH) domain orientation. The scFv format consists of a variable heavy chain and variable light chain linked by a linker. Due to the absence of  $C_L$  in the scFv format for active bond formation, Cys 80 in the  $V_L$  region remains free resulting in the formation of a free thiol group, which can be exploited for conjugation purposes.

Sequencing of the clones selected from initial ranking analysis, showed that 40% of clones had Cys 80 and rest of the clones had Arg 80. The majority of the diversity exhibited between these clones, in both CDRs and FRs, were restricted. This observation was similar to that of Popkov *et al.* (2003) for which it was reasoned that the unpaired Cys 80 on the rabbit scFvs, produced due to the scFv format, reduces the selectable antibody diversity of the scFv population.



Before applying the Cys 80 based scFvs in diagnostic assay platforms it is necessary to characterise the scFv for its specificity, sensitivity, stability, expression and purification features. ScFv with compromised features will hamper the performance of the assay. This novel method of using Cys 80 for antibody conjugation relies on the fundamental aspect of selecting high affinity candidates by phage display possessing ideal features of the antibody along with the reactive Cys residue, which can provide a suitable approach for conjugation. This process eliminates those scFvs from the very beginning, in which Cys affects the stability and affinity of the antibody. However, it is important to understand the role of Cys 80 in influencing scFv features before proceeding further as a quality control that might influence the assay performance.

The expression and purification studies showed that expression and purification of the scFv MG4 (Cys 80) was less as compared to MB6 (Arg 80). The problems associated with expression and purification of MG4 scFv may be due to the potential impact of free thiol group on the scFv which may affect *E. coli* biological system adversely (Proba *et al.*, 1998). Another possibility would be the reaction of free thiol groups with *E. coli* periplasmic proteins or formation of complex aggregates, which are hard to purify (Schmiedl *et al.*, 2000).

The performance of MG4 scFv with the Cys 80 in expression and purification studies led to the idea of formatting the antibody fragments. This analysis was carried out to study two basic concepts: (i) the impact of changing the position of Cys 80 by changing  $V_L/V_H$  interface, and (ii) to study the effect of different constant domains on the expression of scFv. Constant light chains from three different species (rabbit, chicken and human) were used in this analysis. The basic idea behind using these species was that the rabbit served as a natural source for providing Cys 171 for engaging the Cys 80

by the formation of a disulphide bridge. The chicken was selected owing to its ability provide stable antibodies (Finlay *et al.*, 2006), and the human constant region was used as it is widely used in humanisation of antibodies for therapeutic applications (Popkov *et al.*, 2003).

The analysis of various formats showed that changing the format to scAb significantly increased the expression of the antibody more than 1000-times irrespective of variable domain orientation and the constant region. This may be due to improved translational efficiency, protection from proteolysis or proper folding due to the fusion protein, thus providing stability to the scFv (Glockshuber *et al.*, 1992; Hayhurst, 2000; Wörn *et al.*, 2000). Another reason for all the scAbs (with different constant regions) to show improved expression than scFvs may be the involvement of the free-Cys 80 of the scFv with Cys residues in C<sub>L</sub> regions, contributing to disulphide bond formation providing stability and proper folding to the antibody. ScAbs with rabbit constant domain showed better IC<sub>50</sub> values than other scAbs due to the restoration of natural disulphide bond of Cys 80 with Cys 171 of the C<sub>L</sub> domain of the scAb. However, all of these observations can only be clarified by detailed analysis of antibody structure based on high level modelling of the molecule or crystallisation studies.

Domain orientation showed a significant affect on the Cys-containing scFv expression. The scFv with V<sub>H</sub> (NH<sub>2</sub>)-linker-V<sub>L</sub> (COOH) orientation demonstrated 100-fold better expression than the scFv with V<sub>L</sub> (NH<sub>2</sub>)-linker-V<sub>H</sub> (COOH) orientation. Similar observations were reported by Kim *et al.* (2008). However, reports suggest that it is not necessary that a change in domain orientation affects antibody activity (Olafsen *et al.*, 2004). Such a change in antibody expression may be due to: (i) more stable conformation of the antibody because of changing V<sub>L</sub> and V<sub>H</sub> interface (Langedijk *et al.*, 1998; Ewert *et al.*,

2003; Masuda *et al.*, 2006). Studies reporting the effect of domain orientation on the functionality of proteins also support this data (Schmiedl *et al.*, 2000; Kipriyanov *et al.*, 2003); (ii) orientation of free cysteine inside the scFv structure, which may be restraining it from making unnecessary bonds with *E. coli* periplasmic protein or adversely affecting its biological system and thus, influencing the scFv expression. This supposition was supported by the observations of Albrecht and colleagues that changing the antibody domain orientation of a non-functional antibody, containing reactive cysteine residues, made it functionally active (Albrecht *et al.*, 2006). To verify this, Cys 80 was replaced with other amino acids, Ser and Ala, by site-directed mutagenesis selecting codons known to be well expressed in the *E. coli* system. In many cases, such a substitution can render the antibody unstable due to the loss of domain stability (Glockshuber *et al.*, 1992). The analysis from the comparison of mutated clones supported our hypothesis by showing improved expression of protein in mutated clones comparable to that of the native Cys-containing scFv with V<sub>H</sub> (NH<sub>2</sub>)-linker-V<sub>L</sub> (COOH). The protein was stable for weeks without any affect in sensitivity in the absence of native Cys 80.

Although the understanding of the underlying principle of the results of this study is still incomplete without modelling of the antibodies, the facts presented at various stages of the study construed that robust, stable antibodies can be obtained using the b4 allotype rabbit strain. The expression can be improved by simply rearranging the variable domains, or by introducing point mutations whereas, changing the format to scAb generates high levels of expression. In studies, where the scFv with free Cys is required for various purposes, such as conjugation to surfaces or labels, altering the domain orientation V<sub>H</sub>-V<sub>L</sub> can be an effective option.

# **Chapter 8**

## **Conclusions**

## 8. CONCLUSIONS

Cardiovascular diseases (CVDs) are leading health problems worldwide. Consequently, there is a huge need for identifying, validating and deploying ‘cardiac biomarkers’ in point-of-care platforms (McDonnell *et al.*, 2009). The main objective of this research was production of high performance recombinant antibodies against cardiac biomarkers to facilitate the development of improved cardiac diagnostic assays. Two tissue injury associated biomarkers, hFABP and cTnI, were selected with complementary diagnostic characteristics for antibody development.

The first part of the research project was focused on developing antibodies against human hFABP. Human hFABP has emerged as a promising early diagnostic marker of AMI (Kleine *et al.*, 1992; Glatz *et al.*, 1998) offering several distinct advantages (discussed in section 1.2.4). Chicken was selected as host for generating recombinant antibodies against hFABP owing to the different advantages (discussed in section 3.1.1). Sequence analysis showed 73% identity between chicken and human forms of hFABP. However, the heterogenicity was spread through the sequence length, thus facilitating avian antibody generation.

ScFvs were produced against hFABP and selected by phage display technology. A high percentage of selected scFvs were hFABP positive. The anti-hFABP scFvs were characterised, on Biacore, by an ‘off-rate’-based ranking approach and twenty five scFvs were selected. However, due to similar ‘off-rates’ being exhibited by the selected scFvs, the diversity of the selected scFv repertoire was analysed by fingerprinting analysis. A detailed kinetic analysis was also performed to determine the affinity constants for each individual scFv. The clones exhibited a high degree of diversity in terms of their gene sequences and affinity constants. This plurality of confirmed

antigen-binding candidates can be further exploited to identify clones with varying epitope specificities, for use in sandwich immunoassays. Three scFvs with high affinity (Table 3.4) were selected and investigated for expression, purification and specificity characteristics. The scFvs were found to be highly specific towards the human cardiac isoform of FABP with satisfactory expression levels, yielding up to 8-10 mg/L of purified protein. This is the first report of generation of a recombinant antibody against human hFABP.

An anti-hFABP scFv (3.G9), with low nanomolar range affinity ( $1.53 (\pm 0.25) \times 10^{-9}$  M) was selected and incorporated into an inhibition ELISA-based assay. The assay was highly sensitive with a LOQ of 1.90 ng/mL. The antibody was also tested and validated in an inhibition LFIA format. The LFIA was able to detect 4 ng/mL hFABP. The scFv, 3.G9, demonstrated superior performance in both ELISA and LFIA, in inhibition-assay format, with comparable sensitivity to the prevailing 2-site immunometric conventions available commercially, thus demonstrating the potential to be used as ligands in a single step direct binding biosensor assay.

The high cost of the commercial antigen and problems with batch-to-batch variations led the 'in-house' expression of recombinant hFABP in *E. coli*. A combination of the pET-26b(+) vector and rare-codon expressing BL-21 (DE3) RIPL cells, resulted in over expression of the hFABP gene, yielding sufficient protein (8 mg/L of culture) for further assay optimisation steps.

The determination of the exact peptide sequence (epitope) to which 3.G9 binds was attempted. The epitope was assumed to be linear due to the binding of the 3.G9 scFv with denatured hFABP antigen in Western blotting (section 3.6). Initial efforts to map

the epitope involved the use of a commercial random peptide library. However, peptides specific to the antibody immobilised on the capture beads were selectively enriched rather than peptides specific to paratope of 3.G9 scFv. Therefore, an alternative approach was employed by digesting the hFABP gene randomly and constructing a library from the resulting fragments. This gene-fragment library was specific to the 3.G9 scFv in contrast to commercially obtained Ph.D.<sup>TM</sup>-12 random peptide library. The scFv was converted to scAb format to facilitate its capture on a surface whilst maintaining proper orientation of the antigen binding paratopes. The gene-fragment library was panned against the scAb to enrich for specific peptides. However, no data could be obtained on the epitopes due to the absence of signal in polyclonal phage ELISA. Finally, a more conventional approach was sought, which involved expressing overlapping gene-fragments from the hFABP gene. Initially, peptide walking was carried out from the N-terminal to C-terminal of the gene, by expressing peptides of varying lengths, excluding a part of C-terminal with each consecutive peptide (as depicted in Figure 5.10). The expressed peptides were then checked against the 3.G9 scFv by Western blotting. However, the results obtained by this methodology were not reproducible. New peptides were expressed, by reversing the previous order of peptide walking i.e. by excluding a part of the N-terminal of the gene. On analysing the longest peptide (representing 94% of the gene) against 3.G9 scFv, no binding was observed, thus precluding the epitope region.

Thus in view of the results obtained on epitope mapping, it was concluded that the epitope recognised by 3.G9 scFv may be conformational. Thus there is a need to devise an alternative strategy for successful mapping of the epitope.

Another marker of cardiac tissue injury, focused on in this project was cTnI, considered to be the ‘gold standard’ for AMI diagnosis. It is a highly specific marker for myocardial necrosis recommended by several international scientific bodies, e.g. the European Society of Cardiology (ESC), the American College of Cardiology (ACC) and the American Heart Association (AHA). Research to develop recombinant antibodies against cTnI-specific epitopes was already on going in the lab. Therefore, this work was focussed on generating polyclonal antibodies against cTnI to be used as a tracer (detection) antibody in immunoassays.

A high rabbit serum titre was obtained against cTnI. However, the polyclonal antibodies preparation was not sufficiently sensitive in a sandwich assay format. This led to the need to generate recombinant antibodies. A recombinant antibody library was constructed using the bone marrow and spleen from the rabbit immunised with cTnI. Five rounds of biopanning were carried out to selectively enrich cTnI-specific antibodies which was subsequently analysed by polyclonal and monoclonal ELISAs. Approximately 23% clones showed binding towards cTnI on screening by ELISA. Further characterisation of the antibodies was carried out by analysis against sTnI for specificity, and ‘off-rate’-based ranking on Biacore 3000. All the selected scFvs were specific to the cardiac isoform of TnI. However, there were issues with ranking analysis due to the high charge exhibited by cTnI, leading to non-specific interaction with the sensor surface. Also there were discrepancies in results when different batches of commercial cTnI were used. Consequently, recombinant expression of ‘in-house’ cTnI was successfully carried out to provide a constant source of antigen. Recombinant cTnI was expressed in *E. coli* with a N-terminal fusion protein to facilitate its solubility and expression and further characterised by analysis on Western blotting using antibodies directed against cTnI-specific-epitopes.



Ten anti-cTnI scFvs were selected based on their percentage stability data, obtained by ranking analysis. Fingerprinting analysis was carried out to determine the diversity exhibited by the selected scFv repertoire. The analysis showed a limited diversity in the selected antibody panel which was later confirmed by the sequencing data of the scFv genes. The antibody panel consisted of two major types of scFv sequences. One type of sequence contained cysteine at position 80 of V<sub>L</sub> (Cys 80) indicative of the aberrant cysteine left by the breaking of disulphide bond between Cys 80 of V<sub>L</sub> region and Cys 171 of the C<sub>L</sub> domain, an interesting feature reported in the antibodies expressed by the b4 allotype of rabbit. On analysis, this scFv showed poor expression and purification as compared to the other scFv from the library with another amino acid (arginine) at V<sub>L</sub> 80.

This cysteine at position 80 of V<sub>L</sub> in scFv can severely impinge on the achievable expression levels when rabbit recombinant antibodies are produced in prokaryote systems. The format of scFv with Cys 80 was optimised for better expression and purification by (i) changing the scFv to an scAb format, (ii) addition of different constant domains on the scAb format and (iii) changing the variable domain orientation on scFv and scAb formats. The scFv was converted into an scAb format by cloning the gene into the pMoPac 16 vector. In the scAb format, a constant light chain is added to the variable domains, which was reported to increase antibody stability and expression (Hayhurst, 2000). Constant domains from three different hosts (human, rabbit and chicken) were used to generate scAbs. In total 6 types of scAbs were generated containing two variable domain orientations (V<sub>L</sub>-V<sub>H</sub> and V<sub>H</sub>-V<sub>L</sub>) and three different constant domains. The effect of variable domain orientation on scFv expression was also determined. Changing the scFv to the scAb format resulted in 1000-fold higher

expression of the antibody. By simply changing the position of Cys 80 (in scFv), i.e. by reversing the variable domain orientation of wild-type scFv, the expression was increased approximately 100-fold along with higher purification yields. These results were verified by substituting the Cys 80 in a wild-type scFv with alanine and serine, by site-directed mutagenesis. The results were in concordance with the previous data showing increased expression levels with in the mutated scFvs.

Rabbit-derived recombinant antibodies have traditionally been viewed as intractable molecules due to the presence of an unnatural disulphide bond which renders an unpaired Cys at position 80 in both scFv and chimeric Fab formats. This is the first study that attempted to optimise rabbit antibody format for improving expression. It revealed a dramatical increase in expression and aided purification, thus making rabbit antibodies more accessible to researchers.

The studies undertaken resulted in the development and optimisation of recombinant antibodies to the cardiac biomarkers, hFABP and cTnI, which were fully characterised based on their binding characteristics, kinetics, sensitivity and specificity which are key parameters for evaluation of their potential applicability in immunoassays. Commercial immunoassays utilising mAbs for both the biomarkers exist. The test formats employed were exclusively sandwich immunometric assays, such as the MARKIT-M (DS Pharma Biomedical Co. Ltd., Japan) hFABP and the Architect STAT Troponin-I assay (Abbott Diagnostics, USA) for cTnI. The advantages of recombinant antibodies compared to mAbs were discussed in section 3.10. Two key features among these are: (i) the capacity of recombinant antibodies to undergo genetic engineering allowing improvement in binding characteristics along with selection of the antibody format to suit the specific immunoassay platform, and (ii) use of recombinant antibodies

eliminates problems arising from interferences related to complement, rheumatoid factors and Fc receptors, which are major interference issues in diagnostic assay development, when using complex biological matrices such as serum.

The assays/antibodies developed for hFABP were equivalent to or better than those commercially available based on their specificity and sensitivities. The characteristics of the immunoreagents (antibodies), developed in this study, are completely defined and can be further exploited for developing novel point-of-care systems.

**The key outcomes from the study can be summarised as:**

- Highly specific and sensitive chicken anti-hFABP scFvs were successfully generated and characterised.
- The performance of anti-hFABP scFv (3.G9) was demonstrated efficiently on ELISA and lateral flow-immunoassay format, in inhibition assay format.
- Different approaches were studied for epitope mapping of 3.G9 scFv. However, the results suggest that the epitope might be discontinuous.
- Specific-rabbit anti-cTnI scFvs were successfully generated and characterised.
- Rabbit anti-cTnI antibody configurations were successfully optimised for improved expression.
- Recombinant expression of both hFABP and cTnI was successfully accomplished for utilisation in assay optimisation.

# **Chapter 9**

## **Bibliography**

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### Glossary of terms and definitions commonly employed in immunoassay development and validation procedures:

The terms referred to herein are commonly used to define key parameters necessary for the development and validation of immunoassays. The criteria used for their definition were extensively reviewed elsewhere (ISO 5725-1:1998; ISO 3534-3:1999; Findlay *et al.*, 2000; Willets and Wood, 2000; Mikkelsen and Cortón, 2004; Geng *et al.*, 2005; ISO 3534-1:2006; ISO 3534-2:2006 and Kelley and DeSilva, 2007).

Term	Definition
<b>Accuracy</b>	The closeness of agreement between a reported result and the accepted reference value.
<b>Affinity</b>	Affinity is a thermodynamically defined term representing the strength of interaction of a single site of an antibody (paratope) with its antigen (epitope).
<b>Avidity</b>	Avidity is the sum of all the individual affinities of all the sites (valency) of an antibody (paratopes) binding to the different sites on antigen (epitopes).
<b>Cut-off</b>	Cut-off is defined as a threshold limit of non-specific background (an OD value) calculated by analyzing samples from healthy or naïve individuals.
<b>Limit of detection</b>	Limit of detection (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Usually LOD is $\pm 3$ standard deviations from the blank value.
<b>Limit of quantitation</b>	The limit of quantitation (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Usually LOQ is $\pm 10$ standard deviations from the blank value.
<b>Precision</b>	The closeness of agreement between independent test results obtained under stipulated conditions.

<b>Repeatability</b>	Precision of a test under the conditions where test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.
<b>Reproducibility</b>	Precision of a test under the conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.
<b>Robustness</b>	The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.
<b>Sensitivity</b>	This is the measure of the change in response which corresponds to a change in analyte concentration. Where the response has been established as linear with respect to the concentration, sensitivity corresponds to the gradient of the response curve.
<b>Specificity</b>	The ability of an analytical method to distinguish the analyte to be determined from any other substances present in the sample.

(Adapted from European Medicines Agency, 1995; ISO 5725-1:1998; ISO 3534-3:1999; Findlay *et al.*, 2000; Willets and Wood, 2000; Mikkelsen and Cortón, 2004; Geng *et al.*, 2005; ISO 3534-1:2006; ISO 3534-2:2006; Kelley and DeSilva, 2007 and Crowther, 2009).

(Details on references are included in the bibliography section).