THE PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST ESAT6 OF *Mycobacterium tuberculosis*

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As the candidate's supervisor I have/have not approved this dissertation for submission.

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ABSTRACT

Mycobacterium tuberculosis (*M. tuberculosis*), the causative agent of tuberculosis (TB), was responsible for 9.6 million cases and 1.5 million deaths globally. Therefore, early TB diagnosis remains a high priority to mitigate the consequences of poor health outcomes and continuous disease transmission. The strategy in detecting the TB antigen instead of antibodies elicited against TB is preferred as immunosuppression in patients co-infected with HIV, makes antibody detection unfavourable. Monoclonal antibodies (MAbs) to TB-specific markers are therefore an attractive option for use in ELISAs and lateral flow tests that effectively meet the criteria for a rapid antigen detection test. Consequently, the objective for the present study was to produce TB-specific MAbs that would aid in the development and optimization of an ELISA for the rapid detection of TB. In order to meet these objectives this study focused on the production and characterization of MAbs that specifically target the early secretory antigenic target 6 (ESAT6) protein from *M. tuberculosis*.

PCR was performed on *M. tuberculosis* H37Ra DNA, using primers specific to the *esat6* gene. PCR products were inserted into pGEM-T vector followed by ligation into the expression vector pGEX6P-1 for transformation into *Escherichia coli* (*E. coli*) strain XL-1 Blue. ESAT6 GST protein was expressed and purified by glutathione sepharose affinity chromatography. Thereafter, recombinant ESAT6 GST protein was used to immunise 10 Balb/C mice and stable hybridoma cell lines were generated.

The specificity of three monoclonal antibodies were confirmed and identified as anti-ESAT6 DE2-1, anti-ESAT6 KE10-1 and KE10-2. Hybridomas showing cross-reactivity to non-specific antigens, were excluded from the study. Ouchterloney Double-Diffusion was employed and the three MAbs were subtyped as an IgG and two IgM's respectively. The multi-epitopic nature of ESAT6 is a desirable characteristic in diagnostic assay development. This characteristic was demonstrated by ELISA using anti-ESAT6 DE2-1 as a coating MAb and anti-ESAT6 KE10-1, conjugated to horse radish peroxidase, as the detection antibody.

The generation of anti-ESAT6 MAbs in this study, have demonstrated their potential for use in the development of a rapid TB diagnostic test. This is critical in TB management, treatment of the disease, reducing TB transmission and incidence. Future work must therefore be aimed at the development of a diagnostic test, for use at the point-of-care, which would complement the TB diagnostic algorithm.

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Westville Campus, from January 2012 to December 2016, under the supervision of Professor Balakrishna Pillay.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION – PLAGIARISM

I, Nethi Pillay, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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This thesis is dedicated to mum, dad my dearest brother, Kenny and sister Lynette. My accomplishments, both on a personal and academic level, have been based on their encouragement and on the deep-seeded morals and values that they have instilled in me. There are no words to express my deepest gratitude to my husband Dhavan and my sons, Huveshan and Thrishen, who have always encouraged and stood by me and dealt with my commitment with a smile of support. Your love has taught me about sacrifice, discipline and compromise.

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ABBREVIATIONS

Abbreviation	Description
American type culture collection	ATCC
Antibody-dependant cell mediated cytotoxicity	ADCC
Antigen	Ag
Bacilli Calmette-Guerin	BCG
Biosafety level	BSL
Cell per millilitre	cpm
Centre for Disease Control	CDC
Complete Freund's adjuvant	CFA
Culture fluid protein	CFP
Cyclic adenosine monophosphate	cAMP
Delayed hypersensitivity reaction	DHR
Dimethyl sulphoxide	DMSO
Early secretory antigenic target 6	ESAT6
Enzyme-linked immunosorbent assay	ELISA
Ethylenediaminetetraacetic acid	EDTA
Fetal bovine serum	FBS
Fms-like	FL
Food and Drug Administration	FDA
Foundation for Innovative New Diagnostics	FIND
Gamma interferon	IFNγ
Gamma interferon assay	IGRA
Glutathione sepharose transferase	GST
Guanidine hydrochloride	GuHCl
Guanidinium thiocyanate	GuSCN
Heat shock protein	HSP
Hepatitis B surface antigen	HBs Ag
Horse radish peroxidase	HRP
Horse serum	HS
Human Immunodeficiency virus	HIV
Hypoxanthine aminopterin thymidine	HAT
Immunochromatographic assay	ICA
Immunoglobulin	Ig
Immunohistochemical	IHC
Incomplete Freund's adjuvant	IFC

Interferon gamma release assay	IGRA
Interleukin	IL
Isopropyl β-D-thiogalactopyranoside	IPTG
Lactate dehydrogenase	LDH
Latent tuberculosis infection	LTBI
Line Probe Assay	LPA
Liparabinomannan	LAM
Lateral Flow-LAM	LF-LAM
Liquid nitrogen	LN_2
Luria-bertani	LB
Maltose binding protein	MBP
Microscopic observation drug susceptibility	MODS
Monoclonal antibodies	MAbs
Monocyte derived macrophage	MDM
Multidrug-resistant TB	MDR-TB
Mycobacteria other than TB	MOTT
Non-tuberculous bacteria	NTM
Nucleic acid amplification	NAA
Optical density	OD
Polyethylene glycol	PEG
Peripheral blood mononuclear cells	PBMC's
Phenylmethylsulfonyl fluoride	PMSF
Phosphate buffered saline	PBS
Point of care	POC
Positive/negative	P/N
Pre-bleed	PB
Purified protein derivative	PPD
Polymerase chain reaction	PCR
Recombinant	r
Region of difference	RD
Respiratory syncytial virus	RSV
Roswell Park Memorial Institute	RPMI
Standard Diagnostics	SD
Surface plasmon resonance	SPR
Sustainable development goals	SDG
Test bleed	TB
Toll-like receptor	TLR

Transformation buffer	TSB
Treatment Action Group	TAG
Tuberculin skin test	TST
Tuberculosis	TB
Tumour growth factor	TGF
Tumour necrosis factor	TNF
Ultra-violet	UV
Varicella virus	VZ
World Economic Forum	WEF
World Health Organisation	WHO
Extensively drug-resistant TB	XDR-TB
5-bromo-4 chloro-3-indolyl-β-D-galactoside	X-gal

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INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Tuberculosis (TB) is second only to the Human Immunodeficiency Virus (HIV) as the leading cause of mortality, morbidity and disease prevalence, despite a history of treatment and prevention (WHO, 2016). In 2015, the World Health Organisation (WHO) reported TB as the leading killer of HIV-positive people with one in three HIV deaths that resulted from TB. A further 1.4 million HIV-negative people also died from the disease. The TB burden saw 10.4 million people fall ill with the disease, including 1.2 million cases among people living with HIV. The WHO has stated that global TB prevalence has been under-reported and that less than two-thirds (63%) of TB cases have been accounted for (WHO, 2015).

The latest statistics estimated that 480 000 people have developed multidrug-resistant (MDR) - TB that do not respond to isoniazid and rifampicin, the two standard first-line anti-TB drugs (WHO, 2015). Surveillance statistics have indicated that about 9.4% of the MDR-TB strains are resistant to isoniazid, rifampicin, any fluoroquinolone and at least one of the three second line injectables, amikacin, kanamycin and capreomycin, that is extensively drug resistant (XDR)-TB (Falzon *et al*., 2013). Although XDR-TB has a lower incidence, is regarded as more severe than MDR-TB, especially for people infected with HIV or have conditions that weaken the immune system (CDC, 2016). These individuals have been shown to have a greater chance of developing active TB infection (CDC, 2016; Falzon *et al*., 2013; WHO, 2015).

The cause of drug-resistant TB occurs mainly as a result of the mismanagement of treatment and subsequent disease transmission (WHO, 2015; CDC, 2016). Treatment of drug-resistant TB is lengthy, very expensive and many patients have reported serious side-effects such as depression, psychosis, hepatitis and kidney impairment. Early TB diagnosis is therefore critical, not only in terms of the financial implications in treating drug-resistant TB but for effective patient management and reducing mortality (WHO, 2015; CDC, 2016).

The limitations of current diagnostic tests, including latent TB infection (LTBI) and cross-reactivity with *M. bovis* Bacilli Calmette-Guerin (BCG) vaccinated individuals are further

exacerbated by the need for expensive diagnostic equipment, skilled personnel, a laboratory set-up in a temperature controlled environment and a lengthy turn-around time for the generation of results (de Lange, 2013). Therefore, a test that would produce an accurate and rapid TB diagnosis is critical to ensure prompt administration of treatment, at the point of care (POC) by first-contact health-care workers (Wang *et al*., 2014; Harrington, 2015).

A third of the world's population live with LTBI (Ahmed, S., 2011). When the host immune response is compromised or when an individual is HIV infected, the dormant bacilli have the ability to reactivate and cause disease (WHO, 2015). The lack of effective TB diagnostic tests, for both active and latent forms of the disease contribute to the difficulties experienced with early diagnosis of TB (Lessem, 2015).

Further challenges presented by current TB diagnostic tests include distinguishing between patients harbouring *M. tuberculosis* pulmonary disease from patients bearing pulmonary disease caused by other members of the *M. tuberculosis* complex (*M. bovis, M. africanum, M. canetii, M. microti* and *M. pinnipidae*) and environmental non-tuberculous mycobacteria (NTM) (Johnson and Odell, 2014).

Conventional purified protein derivative (PPD) that is used in the Tuberculin Skin Test (TST) has been used for many years for TB diagnosis. PPD is not ideal as it contains a mixture of mycobacterial antigens from the pathogenic *M. tuberculosis* complex, NTM and BCG. Limitations experienced with PPD can therefore be overcome by the development of a diagnostic test or the production of a diagnostic reagent specific for *M. tuberculosis* (Velayudhan *et al*., 2011).

Studies have shown that assays based on antibody detection, although economical, are not ideal in high burdened areas such as Africa where the prevalence of HIV makes antibody detection an unfavourable approach (Harrington, 2015; Zhang *et al*., 2015). It was demonstrated that antibody detection assays were inconsistent, offered poor sensitivity and specificity and produced cross-reactions and false positive results with NTM. These assays were also unable to differentiate between active TB and LTBI (Ben-Selma *et al*., 2011; Harrington, 2015).

Limitations in research and current diagnostic tests have contributed to the adoption of the Sustainable Development Goals (SDG), by the WHO in 2014. Amongst the aims of the SDG was reduction in TB related deaths by 90% and reduction in the number of new TB cases by 80%

between 2015 and 2030. However, the SDG can only be achieved if the world invests an estimated US$ 270 million per year into research for the development of rapid TB diagnostic tests (Harrington, 2015). Studies have indicated that the development of a test that would effectively identify a combination of TB-specific biomarkers for early detection of both active and latent forms of *M. tuberculosis* from BCG vaccinated individuals, remains critical in reducing the mortality and incidence of the disease (Bekmurzayeva *et al*., 2013).

Although many platforms have been explored for the detection of TB, none have been accurate enough to measure up to the gold standard of liquid and solid culture (Harrington, 2015). Furthermore, the false negative results produced by antibody detection assays has resulted in a policy being drafted by the WHO, discouraging the use of such diagnostic assays (Harrington, 2015; Zhang *et al*., 2015). Previously, studies have focused on the detection of active infection for the diagnosis of TB However, evidence supports the early detection of disease in order to promptly administer treatment in the control of disease transmission. This may be achieved through the diagnosis of LTB.

A study by Tang *et al*. (2014) supported the use of the 6 kDa early secretory antigenic target (ESAT6) target antigen for LTBI diagnosis with potential for early pulmonary and extra-pulmonary TB diagnosis. It was demonstrated that cloned and expressed ESAT6 protein detected LTBI with a higher sensitivity and specificity than TST (Masood *et al*., 2014; Tang *et al*., 2014). The interferon gamma interferon assay (IGRA), a test described as being more accurate than TST, has had restrictions placed on its use, due to inaccuracy of results produced. This has resulted in focus being placed on the ESAT6 antigen, for LTBI effective diagnosis (Harrington, 2015).

It has been recommended that ESAT6 antigen be used in combination with other biomarkers, such as TB7.7 in a test that would complement the existing diagnostic algorithm for early TB diagnosis (Bekmurzayeva *et al*., 2013; WHO, 2015; Zhang *et al*., 2015).

Although *M. tuberculosis* is an intracellular organism, found within macrophages and epithelial cells early in infection, the bacterium is also found extracellularly, within tissues during progressive disease and granuloma formation (Velayudhan *et al*., 2011). Therefore, monoclonal antibodies (MAbs) targeting the extracellular antigen could be useful for diagnosis and treatment of disease (Uvarova *et al*. 2013). ESAT6, a key virulence factor in *M. tuberculosis*, induces a strong T-cell mediated immune response, resulting in host cell lysis and extracellular release of bacteria. The characterization of B-cell epitopes on ESAT6 from *M. tuberculosis*, further supports

the production of MAbs against ESAT6 for diagnosis of the disease (Harboe *et al*., 1998; Teitelbaum *et al*, 1998; Leng *et al*., 2014).

Studies have demonstrated the efficacy of anti-ESAT6 MAbs in an enzyme linked immunosorbent assay (ELISA)-based assay for the rapid immuno-diagnosis of *M. tuberculosis* (Leng *et al*., 2014). Clinical validation of the anti-ESAT6 MAbs demonstrated a high detection rate and sensitivity in sputum culture supernatants and pleural effusion fluid from TB cases. Therefore, the application of anti-ESAT6 MAbs in an ELISA makes it an efficient tool in a laboratory-based assay. Although, Leng *et al*. (2014) has described that sputum samples required bacterial culture prior to testing, Namba (2010) has shown that bacterial culture step is not necessary if the bacteria are inactivated prior to testing (Namba, 2010). This provides evidence that the application of MAbs directed against secretory antigen ESAT6, present in sputum and pleural fluid, has potential for the detection of *M. tuberculosis* at the POC (Dai *et al*., 2012; Leng *et al*., 2014).

Feng *et al*. (2011) described the use of a combination of ESAT6 and 10 kDa culture filtrate protein (CFP10) antigens in MAb production. Anti-ESAT6 MAbs, produced in mice, were shown to have a better positive detection rate of 95.4% and negative detection rate of 100% in comparison to anti-CFP10 MAbs for use in a diagnostic ELISA for TB (Feng *et al*., 2011). The use of anti-ESAT6 MAbs as a tool for the early and rapid TB diagnosis, therefore addresses the limitations of antibody detection assays (Zhang *et al*., 2015).

1.1.1 Rationale for the present study

The rationale for the present study was that the production of murine MAbs to recombinant ESAT6 from *M. tuberculosis* will have potential for early TB diagnosis, discriminating between active, latent and BCG-vaccinated individuals. Anti-ESAT6 MAbs may be used in the development of a rapid test, thereby alleviating the challenge of delays in early TB diagnosis and treatment, a contributing factor to disease dissemination and mortality. Furthermore, anti-ESAT6 MAbs, when used in combination with MAbs against other *M. tuberculosis*-specific biomarkers, are valuable tools for the development of a simple and sensitive test for TB diagnosis, which may be used by first-contact healthcare workers at a community level.

1.1.2 Scope of the present study

The focus of this study was to clone and express multi-epitopic recombinant ESAT6 in order to immunise Balb/C mice for the generation of anti-ESAT6 MAbs using hybridoma technology. The recombinant protein was further used as a screening tool in an ELISA detecting anti-ESAT6 MAbs produced by hybridoma fusion. The anti-ESAT6 MAbs generated in this study was used in the development and optimization of an ELISA for the rapid detection of recombinant ESAT6. Further work is required to validate the sensitivity and specificity of the ELISA through the analysis of TB infected and uninfected clinical samples.

Given the information provided, the present study is a likely solution to the problems experienced with the early and rapid diagnosis if TB. Studies have shown that TB-specific MAbs are tools amenable for use in rapid, antigen-detection assays for the early detection of infection (Leng *et* al., 2014; WHO, 2011b). Dormancy antigen ESAT6 protein from *M. tuberculosis* has therefore been investigated for use as an effective biomarker and an indicator of early TB infection (Velayudhan *et al*., 2011).

1.2 Literature review

1.2.1 The approach that is currently used for TB diagnosis

A recent report by the Treatment Action Group emphasised the need for an early detection, economical and efficient antigen detection test for use at the point-of-care (POC) (Harrington, 2015). However, progress in all areas of tuberculosis (TB) research were hindered as funds that were allocated in achieving the Millennium Development Goals (MDG) were not made available between 2014 and 2015. TB remains a major cause for concern, ranking high in terms of mortality, morbidity and disease prevalence because the diagnostic tests that are currently available are inadequate for the prompt and accurate diagnosis of the disease (WHO, 2015).

1.2.1.1 The benefits and drawbacks of current diagnostic tests

The current tests that are available for TB diagnosis can be classified into four categories, namely, conventional tests, culture-based methods, immunological tests and nucleic acid amplification tests. The benefits and drawbacks associated with each of the tests are highlighted in Table 1.1.

Radiographic results are suggestive and not specific for TB and as many as 15% of patients with primary TB may be missed (FIND, 2012). In such cases, computerised tomography (CT) scan may provide more information by providing more detailed images. Latent TB cannot be differentiated from active TB based on radiography alone and may require additional tests such as TST and interferon gamma release assays (IGRAs) to make a diagnosis (Bhatt *et al*., 2012).

Although the sputum smear microscopy technique is specific, simple and inexpensive, the sensitivity of the test is limited to a bacterial load of less than 10 000 organisms/ml of sputum (Dorman, 2010). Limited resources and large sample numbers in low to middle-income countries has reduced the observation time per slide to less than 60 seconds, further impacting on test sensitivity (Desikan, 2013). Other drawbacks associated with smear microscopy include moderately trained staff and misdiagnoses, as a negative smear result does not necessarily exclude TB disease. Variable sensitivities have been cited and in a study by Dorman (2010) the sensitivity of the test was estimated at 70%. In this study, the sensitivity of smear microscopy was further reduced to about 35% despite the high rates of TB, due to HIV co-infection (Dorman, 2010).

It was further shown that sample processing techniques was one of the factors that played a major role in the sensitivity of smear microscopy (Singhal and Myneedu, 2015). For example, sodium hypochlorite liquefaction produced a 14.7% test sensitivity when compared to phenol ammonium sulphate that produced a sensitivity of 85.5%. Sputum smear microscopy remains the gold standard in resource-limited settings. Studies suggest a greater sensitivity of the test may be improved if the examination time per sputum slide was 2 minutes and 6 seconds (Singhal and Myneedu, 2015).

Although the culture of *M. tuberculosis* is a more sensitive test than smear microscopy, the long doubling time of this organism negatively impacts on the time it takes to obtain results. A stained smear requires approximately 10 000 bacilli per millilitre of specimen whilst a positive culture requires 10 to 100 bacilli per millilitre (Dorman. 2010). The incubation period for liquid culture is 10–14 days using automated systems such as “BACTEC”, “MGIT”, “VersaTREK” and “MBBACT” and 3–4 weeks for solid culture (Dorman, 2010). However, the cost of the test is a

setback in under-resourced laboratories with poor quality assurance (Dorman, 2010; Sun *et al*., 2009). Although the cost of bacterial culture is greater than microscopy, it is not as expensive as molecular assays such as the Xpert MTB/RIF rapid molecular assay (Dorman. 2010). Therefore, WHO recommends the use of liquid cultures for TB diagnosis in areas of high prevalence based on the cost effectiveness and sensitivity of the test (WHO, 2015).

The microscopic observation drug susceptibility (MODS) assay is a cost effective, rapid, liquid culture with high sensitivity and specificity for the detection of *M. tuberculosis* and drug susceptibility testing (Wang *et al*., 2015). Despite its advantages, MODS are not widely used due to biosafety risks, efficiency in handling large numbers of samples, cross contamination of samples and the need for trained lab personnel (WHO, 2013). A further disadvantage is the inability of the test to differentiate between *M. tuberculosis* and NTM (Bwanga *et* al., 2009; Cantazaro, 2013; Wang *et* al., 2015; WHO, 2013).

A meta-analysis of over 1000 participants from India, Moldova and South Africa compared conventional growth-based drug-susceptibility testing to three new rapid tests, evaluating drug resistance to isoniazid, rifampicin, moxifloxacin, ofloxacin, amikacin, capreomycin and kanamycin. The MODS assay took 15 days to produce results, pyrosequencing took 8 days whilst the Line Probe Aassay (LPA) took 5 days to produce results. Although the MODS test is the slowest of the three, it is the cheapest and a viable option in settings having financial restrictions (Cantazaro, 2013).

Delayed hypersensitivity reaction (DHR) is an inflammatory reaction mediated by T-cells and macrophages about 48-72 hours after the patient has been exposed to TB antigens other than purified protein derivative (PPD). A test based on recombinant ESAT6 and culture filtrate protein (CFP)10 was developed to test for DHR as an alternative to tuberculin PPD. Results showed high sensitivity in patients with new and active TB and no false positives with Bacillus Calmette-Guerin (BCG) vaccinated patients(Bekmurzayeva *et al*., 2013). A study performed in guinea pigs, showed that the DHR elicited by seven recombinant antigens (ESAT6; CFP10; Tb10.3; Tb10.4; Mtsp11; Mpt70 and Mpt83) was stronger than the DHR produced by PPD alone. Researchers therefore propose that a combination of several proteins in one test would improve diagnostic accuracy of the test (Malaghini *et al*., 2011).

TST or Mantoux test requires the injection of PPD, a mixture of antigens derived from heat-killed *M. tuberculosis*, intra-dermally on the forearm in order to determine a person's sensitivity to

tuberculin protein. The reaction is evaluated after 48 – 72 hours and if the person has been exposed to TB, the hard, dense, induration that forms, is measured. Although the TB patch test is a POC test, several drawbacks are associated with the test, making it an unfavourable approach for TB diagnosis (Bekmurzayeva *et al*., 2013, Dorman, 2010).

Similar to the TST, diagnostics company Sequella, has developed a patch that delivers Mpt64, an antigen that elicits a positive response in individuals with active TB. It involves intra-cutaneous antigen injection to see whether hypersensitivity occurs (Bekmurzayeva *et al*., 2013). Patients with active TB infection develop erythema or vesicles 3- 4 days at the site of the patch. This test produced 98% sensitivity and 100% specificity (Find, 2012).

Immunohistochemistry has been used in the diagnosis of several diseases including TB. The test utilises polyclonal and monoclonal antibodies in order to detect antigens in formalin fixed or paraffin embedded tissue sections (Ramos-Vara, 2005). One to three weeks are required for sample preparation and pre-treatment and antigen detection takes 3–4 hours thereafter. The sensitivity (89 – 93%) and specificity (95 – 98%) of the test is high, with the advantage of being able to test lymph node aspirates, cerebrospinal fluid and pleural effusions. The drawbacks of the test are cross-reactivity and results that require validation using additional tests such as Polymerase chain reaction (PCR) and acid fast bacilli staining (Mustapha *et al*., 2006).

A biosensor is described as an analytical device that uses an electric current, a sensing element and an electrical transducer to quantify disease molecules (Seong *et al*., 2011). More recent studies describe a waveguide-based biosensor for the detection of liparabinomannan (LAM), ESAT6 and Antigen 85 (Ag85) (Mukundan *et al*., 2012). This test is based on a sandwich immunoassay using a biotinylated capture antibody and a fluorophore labelled reporter antibody on the sensor's surface. Hong *et al* (2011) targeted CFP10 in an immunoassay based on surface plasmon resonance (SPR) spectroscopy. CFP10 immobilised on the gold surface of a SPR chip was used as the sensing surface. Results showed a significant difference in results between healthy and infected patient's urine samples and that there was a linear relationship between CFP10 concentration in urine and the number of acid fast stained bacterial cells in sputum. These results provided evidence that this test has the potential for use as a rapid, label-free test for the early detection of *M. tuberculosis* (Mukundan *et al*., 2012).

Lateral flow tests have been designed for use at point of care, based on the "test and treat" approach (Manabe *et al*., 2014). It is aimed at resource-impoverished settings and is required to

fulfil the "ASSURRED" (affordable, sensitive, specific, user-friendly, robust/rapid, equipment-free, and deliverable to those who need the test / no refrigeration required) criteria. Lateral flow tests may be used to detect either *M. tuberculosis* antigen or antibody (Manabe *et al*., 2014).

The One Step TB Test kit (Health-Chem Diagnostics, USA), the Tell Me Fast TB IgG/IgM Test device (BioCan Diagnostics, Canada) and the SD TB IgG/IgM (SD Bioline, South Korea) are examples of rapid tests that are currently available. Three diagnostic companies have developed assays based on the detection of Mpt64 and each differs slightly in terms of sensitivity and specificity. SD Bioline TB AG Mpt64 rapid test (Standard Diagnostics, South Korea), BD MGIT TBc Identification test (BD Diagnostics, USA) and the WHO endorsed Tibilia TB rapid test (Genesis, China), is able to detect Mpt64 from positive liquid or solid *M. tuberculosis* cultures (Ngeow *et al*., 2011). However, not all manufacturers readily specify the antigens that are immobilised in the assay. The Foundation for Innovative New Diagnostics (FIND) made available the Tibilia test to high TB-burden countries while the remaining two tests are alternatives, available commercially. The sensitivity of rapid tests have been enhanced using combinations of antigens. The double-antigen colloidal gold test that detects ESAT6-16-38 fusion protein and a test that detects Ag85 (Bekmurzayeva *et al*., 2013).

The WHO (2015) has recommended the use of lateral flow lipoarabinmannan (LF-LAM) for the diagnosis of active TB in people infected with HIV. LF-LAM may be used to detect LAM antigen in urine samples at point of care. The objectives of the document is to assess whether this test may be used as a replacement or in combination with other diagnostic tests (WHO, 2015). The LF-LAM test is beneficial in the diagnosis of extra pulmonary TB in HIV positive patients that cannot be diagnosed using conventional sputum tests with a lower sensitivity (Shah *et al*., 2016).

An ELISA is a solid phase assay used to detect the presence of antigen or antibody. Previously, the disadvantage associated with this test was a lengthy sample preparation of 13 weeks as the bacteria required culture on liquid or solid media. In a claim by Namba (2010), samples for ELISA no longer require culture. However, a biosafety level 3 laboratory is still needed for bacterial inactivation of the sample. The disclosure provides evidence that these tests can be carried out on non-cultured samples such as sputum or urine, thus providing non-invasive, rapid and accurate results, safely. Advantages include a reduced risk of infection to laboratory staff that do not have to culture specimens and no expensive equipment or skilled personnel is required to produce accurate results (Namba, 2010).

Gutlapalli *et al*. (2016) evaluated the sensitivity and specificity of multiple ELISAs to quantify antibody responses to 38 kDa, LAM and ESAT6 antigens in the diagnosis of TB in HIV-infected individuals. Patient samples were tested on four separate ELISAs. The first three ELISA plates were coated with a combination of 38 kDa-LAM antigens, with each of the three plates detecting either IgG, IgM or IgA antibodies. The fourth ELISA was used for the detection of anti-ESAT6 antibodies. Results showed that when a single ELISA was used to detect TB, then the sensitivity of the test varied from 39-72%. An optimal sensitivity of 72% and specificity of 95% was achieved when two out of the four ELISAs were positive. This study demonstrated the value in combining two or more TB antigen-based ELISAs for the diagnosis of TB in patients with and without HIV infection (Gutlapalli *et al*., 2016).

IGRAs measure how the body's immune system reacts to the *M. tuberculosis* bacteria (CDC, 2012). The ELISA-based assay measures the concentration of gamma-interferon' (IFNγ) that is released when a patient's fresh blood sample is mixed with TB antigens ESAT6, CFP-10 and TB7.7. Two such assays approved by the United States Food and Drug Administration (FDA) are the Quantiferon–TB Gold in Tube test that uses a mixture of ESAT6, CFP-10 and TB7.7 synthetic peptides and the T-SPOT TB test utilizing synthetic peptides representing ESAT6 and CFP-10 in separate tests (Pai *et al*., 2004).

IGRAs, unlike TST, does not produce a false positive test result in patients previously vaccinated with BCG (Hsia *et al*., 2012). Advantages include a single patient visit to the clinic with results made available in 24 hours. The test is able to differentiate between *M. tuberculosis* and environmental mycobacteria. However, there are several drawbacks in using IGRAs. Blood samples require processing within 8 to 30 hours of collection, due to the limited lifespan of the white blood cells. The test produces inaccurate results for immunocompromised patients with low white cell counts and is unable to predict disease progression. Although IGRA is more accurate that TST, studies have shown that it has limited efficacy in diagnosing LTBI (Ngeow *et al*., 2011 and Bautista and Banaei, 2012). TST is preferred over IGRAs for the diagnosis of LTBI in children under 5 years of age, due to the limited data that is available. Furthermore, the IGRA is expensive and requires skilled personnel, making this test unsuitable for use in resource impoverished settings (Bautista and Banaei, 2012).

Nucleic acid amplification tests (NAA) assays although more sensitive in comparison to other diagnostic tests, cannot differentiate between live and dead tuberculous bacteria and hence cannot quantitate bacterial load before and after treatment (Adekambi *et al*., 2015). NAA products

currently on the market include Amplified TB Direct Test (Gen-Probe) and Amplicore tuberculosis test (Roche Diagnostics). As a result of the drawbacks of the test, TB culture remains the gold standard in the diagnosis of TB disease. The growing bacteria are further used in performing drug susceptibility tests and bacterial genotyping (CDC, 2012).

Pyrosequencing is a DNA sequencing technique used in the diagnosis of drug resistant TB (Bravo *et al*., 2009). The Hain line probe assay (LPA) diagnoses drug-resistant TB using PCR to amplify the region of the gene associated with resistance. Products on the market include Genotype MTBDR*plus* (Hain Lifescience) and INNO-LiPA Mycobacteria by Innogenetics (Cantazaro, 2013; WHO, 2013).

The Xpert MTB/RIF, a rapid molecular assay developed by Cepheid Incorporated (Sunnyvale, CA, USA) using leading edge technology, was WHO-approved for routine TB diagnosis in 2010 (Zeka *et al*., 2011).Thus, the test has been funded by the WHO STOP TB Department and offers high sensitivity in detecting *M. tuberculosis* in pulmonary and non-pulmonary clinical specimens and detecting resistance to rifampicin in less than two hours (Zeka *et al*., 2011).

South Africa has, since 2011, procured more than 50% of the global supply of GeneXpert instruments (Schnippel *et al*., 2013). By the end of June 2013, 1402 testing machines and 3.2 million test cartridges had been procured by 88 out of the 145 countries eligible for concessional prices. The instrument has a high throughput capacity, having the ability to process up to 320 tests a day. Since the introduction of the test, the rate of diagnosis of the disease has increased from 9% to 16%, with 7% of confirmed cases being rifampicin resistant. This allows for early medical intervention and a reduced mortality rate. This assay, however, identifies active pulmonary TB and not the latent disease (Walzl *et al*., 2011).

The Xpert MTB/RIF rapid molecular assay was evaluated with serial sputum samples, obtained over 26 weeks from a cohort of patients in Cape Town, South Africa and Tanzania. The results were compared with smear microscopy and culture. In addition to the drawbacks listed in Table 1.1, the test demonstrated poor specificity and was therefore not replace smear and culture tests (Friedrich *et al*., 2013).

The GeneExpert has revolutionised TB diagnosis and DST by simultaneously detecting M. *tuberculosis* and rifampicin resistance in less than 2 hours (Boehme *et al*., 2010). Although the test has characteristics of a POC test, there is still a need for electricity as a machine is required

for the running of the PCR (Evans, 2011). Furthermore, the machines need calibration and servicing. Therefore, the system does not meet the need for sustainable long term provision for low income settings (Evans, 2011).

Table 1.1: Benefits and drawbacks of currently WHO endorsed and other tests for the diagnosis of TB.

Diagnostic test	Benefits	Drawbacks
	Conventional Diagnostic tests	
Smear microscopy for acid fast bacilli	[1]Rapid results-2 day turnaround Minimal equipment. Moderately trained personnel. Low cost Non-invasive sputum sample Detects active TB No cross reaction with BCG Reliable results with immunocompromised individuals	[1]Negative result does not exclude disease. Poor sensitivity. Low through-put Require lab setup Incorrect smear negative result can lead to patient mismanagement and death. Cannot detect LTBI Cross reaction with NTM
Chest radiography	[4]Rapid results	[4]Low sensitivity and specificity of test. Expensive equipment required. Skilled personnel required.
Computerised Tomography (CT)	[4]More detailed scan than radiography	[4]More expensive than radiography. Cannot differentiate between latent and active TB. Negative result does not exclude TB.
	Culture-based methods	
Liquid culture	[1]Cost effective. High sensitivity Shorter incubation time than solid culture. Non-invasive sputum sample	[1]Culture incubation 10-14 days. Automation adds to expense. False positives due to cross reaction with other mycobacteria.
Solid culture	[1]Cost effective. More sensitive than smears.	[1]Longer incubation time than liquid culture. False positives produced by cross reactivity with other non-tuberculous mycobacteria.

Diagnostic test	Benefits	Drawbacks
Microscopic observation drug susceptibility (MODS)	[23,24,25]Cheapest compared to line probe assay and pyrosequencing.	[23,24,25]15 days to produce results (longest time to produce results).
	Immunological tests	
Delayed Hypersensitivity Reaction	[2,5,6]High sensitivity for active TB. No cross-reaction with BCG.	[2]Invasive test. Follow up visit required. [1,3]Poor sensitivity in immunocompromised patients.
TST	[1,2]Preferred over IGRA's in children under 5 years of age. [4]Simple test. No sample preparation.	Cross reaction with BCG and patients infected with other Mycobacterium species. Cannot differentiate between latent and active TB.
TB patch test that delivers Mtp antigen.	No expensive equipment required. Skilled staff not required. Diagnose active TB. No cross-reaction with BCG or patients infected with other mycobacteria.	[1]Follow up visit required. Invasive test.
Biosensor	[18,19,20]Rapid results. Early disease detection. High sensitivity and specificity. Reusable. No cross reaction with BCG.	[21]Expensive
Immunochromatographic assay: Lateral Flow Assay	[12,26]Rapid. Ease of use. Cost effective. Can differentiate between *M. tuberculosis* and mycobacteria other than tuberculosis) MOTT.	[30]Does not discriminate between active and latent TB. Antibody detection tests are not favoured because false positives may result from environmental mycobacteria and false negatives resulting from immunocompromised patients leads to untreated patients and disease transmission. Liquid or solid culture of bacteria therefore special laboratory setup and incubation time needed.

Diagnostic test	Benefits	Drawbacks
		Gene mutations can result in false negatives. Cannot distinguish between *M. tuberculosis* species. Drug susceptibility cannot be characterised. [28]Some assays require lengthy 13 week sample preparation. Biosafety level 3 (BSL-3) safety cabinet required. Liquid or solid culture of bacteria therefore special laboratory setup and incubation time needed.
Enzyme linked immunosorbent assay (ELISA)	[26,27,29]High sensitivity and specificity for detection of select antigens. Cost effective when compared to PCR. Does not require expensive equipment. Can differentiate between *M. tuberculosis* and MOTT. Convenience and simplicity of use.	Gene mutations can result in false negatives. Cannot distinguish between *M. tuberculosis* species. Drug susceptibility cannot be characterised.
Nucleic acid amplification tests		
Xpert MTB/RIF Assay	[9,10]Greater sensitivity Than sputum smear microscopy. Detects resistance to rifampicin in 2 hours. Processes up to 320 specimens a day. Detects active TB.	[11,12,13]Does not detect latent TB. Expensive. Require skilled personnel. Require electricity and temperature controlled environment. Lacks specificity.
Haine Line Probe Assay	[23]5 days to produce results – quickest of the 3 methods.	[23]Labour intensive. Cross-contamination. Labour intensive.
Pyrosequencing	[22]Does not take as long as MODS to produce results.	[22]8 days to produce results.

[a]

[a][1]Dorman, 2010; [2]Bekmurzayeva *et al*., 2013; [3]Ligher and Rigaud, 2009; [4]FIND, 2012; [6]Malagini *et al*., 2011; [7]Mustapha *et al*., 2006; [8]Ramos-Vara, 2005; [9]WHO, 2010; [10]Zeka *et al*., 2011; [11]Walzl *et al*., 2011; [12]Harrington, 2015; [13]Friedrich *et al*., 2013; [14]CDC, 2012; [15]Hsia *et al*., 2012; [16]Ngeow *et al*., 2011; [17]Bautista and Banaei, 2012; [18]Wang and Liu, 2009; [19]Mukandan *et al*., 2012; [20]Hung *et al*., 2011; [21]Seong *et al*., 2011; [22]Bravo *et al*., 2009; [23]Cantaro, 2013; [24]WHO, 2013; [25]Zhang *et al*., 2014; [26]Namba, 2010; [27]Pai *et al*., 2010; [28]Dayal *et al*., 2008; [29]Vadwai *et al*., 2012; [30]Ben-Selma *et al*., 2011. [a]

1.2.1.2 Benefits and drawbacks associated with TB biomarkers

TB Biomarkers are not only indicators of disease presence, stage and severity but are also used to monitor the efficacy of anti-TB drugs. These biomarkers may either be derived from the host, for example immunological markers such as IFNγ or from the bacterium, for example ESAT6 (McNerney *et al*., 2012). In order for pathogen-derived biomarkers to be considered as candidates for antigen detection assays, they must be able to reach sample matrices such as sputum, urine and plasma, and be specifically detected in infected patients (Bekmurzayeva *et al*., 2013).

Targeting circulating antibodies for TB diagnosis has not been regarded as a favourable approach. The reason is that antibody profiles are known to vary from one patient to another. Despite intense research, little is known about host associated variables and how much of the TB proteome is targeted by human antibody (Velayudhan *et al*., 2011). The host specific antibody response in the diagnosis of TB, unlike with other infectious diseases, is not well understood and poses a challenge (McNerney *et al*., 2012).

HIV co-infection resulting in loss of immune control with the immunosuppression or destruction of T and-B cells make the diagnosis of TB by targeting antibodies, an unfavourable approach. Despite the economical option of developing antibody detection assays for use in impoverished settings, this type of assay is not recommended (Harrington, 2015). Therefore, pathogen-derived biomarkers are preferred candidates for use in a rapid, POC test (McNerney *et al*., 2012).

Current antigen detection methods use TB Biomarkers based on antigenic specificity and their presence in detectable levels in clinical samples, such as sputum, urine and plasma. Antigen from infected tissue, reaches the bloodstream and may be shed in urine. Therefore, urine has been regarded as a practical sample as it is obtained safely, non-invasively and is easily obtained from both adults and children (Tucci *et al*., 2014). More importantly, urine based assays make TB diagnosis in HIV co-infected patients with low bacterial loads in sputum, an assay of choice as it avoids invasive procedures in patients with extra pulmonary TB (WHO, 2009). The detection of TB biomarkers in urine samples using a simple and rapid antigen detection test, are beneficial for the diagnosis of TB (Tucci *et al*., 2014).

Several studies have suggested the use of monoclonal antibodies produced against secretory proteins, Mpt64, ESAT6, CFP10 and Ag85 complex for use as tools in the diagnosis of TB (Bekmurzayeva *et al*., 2013, Fabre *et al*., 2011, Shi *et al*., 2004). Immunodominant, secreted proteins of TB are associated with active TB. They are responsible for virulence and induce a

strong immune response in infected individuals (Velayudhan *et al*., 2010). Therefore, secretory proteins from *M. tuberculosis* have been used in the development of several diagnostic assays such as the skin patch test, biosensor analyses, immunochromatographic assays, immunohistochemical assays, PCR and ELISA (Bekmurzayeva *et al*., 2013).

Another finding critical in the development of a diagnostic test for TB, is the use of a combination of biomarkers. Raja *et al* (2008) demonstrated that about one third of all TB patients are seronegative when tested against a single antigen marker. This was a significant finding as it suggests that for a diagnostic test to be sensitive and efficient for the diagnosis of TB, combinations of proteins used either separately or as fused proteins, are required (Raja *et al*., 2008; Kalra *et al*., 2010; Pollock *et al*., 2013).

Velayudhan *et al*. (2011) suggested the development of a multi-analyte diagnostic assay if advancements in immunodiagnosis are to be made (Velayudhan *et al*., 2011). ESAT6 and CFP10 were reported as strong gamma interferon inducers with potential for use in diagnostic tests for active and latent TB. It was shown that when CFP10 was used as a single diagnostic marker, the test did not produce a strongly reactive test signal. The same results were produced when ESAT6 was used on its own. However, a combination of these antigens in a single diagnostic test improved the sensitivity of the test for TB (Tavares *et al*., 2007, Hemmati *et al*., 2011).

A study done on guinea pigs in the development of a diagnostic skin test also showed that a combination of ESAT6 / MPT64 was more reactive than when ESAT6 was used alone (Elhay *et al*., 1998). Tavares *et al* (2007) conducted a study in Brazil to determine IFN-γ T-cell responses in patients exposed to the combinations 38 kDa/CFP10, 38 kDa/MPT64, ESAT6/CFP10 and each of the individual proteins. Results from this study indicated that antigens used in combination as opposed to single antigens were more suitable for TB diagnosis. Patients that presented with latent infection demonstrated a higher INFγ response to the ESAT6 / CFP10 combination of proteins (Tavares *et al*., 2007).

Target antigens have been selected for detection methods, based on their role in *M. tuberculosis* infection. The benefits and drawbacks associated with each of these biomarkers, have been listed in Table 1.2 below. Several studies have indicated that prominent biomarkers CFP10 and ESAT6, located in the RD1 region, are two of the most abundant *M. tuberculosis* secretory proteins, responsible for bacterial virulence. These proteins are absent from BCG and most MOTTs (Nguyen *et al*., 2012). CFP10 helps attachment of the bacteria to the cell's surface while both

ESAT6 and CFP10 stimulate T-cells and are therefore used in IGRA's (Majlessi, *et al*., 2005; Renshaw *et al*., 2005; Bautista and Banaei, 2012).

CFP10 proteins encoded in the ESX region of *M. tuberculosis* are responsible for immunodominance and have the ability to evade the host's immune system. Diagnostic tests such as QuantiFERON and T-SPOT.TB use EsxA and EsxB genes from *M. tuberculosis* as T-cells from infected individuals respond strongly to the membrane associated and secretory proteins encoded in these genes (Velayudhan and Porcelli, 2013).

Mpt64 multi-epitopes are recognised by T-cells and is therefore significant in TB diagnostics (Koo *et al*., 2005). However, the INF-γ response produced by Mpt64 is not as strong as that produced by ESAT6 and CFP10 (Chaves *et al*., 2010). Mpt64 produces a strong delayed type hypersensitivity (DTH). This makes Mpt64 the ideal vaccine candidate, either on its own or in combination with ESAT6 (Brodin *et al*., 2004).

Ag85, another immunodominant, secreted protein, is responsible for cell wall biosynthesis and immunogenicity of the bacterium. It is found on the external cell wall of the bacterium and in blood. Ag85 binds to plasma fibronectin, altering the host's immune response by reducing the phagocytosis of TB thereby promoting infection. Expression of this protein is important for the survival of TB within the macrophage and evading the host immune system (Ronning *et al*., 2000). Ag85 may be used in a cost-effective immunoassay for the diagnosis of pulmonary and extra-pulmonary TB (Kashyap *et al*., 2013). However, it has been suggested that Ag85 may be used in an ELISA and therefore does not qualify as a POC test (Kashyap *et al*., 2007).

As already discussed, the detection of LAM is useful in HIV coinfected patients (Lawn *et al*. 2013). LAM, a cell wall protein shed in urine, facilitates the diagnosis of TB in children especially as it is difficult to obtain sputum samples from them. The additional benefit of obtaining a urine sample is that it avoids the risk of disease transmission during sample collection (Lawn *et al*. 2013).

The Alere Determine TB LAM Ag rapid test (Waltham, Massachusetts, United States of America), is used to detect active TB in urine samples of HIV positive patients. Although this assay accurately diagnoses TB in HIV positive patients, it is not recommended in a HIV negative population or in HIV positive patients with a high CD4 count. The reason for this is that the sensitivity of the LAM assay increases as the patient's CD4 count decreases and test sensitivity

increases with increased severity of the disease. The test does not allow for early diagnosis of the disease and effective patient management. Another disadvantage is that there is no recorded data for trials done on children (Minion *et al*., 2011). There is also reactivity of LAM with other NTM. Therefore, the Determine TB-LAM test cannot be used as a stand-alone POC test (Lawn *et al*. 2013).

The TB7.7 protein (corresponding to Rv2654), is found in the RD11 genomic region of *M. tuberculosis*. It is an immunodominant marker predictive of recent TB infection and is present in the newer version of the QFN-TB Gold, called QuantiFERON-TB-Gold-In-Tube (Cellestis Ltd., Carnegie, Australia; Wang *et al*., 2013). The new test uses peptides of the antigens as opposed to whole ESAT6 and CFP10. The application of the test is the diagnosis of LTBI in countries with low incidence of the disease and has contributed to further reduction in incidence (Ahmad, 2011).

Heat Shock Protein (16 kDa), also referred to as HSPX, Rv2031 and alpha-crystalline chaperone, is one of the most immunogenic latency proteins. Belay *et al.* (2015) showed that pro-inflammatory cytokines, IFNγ, tumour necrosis factor alpha (TNFα) and anti-inflammatory interleukin-10 (IL-10) were significantly higher in latent TB infection. This indicates that these markers may offer protection against TB. The findings of this study, further indicate that these biomarkers have the potential to diagnose the absence of active TB. Rv2031-based vaccine administered to pigs showed an increased production of IL-12, IL-10 and tumour growth factor (TGF) β, indicating protection against TB. IFNγ, TNFα and IL-10 were also measured against ESAT6 / CFP10 antigens, specific to *M. tuberculosis*, and this cytokine response correlated with specific cytokine response towards Rv2031, suggesting that Rv2031 is specific to *M. tuberculosis*. IFNγ, TNFα and IL-10, may therefore have the potential for use as biomarkers of protective immunity in patients with latent TB infection (Belay *et al*., 2015).

The 38 kDa lipoprotein is also referred to as Rv3082, PstS-1 and MT3167. The extracellular, secretory, phosphate-binding lipoprotein is known to induce strong T and B-cell responses and serves as a receptor for active transport of a variety of nutrients (Velayudhan *et* al., 2011; Sanchez *et al*., 2008). Although the protein is apoptogenic for human monocyte derived macrophages (MDM), 38 kDa-induced apoptosis in infected macrophages is significantly reduced in toll-like receptor 2 (TLR-2) deficient macrophages. TLR's form the initial line of defence against invading pathogens. Therefore, although 38 kDa induced apoptosis results in release of the bacilli from the host reservoir resulting in progression of the disease, this may benefit the host by producing an

adaptive anti-TB immune response. The role of the 38 kDa protein is not only for diagnosis of the disease but has potential for use as a novel vaccine (Lim *et al*., 2015; Chang *et al*., 1994).

Although the protective immune response in *M. tuberculosis* is primarily cell mediated (Talaat *et al*., 2010), mycobacteria also elicits a humoral immune response (Wu *et al*., 2010). Diagnostic test manufacturers have therefore focused the development of antibody detection tests for the sero-diagnosis of TB as opposed to tests for cell mediated responses that are more expensive to produce. A lateral flow test for the detection of serum antibodies against a 38 kDa antigen was developed for the diagnosis of *M. tuberculosis* in developing countries (Grobusch *et al*., 1998).

Standard Diagnostics, South Korea, developed a rapid test to detect serum immunoglobulins raised against the 38 kDa, 16 kDa and 6 kDa antigens in order to facilitate the early diagnosis and prevent transmission of *M. tuberculosis*. This type of antibody detection test would work in countries that have a low prevalence of TB, but would be less effective in countries such as Africa where not only is the prevalence of TB high but that TB exists as a disease secondary to HIV. False negative results would be the result of patients having a low or no antibody mediated response (WHO, 2015).

Pollock *et al.* (2013), suggests that Rv1681, detected in urine, may be used as a diagnostic marker for active pulmonary TB in a non-invasive POC test. The protein is able to distinguish between latent and active TB infection. There is evidence that Rv1681 is a more sensitive marker than LAM and therefore has the potential for development of a POC test (Pollock *et al*., 1997; *Pollock et al*., 2013).

Table 1.2: The benefits and drawbacks associated with biomarkers for TB that are used in current detection methods.

Biomarker	Benefit	Drawback	Detection method
ESAT6	[1,2,3]Not present in BCG and most MOTTs. Stimulates T-cell response. Vaccine candidate in combination with Ag85.	[1]Requires that it be used in combination with other biomarkers if considered for use in a diagnostic assay.	IGRAs Immunohistochemistry Biosensor DHR ICA's
Mpt64	[1,4]Not present in BCG and MOTT. Associated with early TB infection. Stimulates gamma interferon release. Potential vaccine candidate.	[5]Solid or liquid culture sample required for use in ICA's. Requires that it be used in combination with other biomarkers if considered for diagnostics. False negatives may result from gene mutations. Need to investigate whether patch test can differentiate between latent and active TB infection.	ICA's. TB patch test (Sequella). Immunohistochemistry. ELISA PCR Biosensor
CFP10	[8]Not present in BCG, most MOTTs. Stimulates T-cell response.	[8]Requires that it be used in combination with other biomarkers if considered for diagnostics.	IGRAs Biosensor DHR ELISA
Ag85	[1,6]Differentiates between pulmonary and extra-pulmonary TB. No cross reaction with non-tuberculous mycobacteria	[7]Cannot be considered for POC test because of detection method and requires use in combination with another biomarker.	PCR Biosensor Lateral flow test
LAM	[9]Present in urine, diagnostic sample of choice. Rapid results.	[10]Used for patients with advanced immunodeficiency. Require infrastructure for performing ELISA. Cross reaction with non-mycobacterial species. Does not detect early TB infection therefore not beneficial for patient management. No documented studies carried out on children.	Biosensor ICA's

Biomarker	Benefit	Drawback	Detection method
TB7.7	[11,12]No cross reactivity with BCG and non-tuberculous mycobacteria	[11]Requires that it be used in combination with other biomarkers if considered for diagnostics.	IGRA
16 kDa	[13]Rv2031-based vaccine has potential to offer protection against TB. Specific to *M. tuberculosis*	[13]ELISA not the ideal rapid test. Cannot be used in antibody detection assays due to false positives arising from hyperglobulinemia	ELISA PCR
38 kDa	[14,17]Antibodies can reduce host macrophage apoptosis.	[14,15]Cross reaction with BCG	Culture ELISA
Rv1681	[16]Present in urine. Distinguish between latent and active TB. More sensitive than LAM.	[16]Low ELISA sensitivity. Requires use in combination with other biomarkers.	ELISA

[1]Bekmurzayeva *et al*., 2013; [2]Baumann, 2012; [3]Tang *et al*., 2014; [4]Martin *et al*., 2011; [5]Tavares *et al*., 2007; [6]Kashyap *et al*., 2013; [7]Kashyap *et al*., 2007; [8]Velayudhan and Porcelli, 2013; [9]Lawn *et al*., 2013; [10]Minion *et al*., 2011; [11]Wang *et al*., 2013; [12]Ahmad, 2011; [13]Belay *et al*., 2015; [14]Velayudhan *et al*., 2011; [15]Sanchez *et al*., 2009; [16]Pollock *et al*., 2013; [17]Lim *et al*., 2015.

Some of the challenges in producing an effective rapid, cost effective diagnostic test is the discrimination between latent and active TB, pulmonary and extra-pulmonary TB, T-cell and B-cell responses and the identification of biomarker expression during infection. Prompt TB diagnosis remains a critical problem and is the catalyst in research into new diagnostic assays. Proteins secreted by *M. tuberculosis* that are abundant in culture and are not found in BCG or mycobacteria other than TB have the potential for early detection of active TB (FIND, 2014).

1.2.2 The relevance of developing new diagnostic assays

1.2.2.1 Significance and challenges associated with the detection of LTBI

It is estimated that a third of the world's population live with LTBI that may potentially develop into active TB (Walzl *et al*., 2011). During initial infection, mycobacteria enter inactivated macrophages, where they rapidly multiply resulting in a rapid growth phase of the infected macrophages. Uninfected macrophages are activated and surround the infected macrophages, resulting in the formation of a granuloma. The granuloma is made up of an infected macrophage core, surrounded by activated macrophages, followed by T-lymphocytes, surrounded by fibroblasts and collagen. B-lymphocytes are also found in abundance in the granuloma. This stage of the disease is known as latent TB. Usually, reported cases of TB are the result of "reactivation" of the latent bacilli (Walzl *et al*., 2011).

Most of the current detection methods focus on active TB whilst the diagnosis of LTBI remains difficult without a reliable and rapid test for the diagnosis of *M. tuberculosis* infection (WHO, 2015; Walzl *et al*., 2011). TST using PPD has been the standard in the diagnosis of LTBI, despite its limitations in terms of accuracy and reliability (Dorman, 2010; Leng *et al*., 2014). IGRA's use *M. tuberculosis*-specific antigens encoded by the region of difference 1 and 11 (RD1 and RD11) resulting in no cross-reactivity with the BCG vaccine. IGRA's, although more accurate than TST, have limitations in that continued macrophage activation during disease becomes pathological as opposed to offering protection from *M. tuberculosis* infection. Therefore, the total amount of INF-γ does not accurately reflect the effectiveness of the immune response (Bautista and Banaei, 2012). At presently, no single diagnostic test is able to accurately differentiate LTBI from active TB and asymptomatic forms of the infection that are associated with a high risk of disease progression. It is therefore imperative to develop a new diagnostic assay that may address these shortfalls (Velayudhan and Gennaro, 2011).

Tang *et al.* (2014) conducted a study using ssDNA aptamers CE24 and CE15 bound to ESAT6 and CFP10 respectively, and was able to detect both these proteins in serum samples from patients with active pulmonary TB, extra-pulmonary TB but not from donors uninfected by TB. Both ESAT6 and CFP10 are therefore significant as they are secreted by virulent *M. tuberculosis* and are not found in the attenuated non-virulent BCG vaccine. These results correlated well with the T-Spot TB assay. The study concluded that ESAT6 and CFP10 have potential as early diagnostic markers not only for active pulmonary TB and extra-pulmonary TB, but also for the detection of latent TB and TB with HIV co-infection (Tang *et al*., 2014; Shu *et al*., 2015).

It has further been suggested that ESAT6-derived peptides enhance the discrimination between active disease and LTBI (Velayudhan *et al*., 2011). During latent infection, membrane bound proteins may become extracellular as a result of low numbers of live bacilli, dead bacilli or from macrophage-excreted exosomes. Also, with an increase in the bacillary burden, proteins are secreted from the metabolically active bacteria and in both these instances, these extracellular proteins become potential diagnostic targets (Velayudhan *et al*., 2011).

In the past, infection with *M. tuberculosis* was thought to have a dual outcome (Velayudhan *et al*., 2011). The first was the characterisation of LTBI by a positive TST/IGRA result, negative chest X-ray and without presentation of clinical symptoms. The second outcome was active disease whereby tubercle bacilli and bacillary products are detected in clinical samples such as sputum. It is now evident that *M. tuberculosis* infection is not as simple. LTBI may now be differentiated according to the risk of disease reactivation. For example, the risks are reduced in immunocompromised individuals over a period of time whereas the risks are considered greater in asymptomatic LTBI patients having abnormal X-rays and past TB infection as opposed to LTBI patients without prior infection. The diagnosis of active disease was dependent on bacillary burden and as a result, patients having a low bacillary burden were missed. With administration of targeted treatment by the clinician, for example LTBI patients at high risk for disease reactivation, it is critical that the correct diagnosis of disease is made (Velayudhan *et al*., 2011).

It has been suggested that a dynamic approach be taken for the identification and treatment of symptomatic and asymptomatic TB infection. Immune biomarker research has been proposed in overcoming these challenges. It has been suggested that in order to successfully control the spread of *M. tuberculosis* infection, it is critical to identify and treat individuals who progress to disease and are able to spread infection. Therefore, the clinician's approach in controlling the spread of disease is targeted treatment for LTBI. The IGRA's, although more accurate than PPD, does not

facilitate the clinicians' decisions regarding targeted LTBI treatment. New diagnostic tools are therefore required as it has been shown that current diagnostic methods, by measuring INFγ, are unable to accurately diagnose active TB infection and are inappropriate for low-income and low-technology settings that actually require these tools (Velayudhan.*et al*., 2011).

1.2.2.2 The role of ESAT6 in the diagnosis of TB

Studies have identified thatRD1 is present in virulent *M. tuberculosis* and *Mycobacterium bovis* (*M. bovis*) strains but is absent from BCG. RD1 encodes the protein secretion system ESX-1, responsible for virulence of *M. tuberculosis* and secretion of several *M. tuberculosis* proteins, one of them being ESAT6 (encoded by *esxA*). Virulence of the ESX-1 proteins have been demonstrated by the deletion of RD1 and disruption of ESAT6 from the TB genome that produced deleted virulence in cultured macrophages and in mice. When RD1 genes were introduced in BCG, results showed virulence and granuloma formation in immune deficient mice while morphology of *M. tuberculosis* colonies in culture were altered (Ahmad, 2011).

An effective biomarker for TB diagnosis should be virulent, immunogenic and present in culture fluids (Bekmurzayeva *et al*., 2013). ESAT6 is one of several antigenic biomarkers that display these characteristics. ESAT6 is encoded in the Rv3875 gene, located in the RD1 region and is present only in virulent or pathogenic strains of *M. tuberculosis* (Farshadzadeh *et al.,* 2011). It was found that although the tubercle bacilli expressed different immunodominant antigens at different TB growth states, ESAT6 expression was high in both active and inactive states, concluding that this marker has potential for use in the diagnosis of active and latent forms of the disease (Davidow *et al*., 2005).

ESAT6 forms pores in the phagosome membrane thereby enabling *M. tuberculosis* to escape into the cytoplasm and induces apoptosis of macrophages via the extrinsic pathway (Ahmad, 2011). The ESX-1 secretion system and ESAT6 are responsible for macrophage infection and TB disease dissemination by releasing the bacilli from the macrophages. It is during this time that ESAT6 may be targeted for early diagnosis of *M. tuberculosis*. A study by Wang *et al* (2009) showed that ESAT6 promoted the spread of intercellular *M. tuberculosis* by decreasing T-cell IFNγ transcription and inhibiting IFNγ secretion. It has been demonstrated that purified TB proteins CFP10, Ag85, Mpt64 and alpha-crystalline did not have the same ability as ESAT6 in apoptosis induction (Ahmad, 2011; Derrick and Morris, 2007; Wang *et* al., 2009).

Davidow *et al* (2005) described a clear correlation that existed between the production of *M. tuberculosis* antibodies in an infected mouse model and the corresponding human antibody profiles. The study showed that the tubercle bacilli expressed different immunodominant antigens at different TB growth states and that ESAT6 expression was high in both active and inactive states, supporting the finding that ESAT6 is a dormancy antigen (Davidow *et al*., 2005; Ahmad, 2011).

Harboe *et al*. (1998) demonstrated that ESAT6 has both a B-cell and T-cell epitope and that the B-cell epitope on the surface-exposed, unfolded part of the polypeptide chain, was responsible for immunodominance of the protein (Harboe *et al*., 1998; Jiang *et al*., 2013). The study demonstrated the production of antibodies to two B-cell epitopes at positions 30 to 38 and 51 to 59 on the *esxA* gene. The surface exposed peptides were coupled to keyhole limpet hemocyanin for the immunisation of rabbit in MAb production. An ELISA was developed using HYB76-8 as the capture antibody, antigen for testing was used in the second layer and anti-peptide antibody was used in the third layer. The assay showed no cross reactivity with *M. bovis* BCG culture fluid or with MPT64 or Ag85. The study successfully characterised B-cell epitopes on ESAT6 that was used to develop a quantitative ELISA (Harboe *et al*., 1998).

However, the presence of ESAT6 in some NTM's, suggests that this marker be used in combination with another marker to achieve specificity and sensitivity of an assay in the diagnosis of *M. tuberculosis* (Ahmad, 2011). Animal models have demonstrated that species specific ESAT6 was targeted by IFNγ-secreting CD4 T-cells that were detected by ELISA (Baumann *et al*., 2012).

Soleimanpour *et al*. (2016) used recombinant ESAT6 tagged with the Fc region of human immunoglobulin (Ig) G1 to produce a selective delivery system in order to promote cellular immunity. ESAT6 was produced in frame with the Fc domain of IgG1. The T-vector and PDR2EF1α eukaryotic expression vector have the sequence encoding for the Fc region of human IgG1 (about 230 amino acids). Fc –tagged ESAT6 showed potential for use in targeting antigen presenting cells to induce an immune response and for application in diagnostic IGRA's (Soleimanpour *et al.*, 2016).

Farshadzadeh *et al.* (2010) cloned and expressed ESAT6 protein of *M. tuberculosis* in *Escherichia coli* as results from previous studies have indicated that RD1 gene based diagnosis was more accurate than TST in countries with low prevalence of TB (Dinnes *et al*., 2007). PPD produced a

high positive rate while some active TB cases tested negative using this technique. Cloned and expressed ESAT6 from this study detected latent TB with more sensitivity and specificity than TST (Dinnes *et al*., 2007; Masood *et al*., 2014).

ESAT6 is currently used in IGRA's, immunohistochemistry, biosensor assays, DHR and ELISA (Bekmurzayeva *et al*., 2013). ESAT6 was selected for the development of a rapid test, based on its characteristics of antigen virulence and immunodominance (Bekmurzayeva *et al*., 2013). It is a potent IFNγ inducer, a marker for early disease detection and disease dissemination (Ahmad, 2011). Evidence suggests that this dormancy antigen has the potential for the diagnosis of LTBI and active TB infection (Davidow *et al*., 2005). The identification of a B-cell epitope on the surface of ESAT6, is significant as a target for MAbs (Harboe *et al*., 1998; Leng *et al*., 2014).

The detection of ESAT6 in sputum and pleural effusion further favours the use of this biomarker for rapid and early diagnosis of TB. ESAT6 is therefore a promising candidate for use in a non-invasive diagnostic test, ideal for use by first-contact healthcare workers (Leng *et al*., 2014; Feng *et al*., 2011).

Literature has supported the use of ESAT6 in diagnostic assays that utilise contrasting test principles. Harboe *et al*. (1998) demonstrated the production of antibodies to two B-cell epitopes on ESAT6. These antibodies against ESAT6 may be detected in an ELISA. The second diagnostic assay principle is based on the detection of IFNγ. ESAT6 is a potent IFNγ inducer and animal models have demonstrated that species specific ESAT6 was targeted by IFNγ-secreting CD4 T-cells that were detected by ELISA (Baumann *et al*., 2012). In addition, ESAT6 expression has been shown as high in both the active and inactive states of TB. Therefore, detection of ESAT target antigen has potential for use in the diagnosis of active and latent TB (Davidow *et al*., 2005). Therefore, the utility of ESAT6 in an ELISA based assay has been investigated in this study.

1.2.2.2.1 Recombinant DNA technology used in the production of recombinant ESAT6, required as an immunogen and for the development and optimization of an ELISA in detecting anti-ESAT6 MAbs

Studies have shown that transformation of *E. coli* is the most common method described in the production of recombinant ESAT6. Restriction enzymes are used to cut the required DNA sequence from genomic DNA. The insert DNA is then ligated into vector, such as pGEX6P-1, using DNA ligase. The vector has in its sequence, an antibiotic selection marker and is inserted into a host cell (e.g. *E. coli*) using a process known as transformation. Therefore, host cells with

vector and the selection marker have antibiotic resistance and is able to survive when grown in media with the corresponding antibiotic. During this process, host cells without the vector and resistance marker die. The *E. coli* host then expresses the required protein from the recombinant gene.

The production of recombinant ESAT6 following transformation of *E. coli*, was described in a study conducted by Farshadzadeh *et al*. (2010). DNA extracted from *M. tuberculosis* H37Rv was PCR amplified using gene specific primers. Recombinant plasmids were produced when PCR product was inserted into pET102/D vector and transferred into *E. coli* strain TOPO10 and then transferred into *E. coli* strain BL21. The expressed fusion protein was in a soluble form and purified on Ni-NTA column. It was suggested that the recombinant ESAT6 could be used in an ELISA for TB diagnosis (Farshadzadeh *et al*., 2010).

Evidence suggested that recombinant ESAT6 may be produced using different bacterial and viral vectors. The cloning of a gene fragment containing three T-cell epitopes of ESAT6 into a pIRES plasmid together with the fms-like tyrosine kinase 3 ligand (FL), an adjuvant that induces a potent immune response, producing a novel recombinant vaccine encoding ESAT6. Data from the study indicated that the recombinant plasmid is useful as a vaccine for preventing TB infection (Jiang *et* al., 2013).

Wang *et al* (2005) inserted ESAT6 into a pQE30 vector that was transferred into *E. coli* strain TG1. However, expressed fusion protein purified through a Ni-NTA column, produced an insoluble protein in the cell lysate (Wang *et al*., 2005). Cloning procedures that produced insoluble protein (in inclusion bodies) required that the inclusion bodies be solubilised with 8M urea or 6M guanidine-hydrochloride at pH 7.4. Studies have shown that recombinant ESAT6 is required in a soluble form, for use in downstream processes (Jiang *et al*., 2013).

1.2.2.2.2 Hybridoma technology used in the generation of anti-ESAT6 MAbs for development and optimization of an ELISA for the detection of recombinant ESAT6

MAbs are monovalent antibodies, produced by a single B-lymphocyte clone, that bind to a specific antigenic epitope (Liu, 2014). Since the development of Hybridoma technology in 1975 by Kohler and Milstein, MAbs have revolutionised research in areas of disease prevention, detection and treatment, vaccine production, identifying antigens involved in disease and understanding host immune responses (Yang *et al*., 2012). Current FDA-approved MAbs are used in humans for the treatment of diseases and conditions such as cancer, chronic inflammatory

diseases, cardiovascular diseases and infectious diseases (Liu, 2014). MAbs act as angiogenesis inhibitors, while radio-labelled MAbs are used in imaging and bind receptors on platelets to prevent them from clumping. MAbs are also used in Neurology to treat diseases such as multiple sclerosis (Acharya *et al*. 2011).

MAbs are very specific and binds onto the epitope of the target antigen (Siddiqui, 2010). The epitope controls the antibody's function, whether it acts as an agonist, antagonist or whether it is non-modulatory. It may also influence the antibody's ability to induce antibody-dependent cell mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). MAbs function using the different mechanisms of direct control of the target antigen, CDC, ADCC and the delivery of toxins to the target cells. Although MAbs have the ability to specifically target antigens associated with disease, it has taken several years for them to be applied in diagnostics and drug development. This is attributed to the development and market success of MAbs, their drug safety profiles and the technological advancements in discovering new MAbs (Siddiqui, 2010).

MAbs were first generated in mice in 1975, using hybridoma technology (Kohler and Milstein, 1975). Hybridomas are generated by immunising an animal species, such as mice or rats, against a specific epitope on an antigen (Liu, 2014). Mice are selected as the laboratory animal of choice as they are easy to house and the mouse myeloma cell line is easily available (Johnson, 2011). Blood samples are collected from the Balb/C mice prior to immunisation with an emulsion of recombinant ESAT6 and complete Freund's adjuvant (CFA). A month later immunisation was enhanced with an emulsion of recombinant ESAT6 in incomplete Freund's adjuvant (IFA). After a month blood samples from the mice were tested by ELISA in order to determine the mouse having the best immune response to the antigen. The best immune responding mouse was then administered an immunisation boost a few days prior to the hybridoma fusion.

The B-lymphocytes that are harvested from the spleen of the immunised animal are then fused by either chemical, viral or electro-fusion to an immortal cell line, of the same species lacking the hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) gene. Hybridomas that result from the fusion of primary B-cells to myeloma cells, are selected by *in vitro* culture using hypoxanthine-aminopterin-thymidine (HAT) media. The result is that only the hybridomas survive as they have inherited the immortality from the myeloma cells and selective resistance from the B-cells. Unfused myeloma cells die in the selective media as they lack the HGPRT gene and are therefore unable to synthesise nucleotides via the *de novo* pathway as this is inhibited by

aminopterin in the culture media. MAbs are then produced from polyclonal antibodies following dilution of hybridoma cells by limiting dilution. An ELISA is then used to confirm the specificity of the MAb (Figure 1.1).

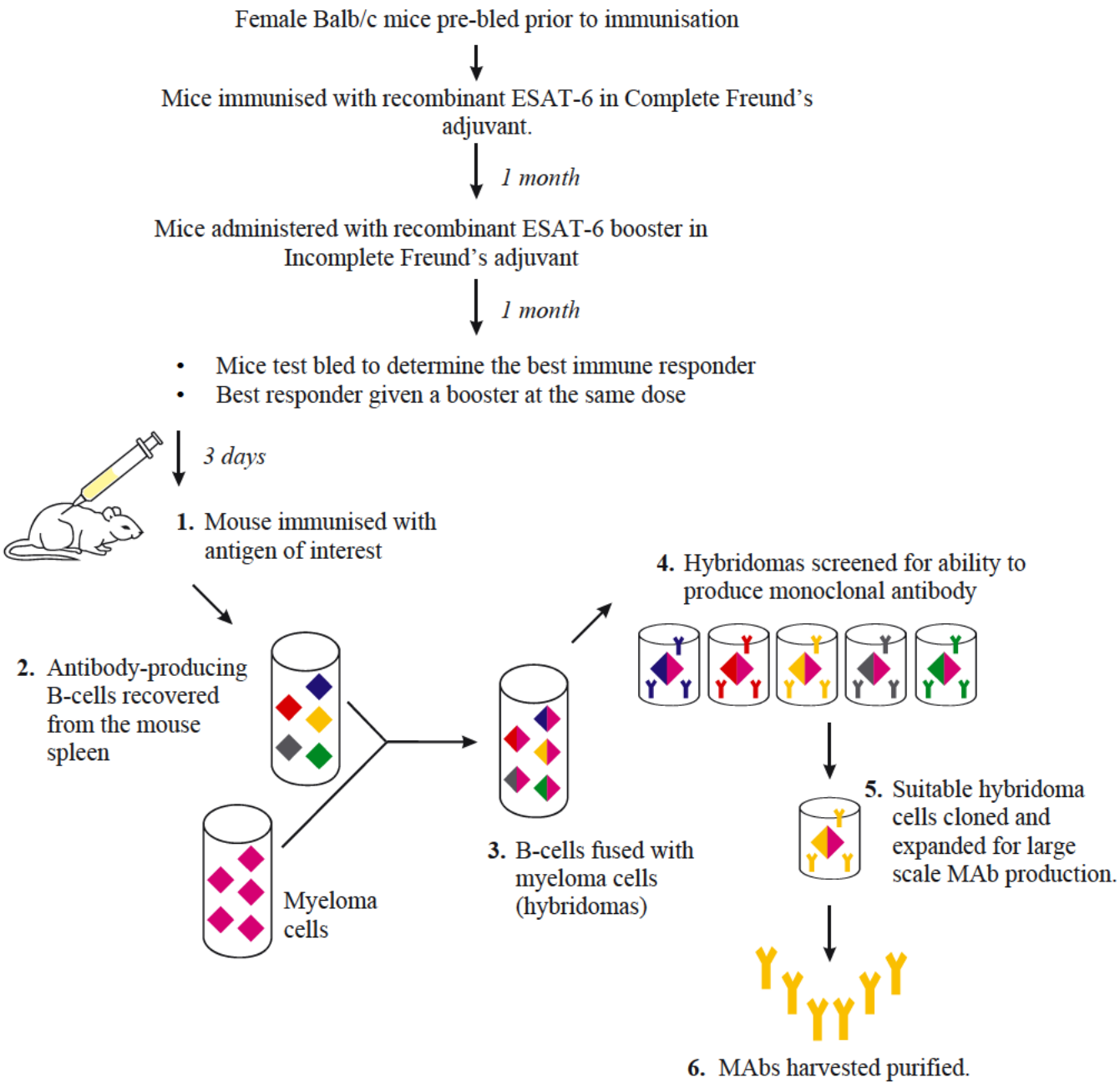

Figure 1.1: Overview of murine monoclonal antibody production

Recombinant DNA technology has been used in the reconstruction of MAbs to produce chimeric and humanised antibodies for therapeutic use. Characteristics that favour the use of MAbs are their specificity, immortality, the production of unlimited homogenous populations and fewer problems that are experienced with cross reactivity (Siddiqqui, 2010). MAbs have therefore been utilised in Western blot analysis, immunoflourescent tests, immunohistochemistry, immunoprecipitation, affinity chromatography, lateral flow rapid tests and ELISAs for the diagnosis of disease (Smith, 2012).

The diagnostic potential of monoclonal antibodies against *M. tuberculosis* has been assessed in several studies (Suharti *et al*., 2014; Grobusch *et al*., 1998; Mdluli *et al*., 2014; Mukundan *et al*. 2012; Ihama *et al*., 2012). An ELISA-based MAb diagnostic assay against 20 kDa *M. tuberculosis* protein was compared to PCR and acid fast staining (Suharti *et al*., 2014). Results from the study showed that the sensitivity of the ELISA was higher (81%) than acid fast staining (65%) but lower than PCR (92%). The specificity of the ELISA (79%) was higher than PCR but lower than acid fast staining (100%). The study therefore concluded that an ELISA based assay against 20 kDa had potential for the diagnosis of *M. tuberculosis* in sputum samples (Suharti *et al*., 2014).

A more effective means of diagnosis and prevention of TB transmission is to be able to detect the antigen in TB infected patients. In the development of rapid diagnostic tests suitable for countries with high incident rates of HIV and TB, a capture antibody and a signal antibody to the target antigen is required. These antibodies must recognise different epitopes on the antigen. The pentameric structure of the IgM molecule makes it the ideal capture antibody in lateral flow tests. The signal antibody may be of an IgG nature and is conjugated to metals such as gold or silver, magnetic particles, enzymes or latex beads in order to generate a signal (Mdluli *et al*., 2014). In order to detect the target antigen, antibodies produced against target antigens from *M. tuberculosis* should be coated on the nitrocellulose strip of the rapid test. The target antigen would need to be present in one of the body fluids of the infected person (Mukundan *et al*. 2012).

MAbs have been used to diagnose intestinal TB using immunohistochemical (IHC) techniques. Intestinal TB is difficult to diagnose and differentiate from Crohn's Disease, with false negative results obtained from current diagnostic tests, Ziehl-Neelsen staining and tissue culture. MAbs against diagnostic biomarkers such as 38 kDa in intestinal tissue, illustrated the advantage of IHC techniques in the diagnosis of TB. Results from a study by Ihama *et al*. (2012) showed specificity

of MAbs against the 38 kDa biomarker and this correlated well with the presence of tuberculous granuloma (Ihama *et al*., 2012).

Leng *et al* (2014) used Hybridoma technology to establish a stable hybridoma cell line that produced MAbs against ESAT6. The murine anti-ESAT6 MAbs were used to coat ELISA plates for TB antigen detection from sputum culture supernatant and pleural effusion. Results produced a 92.4% sensitivity and 100% specificity in all TB cases and this indicated that anti-ESAT6 MAb is a potential tool in the diagnosis of TB (Leng *et al*., 2014).

An earlier study demonstrated that mouse MAbs to ESAT6 and CFP10 were used to coat ELISA plates for the diagnosis of pleural TB from pleural culture supernatants and pleural effusion aspirates (Feng *et al*., 2011). The sensitivity and specificity of the ESAT6-specific ELISA was 95.4% and 100% while results for the CFP10-specific ELISA was slightly lower at 81.6% and 92.2%, respectively. These results showed the efficacy of MAbs against ESAT6 for the diagnosis of TB (Feng *et al*., 2011). These studies have thus elucidated the potential of MAbs against ESAT6 in the diagnosis of *M. tuberculosis*.

1.2.3 Research Design

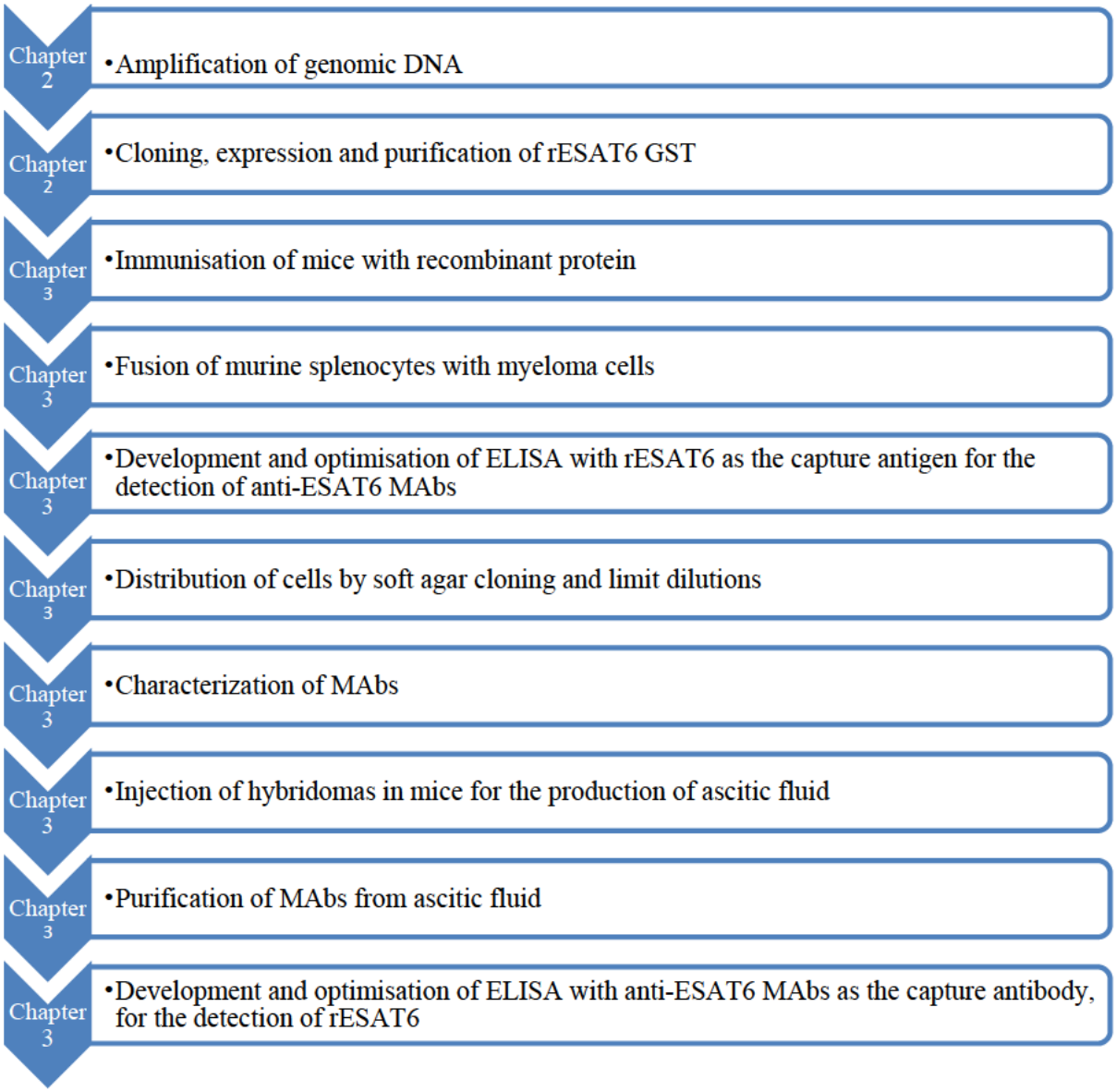

Figure 1.2: Overview of study design

Chapter 2 will describe the amplification of Genomic DNA, cloning of the *esat6* gene into vector, expression of rESAT6 protein in *E. coli* strain XL-1Blue and purification of the protein using affinity chromatography. The production and characterization of anti-ESAT6 MAbs and the development of an ELISA for the detection of rESAT6 will be shown in Chapter 3.

1.2.3.1 Hypothesis

It was hypothesised that recombinant DNA technology may be used to clone, express and purify recombinant ESAT6 from the laboratory strain H37Ra of *M. tuberculosis*.

It was also hypothesised that recombinant ESAT6 may induce an immune response in immunised mice for the production of anti-ESAT6 MAbs.

It was further hypothesised that anti-ESAT6 MAbs may be used to develop and optimise an ELISA for the rapid detection of recombinant ESAT6.

1.2.3.2 Aims

- The aim of the study was to clone and express recombinant ESAT6 for use as an immunogen in the production and characterization of murine MAbs against *M. tuberculosis*-associated ESAT6.
- To use recombinant ESAT6 in the development of an ELISA for the detection and evaluation of anti-ESAT6 MAbs.
- To use anti-ESAT6 MAbs in the development and optimization of an ELISA for the rapid detection of recombinant ESAT6

1.2.3.3 Objectives

In order to achieve these aims, the following objectives were achieved:

- Extraction and amplification of genomic DNA from the laboratory strain H37Ra of *M. tuberculosis*
- Ligation of the amplified DNA into vector and transformation into *E. coli* strain XL-1 Blue cells
- Expression and purification of recombinant ESAT6 GST using Glutathione affinity chromatography
- Immunisation of Balb/C mice with recombinant ESAT6 GST protein
- Fusion of splenocytes from the best immunologically responding mouse with NS0-1, mouse myeloma cells
- Development and optimization of an ELISA for identification of hybridomas produced from the fusion
- 1.2.3.3.7 Distribution of cells by limit dilution and soft agar cloning for the generation of anti-ESAT6 MAbs
- Injection of MAbs into the peritoneal cavity of Balb/C mice for the production of

ascitic fluid

- Purification of MAbs from the ascitic fluid using affinity chromatography Development and optimization of an ELISA, using anti-ESAT6 MAb as coating antibody, for the rapid detection of recombinant ESAT6

CHAPTER 2
THE PRODUCTION OF RECOMBINANT ESAT6 FOR THE DEVELOPMENT AND OPTIMIZATION OF AN ELISA AND FOR THE IMMUNISATION OF BALB/C MICE IN THE GENERATION OF HYBRIDOMAS

2.1. Introduction

Rapid and early detection of *M. tuberculosis* is important in controlling the spread of the bacilli and disease progression (Harrington, 2015). Conventional tests, such as chest X-ray and smear microscopy for acid-fast bacilli and culture based methods have limitations in terms of sensitivity, specificity and the time that is required to produce results. Nucleic acid amplification (NAA) on the other hand is expensive, requiring skilled staff to operate complex equipment. Such assays are therefore unsuitable for routine testing in developing countries. Rapid tests such as Enzyme Linked Immunosorbent Assay (ELISA) and lateral flow tests are simple and economical, with the latter being suitable for use at the point-of-care (POC) (Bekmurzayeva *et al*., 2013). Recombinant DNA technology has therefore been the focus of extensive research, as it was shown that the specificity of recombinant *M. tuberculosis* antigens have potential for the development of rapid tests such as ELISA and lateral flow tests (Leng *et al*., 2014).

Prior to recombinant DNA technology, there were challenges regarding the production of large quantities of mycobacterial proteins (Wang *et* al., 2005). Pathogenic mycobacteria such as *M. tuberculosis* required special facilities for culture. Some mycobacterial organisms such as *M. tuberculosis* grew very slowly whereas other mycobacterial species such as *M. leprae* are unculturable. Besides the problems that were experienced in culturing these organisms, it was difficult to isolate the different antigens from culture as it required using complicated biochemical procedures. Furthermore, it was shown that the use of guanidinium thiocyanate in the cell lysis buffer as opposed to sonication procedures, improved the quality of DNA by reducing the chance of DNA shearing (Boom *et al*., 1990). These are some of the factors that have had an adverse effect on the yield of protein required for tuberculosis (TB) research and has therefore encouraged studies in the area of recombinant DNA technology (Wang *et* al., 2005).

Farshadzadeh *et al*. (2010) successfully demonstrated the use of recombinant DNA technology for the development of a rapid test in the diagnosis of *M. tuberculosis*. The study was aimed at the cloning and expression of Early secretory antigenic target 6 (ESAT6) as this dominant target was able to differentiate between latent and active TB. DNA extracted from *M. tuberculosis* strain H37Ra was polymerase chain reaction (PCR) amplified, inserted into pET102/D vector and transferred into *E. coli* strain TOPO10. Soluble recombinant ESAT6 protein was effectively expressed from *E. coli* strain BL21 and purified by Ni-NTA column. Thus it was demonstrated that ESAT6 is amenable to cloning, expression and purification in *E.coli* cloning systems. It was concluded that recombinant ESAT6 can be useful for the diagnosis of both active and latent TB by ELISA (Farshadzadeh *et al*., 2010).

Recombinant DNA technology used in a recent study by Yindeeyoungyeon *et al*. (2015), was aimed at the development of a more sensitive diagnostic test as the purified protein derivative (PPD) test that is widely used, often produces inaccurate results. Genes encoding MPT64, CFP10 and ESAT6 were therefore cloned, expressed and purified by immobilised metal ion affinity chromatography as it was demonstrated that recombinant antigens, when used in combination, produced a stronger delayed hypersensitivity reaction (DHR) reaction in guinea pigs infected with TB. The results however, showed that purified recombinant proteins did not produce a DHR that was as strong as PPD. However, the lack of the delayed hypersensitivity using the skin test showed that these antigens may be more useful in another diagnostic platform other than the tuberculin skin test (TST) that was investigated and may lead to more accurate diagnoses (Yindeeyoungyeon *et al*. 2015).

A similar study conducted by Malaghini *et al* (2011), used recombinant DNA technology to improve results produced by PPD. Genes encoding ESAT6, CFP10, TB10.3, TB10.4, MTSP11, MPT70 and MPT83, were successfully cloned and expressed. These seven recombinant proteins, when used in combination, showed a higher intra-dermo reaction in *Cavia porcellus* than the standard PPD test (Malaghini *et al*., 2011).

Wu *et al.* (2008) showed that as little as 1.0 µg of recombinant ESAT6 elicited a positive skin reaction in both animal and human test subjects. This demonstrated that recombinant ESAT6 was more specific to *M. tuberculosis* infection than TST, as individuals and guinea pigs vaccinated with BCG and *Mycobacteria* that did not contain the ESAT6 gene showed no skin responses. Data obtained from the study by Wu *et al* (2008) supported the use of recombinant ESAT6 as a

skin test in the diagnosis of TB. It had been reported that ESAT6, together with CFP10, can be found in more than 70% of people infected with TB (Wu *et al*., 2008).

In addition to its diagnostic value, recombinant DNA technology has successfully been used in vaccine development. BCG, the vaccine that is currently available for human use, is attenuated from *Mycobacterium bovis*. However, its use is controversial because of its efficacy in childhood TB with insufficient protection against adult pulmonary disease (Lu *et al*., 2012). With efforts in place to develop a new vaccine several antigens have been evaluated, one of them being ESAT6 against *M. tuberculosis*. Lu *et al*. (2012) designed a study aimed at the development of a vaccine that offered greater immunogenicity by incorporating cell mediated immunodominant antigens ESAT6, antigen 85B (Ag85B) and Rv2608. The study showed that the design of a recombinant BCG strain secreting Ag85B-ESAT6-Rv2608 produced a more efficient immune response in C57BL/6 strain of mice (Lu *et al*., 2012).

Studies investigating the development of diagnostic tests to vaccine production, have based their investigations on the application of recombinant DNA technology. Zhang *et al*. (2010) used recombinant DNA technology to produce recombinant *Mycobacterium smegmatis* expressing fusion protein ESAT6-CFP10 that offered protection to mice challenged with *M. tuberculosis*. The reduced numbers of *M. tuberculosis* colony forming units in mice immunised with recombinant *M. smegmatis*, indicated the potential of recombinant fusion protein ESAT6-CFP10 in vaccine production (Zhang *et al*., 2010).

Research into developing new assays, requires the production of reliable and accurate diagnostic reagents and specific and effective recombinant TB antigens for the identification of *M. tuberculosis*. This chapter therefore described the identification, isolation and characterization of recombinant ESAT6 from *M. tuberculosis*. DNA was extracted from *M. tuberculosis* H37Ra and PCR was performed using gene-specific oligonucleotide primers. The PCR products were inserted into pGEM-T cloning vector and transferred into *E. coli* strain XL-1 Blue. The recombinant plasmids ligated into expression vector pGEX6P-1 were transferred into *E. coli* strain XL-1 Blue and effectively expressed. The expressed fusion protein recombinant ESAT6 GST (32 kDa on sodium dodecyl sulphate polyacrylamide gel electrophoresis; SDS-PAGE) found in the soluble form, was purified on a glutathione sepharose column (Appendix B: B11). Recombinant ESAT6 was used to develop and optimise an ELISA for the identification of anti-ESAT6 MAbs and to immunise Balb/C mice for the generation of hybridomas.

2.2. Materials and methods

2.2.1 Extraction of genomic DNA from *M. tuberculosis*

Genomic DNA was obtained from H37Ra cultures of *M. tuberculosis* grown on Lowenstein Jenson culture media (Appendix A1) using the chaotropic property of guanidinium thiocyanate (GuSCN), as described by Boom *et al* (1990). The starting TB isolate was obtained from Ampath Laboratories. Single bacterial colonies were picked using a sterile loop and transferred to each of two eppendorf tubes containing 500 µl of 0.05M Phosphate – 0.15 M NaCl buffer (PBS), pH 7.4. The tubes were incubated for 30 seconds in an 80°C water bath, and then centrifuged at 15000 x g for 5 minutes and the supernatant discarded. The samples were washed with Guanidinium thiocyanate (GuSCN) containing lysis buffer (Reagents L1 and L2 described in Appendix A1) and 20 µl of silica. The nucleic acid that was released by the bacteria bound to the silica carrier. Complexes that were formed were washed twice with GuSCN containing wash buffer, twice with 70% ethanol and once with acetone. After the silica had dried at 56°C for 10 minutes, the DNA was eluted from the silica with the addition of 50 µl of water to each of the samples. The samples were incubated for 10 minutes at 56°C. The supernatant containing DNA was quantified by ultraviolet spectrophotometry (UV) at OD_{260} nm and stored at -20°C for PCR amplification (Boom *et al*., 1990).

2.2.2 PCR using *esat6*-specific oligonucleotide primers

The ESAT6 DNA sequence is located in the RD1 region and is encoded in the Rv3875 locus of the EsxA gene. The DNA fragment encoding the ESAT6 consists of 288 nucleotides (Gene Bank Gene I.D**.** 886209). Table 1.3 shows the sequence of *esat6*-specific oligonucleotide primers that were designed and synthesised on the Beckman Oligo 1000M DNA Synthesiser at The Department of Molecular and Cellular Biology, University of Cape Town.

Table 1.3: *esat6*-specific forward and reverse primer oligonucleotide sequence

Name of primer	5'-3' sequence
Forward primer	5' tat aaa gga tcc atg aca gag cag cag tgg aat ttc
Reverse primer	5' cta gag gaa ttc cta tgc gaa cat ccc agt gac gtt gcc

The PCR mixtures for each of samples 1 and 2 contained 100 ng/µl genomic DNA, 10 pmol/µl forward and reverse primers, $MgCl_2$ (1.5 mM), 10 X PCR buffer, 0.1 mM dNTP mix and 0.5 µl

DNA polymerase. The PCR cycling procedure was performed on the thermocycler Perkin Elmer GeneAmp 96000 System using a method modified from Sambrook *et al.* (1989).

Table 1.4 describes the PCR conditions optimised for the amplification of ESAT6 DNA.

Table 1.4: PCR conditions and reaction mixtures used for the amplification of ESAT6 DNA

Temperature	Time	Comment
95°C	10 min	Fast start- Denaturation temperature
95°C	10 sec	
50°C	10 sec	45 cycles – Annealing temperature
72°C	20 sec	
72°C	10 min	Extension temperature- 2 holds
4°C	Infinity	

2.2.3 Extraction of PCR product from agarose gel for ligation into pGEM-T vector system

PCR products were electrophoresed on a 1% Sigma Type 11 agarose gel (with 10 µl ethidium bromide; Sigma Aldrich) in Tris Acetate EDTA electrophoresis buffer (Appendix A2.1), alongside a 1 kB Plus DNA ladder (Invitrogen). The gel was stained using ethidium bromide and analysed by UV transillumination. The desired band was excised and purified by Qiagen's PCR product DNA extraction kit (according to manufacturer's instruction) and the concentration spectrophotometrically analysed. ESAT6 DNA (from sample 2) with band size of about 288 base pairs was ligated into pGEM-T cloning vector system (Promega) using T4 DNA ligase. The ligation reaction was incubated overnight at 4°C and the reaction was heated at 70-72°C for 10 minutes to denature ligase. Samples were stored at -20°C for use in the transformation reaction.

T-vector ligation reactions were run using 10 µl and 20 µl volumes with the molar ratio of the positive control to pGEMT vector at 1:1 and the molar ratio of ESAT6 to pGEMT vector at 1:3. pGEMT vector was used at a concentration of 50 ng/µl and insert DNA concentration was calculated according to the formula below. Dilutions were made in RNAse free water at an optimised ratio of 1:4.

$$\text{ng insert} = \frac{\text{ng of vector X kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio (insert: vector)}$$

ESAT6: T-vector was used as a 1:1 molar ratio (Promega pGEM-T kit instructions). The ligation mixture contained the final concentration of 2x ligation buffer, 10 ng/µl T-vector, 10 ng/µl insert DNA, 1 µl (400 000 U/ml) T4 DNA ligase in a final volume of 10 µl. The ligation was performed on the Perkin Elmer Geneamp 96000 System overnight at 4°C. The ligase was denatured by heating the reaction at 71°C for 10 minutes (Sambrook *et al.,* 1989).

2.2.4 Transformation of recombinant plasmid into *E. coli* strain XL-1 Blue

Luria-bertani (LB) agar plates were prepared with the addition of ampicillin at 50 mg/ml, isopropyl β-D-thiogalactopyranoside (IPTG) 0.5 mM and 5-bromo-4 chloro-3-indolyl-β-D-galactosidase (X-gal) at 80 µg/ml. LB media containing 5 µl of tetracycline at 5 mg/ml was inoculated with 80 µl of *E. coli* XL-1 Blue cells and cultured overnight by rocking at 37°C (Appendix A11; Sambrook *et al*., 1989). This indicated that steady state growth ceased at OD_{600} 0.3. The 16 hour incubation at 37°C was followed by a 1/500 dilution of the overnight culture of *E. coli* XL-1 Blue cells in 20 ml of LB media that was left to grow with vigorous shaking at 37°C to early log phase OD_{600} 0.3 – 0.6.

The *E. coli* culture was pelleted by centrifugation at 1600 x g for 30 minutes at 4°C and resuspended in cold bacterial transformation buffer (TSB) on ice for 10 minutes (Appendix A3). Ampicillin sensitive *E. coli* XL-1 Blue cells were made competent by the addition of DMSO in the TSB. Addition of the ligation mixture thereafter, enabled transformation of the ampicillin resistant, T-vector/ESAT6 constructs into *E. coli* XL-1 Blue cells. The mixture was incubated on ice for 30 minutes (Chung *et al*., 1989).

A 900 µl volume of TSB was added to all tubes and incubated at 37°C for 1 hour. The transformation mixture was plated onto LB agar and incubated at 37°C, overnight, to allow the growth of *E. coli* XL-1 Blue colonies (Appendix A3; Sambrook *et al.,* 1989).

2.2.5 The isolation of plasmid DNA from bacterial DNA

Transformants were cultured in 5 ml of LB media incubated overnight on a rocker at 37°C. The protocol of the High Pure Plasmid Isolation kit by Roche was followed, to recover the purified plasmid DNA from the bacterial DNA. The plasmid DNA was eluted into a small volume of low salt buffer and placed on ice (Appendix A4). Glycerol stocks of the individual colonies were prepared by adding 665 µl of the overnight cultures to 335 µl of 80% glycerol (Sigma Aldrich) (Sambrook *et al.,* 1989).

2.2.6 ESAT6 DNA sequencing following pGEM-T vector cloning

Purified DNA from T-vector cloning was sent for DNA sequencing to The Department of Molecular and Cell Biology, at the University of Cape Town. The results were compared to the GI 90811459 sequence available on the NCBI website (www.ncbi.nlm.nih.gov). The sequence was analysed using the BLAST alignment available as part of the above database and the BioEdit software for sequence alignment was used to confirm the presence of the ESAT6 DNA.

2.2.7 Ligation of plasmid DNA into pGEX6P-1 expression vector

Electrophoresis of purified plasmid DNA from T-vector cloning was performed on a 1% agarose gel, alongside a 10 kb ladder (Invitrogen). Qiagen's silica membrane assembly for the binding of DNA in a high salted buffer, with elution using a low salt buffer, was used to extract the DNA from the agarose gel (according to manufacturer's instructions). The plasmid DNA extracted from the excised agarose, was quantified spectrophotometrically at OD_{260} and digested with endonuclease *EcoR1* and *BamH1*, for 2 hours at 37°C. The ESAT6 gene sequence was ligated into the *BamH1* and *EcoR1* sites of GST 5kb expression vector pGEX6P-1 (Appendix A13; GE Healthcare) for the controlled expression of ESAT6 by IPTG induction and transformation into *E. coli* strain XL-1 Blue. The concentration of insert DNA at molar ratios 1:1 (insert: vector), was calculated as follows:

$$\text{ng insert} = \frac{\text{ng vector} \times \text{kilobase (kb) size of insert}}{\text{kilobase size of vector}} \times \text{molar ratio } \left({}^{\text{insert}}/_{\text{vector}}\right)$$

$$= \frac{100 \times 0.288 \times 1}{5}$$

$$= 5.76 \text{ ng for molar ratio 1:1.}$$

The concentration of the linear expression vector pGEX6P-1, for the ligation reaction was calculated as 28.75 ng/µl and 3 units/µl ligase (Promega) was used. The ligation reaction mixture was incubated at 4°C overnight followed by denaturation of the ligase at 70°C for 10 minutes. Competent *E. coli* XL-1 Blue cells were transformed with pGEX6P-1 / insert using the DMSO method of transformation.

Ten Lysogeny broth plates were prepared with the addition of 500 µl of 50 mg/ml of ampicillin. An overnight culture of *E. coli* strain XL-1 Blue was prepared with the addition of 5 µl of tetracycline at 5 mg/ml and 80 µl of *E. coli* strain XL-1 Blue to 5 ml of LB media. Volumes of

300 – 500 µl of the transformation reactions at molar ratios 1:1, 3:1 and 5:1 were plated onto LB agar containing ampicillin. From the colonies that grew following overnight incubation at 37°C, 24 colonies were picked and cultured overnight in 5 ml of LB media containing 5 µl of ampicillin at 50 mg/ml and 5 µl of tetracycline at 5 mg/ml (Appendix A14). The Roche High Pure Plasmid Isolation kit was used to recover the purified plasmid DNA from the bacterial DNA. The plasmid DNA was eluted into a small volume of low salt buffer and spectrophotometrically quantified. Glycerol stocks were prepared and stored at -80°C (Sambrook *et al.,* 1989).

2.2.8 The Transformation of pGEX6P-1 into competent *E. coli* strain XL-1 Blue

A culture of *E. coli* strain XL-1 Blue cells was prepared in 5 ml of LB media containing tetracycline at 5 mg/ml. The culture was placed on a rocker, overnight at 37°C. LB media was used to prepare transformation plates with ampicillin at 50 mg/ml. The overnight culture was diluted 1/50, in 20 ml of LB media and grown to an early log phase (OD_{600} 0.3 – 0.6) with vigorous shaking at 37°C. The *E. coli* strain XL-1 Blue cells were made competent with DMSO for the transformation of pGEX6P-1/ESAT6 constructs into *E. coli* strain XL-1 Blue. The mixture was incubated on ice for 30 minutes (Chung *et al*., 1989). TSB, 900 µl, was added to all tubes and incubated at 37°C for 1 hour. The transformation mixture was plated onto LB agar and incubated at 37°C, overnight, for growth of *E. coli* strain XL-1 Blue colonies. Colonies were cultured overnight at 37°C in LB media with ampicillin at 50 mg/ml (Sambrook *et al.,* 1989).

2.2.9 ESAT6 GST protein expression and purification by affinity chromatography

Two hundred microliters overnight culture of *E. coli* strain XL-1 Blue cells containing the recombinant plasmid pGEX6P-1 was inoculated into 100 ml LB media with ampicillin (50 mg/ml). The cells were grown overnight at 37°C with shaking. The overnight culture was diluted 1:10 in sterile LB media with ampicillin at 50 mg/ml and cultured at 37°C with shaking (Appendix A12). Protein expression was induced with 1mM IPTG at 21°C, with shaking. The bacterial cells were harvested by centrifugation at 1400 x g for 30 minutes and the pellet treated with 100 mg/ml lysozyme and the solution was slowly stirred on a magnetic stirrer in the cold room for 30 minutes to soften the bacterial cell wall. The bacterial suspension was treated with 1/100 v/v phenylmethylsulphonyl fluoride (PMSF). The cells were placed on ice and sonicated and the lysate cleared by ultracentrifugation at 19000 x g for 30 minutes at 4 - 10°C. The pooled, cleared lysate was passed through a 1.2 µ filter and then a 0.45 µ Millipore filter.

Recombinant ESAT6 was purified from the cell-free supernatant by Glutathione Sepharose affinity chromatography on a Glutathione Sepharose column (Appendix A5), within the binding

capacity of the column i.e. 5-10 mg of glutathione S transferase per ml of resin. The column was rinsed with Tris saline pH 8.0 buffer containing 0.02% sodium azide (Appendix A4). The peristaltic pump speed was set on 100 and the fraction collector on 150 drops per fraction. The filtered lysate was loaded onto the column and the fractions that were collected were read at OD_{280}. When all the lysate had been loaded on the column, the column was washed with Tris saline on pump setting 150 until OD_{280} fell below 0.03.

The bound protein was eluted from the column with Tris saline containing 10 mM reduced glutathione (pump setting 125) and the collected fractions were analysed spectrophotometrically at a wavelength of 280 nm. When the protein peak had eluted and the spectrophotometric reading fell below OD_{280} 0.03, the column was washed with Tris saline. The fractions with the highest OD_{280} readings, containing recESAT6 were pooled and spectrophotometric analysis was used to calculate the concentration of the purified protein at an extinction co-efficient of 1.

$$Protein\ yield = concentration\left(\frac{mg}{ml}\right) \times volume(ml\ after\ dialysis)$$
$$= protein/culture(mg)$$

The protein was then dialysed against 2 L Tris saline pH 8.0 at 4°C overnight. This buffer aided in the removal of free glutathione and smaller molecular weight contaminants in the sample. The molecular weight cut off of the dialysis tubing used was 3,500.

2.2.10 ESAT6 protein identification by SDS-PAGE

The Laemmli method (Laemmli, 1970) of SDS-PAGE was used to determine the molecular mass of expressed recombinant protein ESAT6 (Appendix A15). A 15% gel was prepared and samples were loaded in wells alongside a protein ladder SeeBlue Plus 2 (Invitrogen) pre stained standard 4-250 kDa, used for sharply resolved bands. The gel was run from negative to positive at 20 mA, until the front of the dye had reached the end of the running gel.

When electrophoresis was complete the gel stack was carefully dismantled and the gel was removed for staining. The gel was rinsed in distilled water for 5 seconds and submerged in Coomassie Blue, overnight (Appendix A: A15). The next day, the gel was destained in destaining solution to enable visualization of the bands. For the first 1.5 hours the destaining solution was changed at half hour intervals and thereafter after every few hours for the rest of the day until the Coomasssie Blue had destained (Sambrook *et al.,* 1989).

2.2.11 Enzymatic cleavage of GST using PreScission Protease

Removal of the GST tag from ESAT6 was necessary to avoid the large GST enzyme (220 amino acid residues), from masking the 6 kDa ESAT6 antigen when used as a coating antigen in an ELISA, for the detection of antibodies specific to recombinant ESAT6. Precision Protease optimised for low temperature cleavage, was selected. Protease cleavage was carried out according to the manufacturer's instructions (GE Healthcare; Appendix A16). ESAT6 GST was extensively dialysed in cleavage buffer for 48 hours at 4°C to remove the free glutathione before being treated with protease (Appendix A7). The spectrophotometric concentration of the protein following dialysis in cleavage buffer was calculated at 218 µg/ml. To a 20 ml sample volume, 43.6 µl of protease was added and the sample was placed on a rocker at 4°C for 5 hours. Once the digestion was complete, the sample was applied to a washed and equilibrated Glutathione Sepharose column to remove the GST moiety of the fusion protein and the precision protease from the protein of interest. The unbound cleaved ESAT6 protein was collected and dialysed for 48 hours in TRIS saline at pH 8.0 with 0.02% NaN3 (Appendix A7; GE Healthcare Precision Protease package insert).

2.3. Results

2.3.1 PCR amplification of *esat6*

The silica method of DNA extraction yielded 65.9 µg of genomic DNA from the H37Ra strain of *M. tuberculosis*. Amplification of the DNA using specific forward and reverse primers for *esat6* to produce a 288 base pair fragment. The PCR product was electrophoresed on a 1% agarose gel alongside a 1 kb DNA ladder (Figure 2.1).

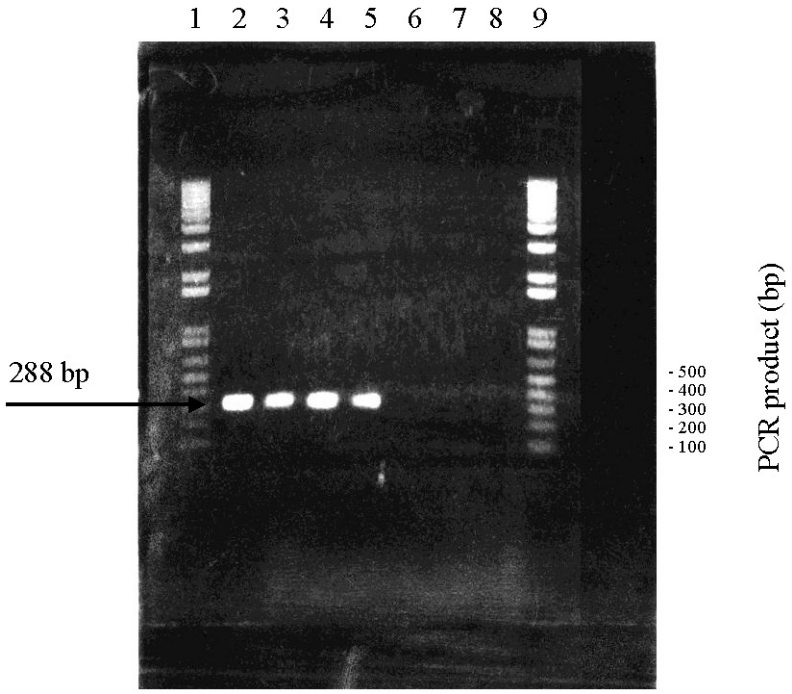

Figure 2.1: Agarose gel electrophoresis confirming the *esat6* gene amplification by PCR. Lanes 2 to 5 showed 288 base pair band corresponding to the ESAT6 gene. The 1 kb DNA ladder is represented in lanes 1 and 9.

PCR reaction mix containing no genomic DNA was used as a negative control and was loaded in wells from lanes 6 to 8. As expected, no bands were visualised (Figure 2.1).

2.3.2 Ligation of ESAT6 into pGEM-T vector

ESAT6 PCR product was successfully cloned into pGEM-T vector and was transformed into *E. coli* XL-1 Blue strain for the propagation of the plasmid. The *E. coli* strain XL-1 Blue cells made competent by the DMSO method, improved the transformation efficiency of the cells. Recombinant plasmid (ESAT6/T-vector) were isolated and electrophoresed on a 1% agarose gel alongside a 10 kb DNA ladder. The molecular weight of pGEM-T vector was 3 kb and the weight of the insert DNA was 288 base pairs. These bands were separated by gel electrophoresis on a 1% agarose gel, shown in Figure 2.2.

The negative transformation plate produced no colonies while 12 transformants grew on the positive transformation plate and this indicated that there was no cross contamination during the transformation process. ESAT6/T-vector constructs, at molar ratios 1:1, 1:3 and 1:5, produced several transformants, of which 24 were selected for DNA extraction. Plasmid DNA was extracted

from the bacterial cells using the Roche High Pure Plasmid Isolation kit. The DNA concentrations of each of the 24 transformants were spectrophotometrically quantified.

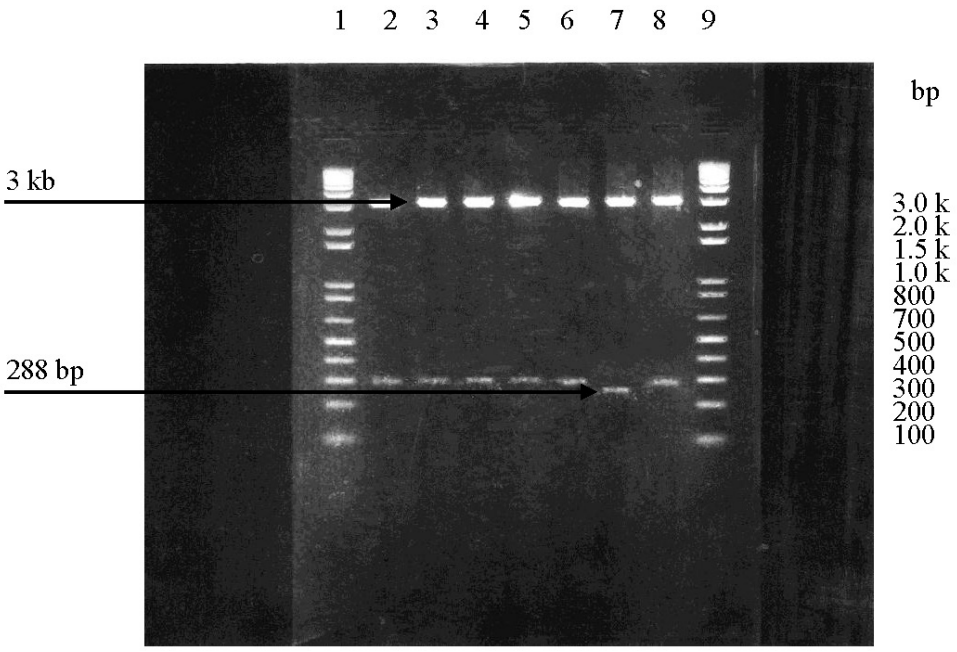

Figure 2.2: Agarose gel electrophoresis confirming insertion of ESAT6 DNA into pGEM-T vector. Lanes 1 and 9 represented the 10kb DNA ladder. Lane 7 showed the ESAT6 288 base pair band on a 1% gel. The 3 kb band in lanes 2 to 8 showed the presence of pGEM-T vector.

Restriction digests were done using *BamH1* and *EcoR1* restriction enzymes (Appendix A: A13) and the samples were electrophoresed. The concentration of the 288 base pair insert extracted from Lane 7 (Figure 2.2) from the gel measured OD_{260} = 1.16µg/µl. The DNA sequencing was done at Inqaba Biotech and verified using the M13 forward primer (GTAAA ACGAC GGCCA GT). Sequencing results showed that the insert corresponded to the 288 base pairs encoding ESAT6. The sequence obtained from Inqaba Biotech was searched for using NCBI blast software. Protein sequencing confirmed ESAT6 sequence in FASTA format (Appendix A19).

Gene Bank Accession number BX842584
M. tuberculosis H37Ra / RV3875 / *esxA* (gene name)
MTEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQGVQQK
WDATATELNNALQNNLARTISEAGQAMASTEGNVTGMFA

2.3.3 Construction of expression vector pGEX6P-1 for transformation of *E. coli* strain XL-1 Blue

Plasmid DNA was extracted from the bacterial cells using the Roche High Pure Plasmid Isolation kit. The DNA concentration of the 24 transformants (ESAT6/pGEX6P-1 constructs) that were selected was listed in Appendix A14. Of these, 14 samples were selected based on DNA concentration and gel electrophoresed (Figure 2.3).

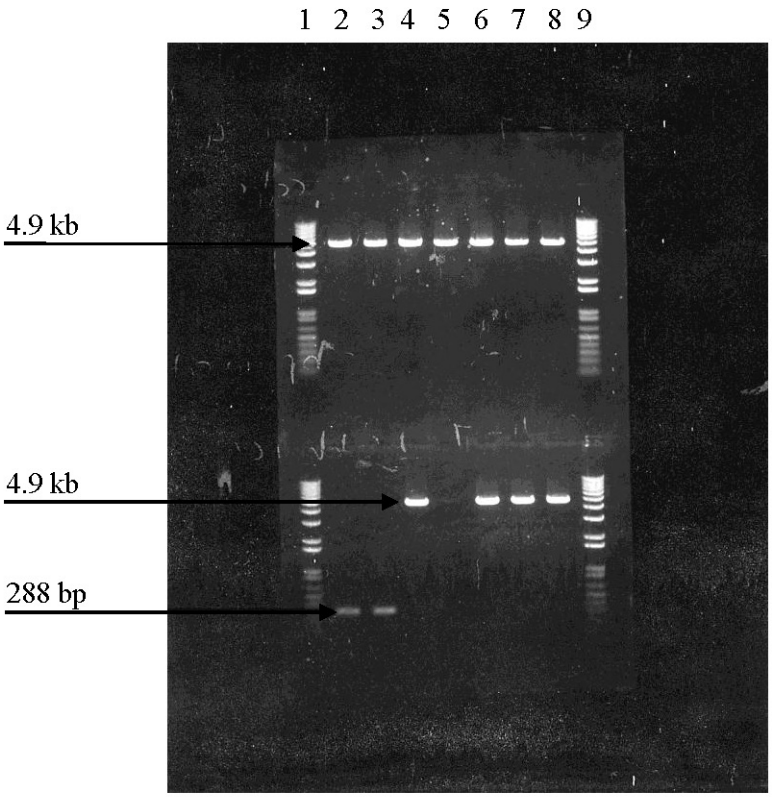

Figure 2.3: Agarose gel electrophoresis confirming insertion of ESAT6 DNA into plasmid pGEX6P-1. The 4.9 kb bands indicated in lanes 2 to 8 (top row) and lines 4, 6, 7 and 8 (bottom row), represented the pGEX6P-1 vector. Lanes 2 and 3 (bottom row) showed the 288 base pair ESAT6. A clear difference in electrophoretic mobility between pGEX6P-1 and ESAT6 on a 1% agarose gel was evident. Bands shown in lanes 1 and 9 (top and bottom rows) represented the 10 kb DNA ladder.

The 288 base pair clone shown in lane 11 (Figure 2.3), was sent for sequencing analysis to Inqaba Biotech and the result was confirmed in Gene Bank of NCBI BLAST. The sequence matched

completely with the ESAT6 sequence (Appendix A20; Gene Bank Accession number BX842584).

2.3.4 ESAT6 GST Protein expression and purification by affinity chromatography

The predicted molecular size of ESAT6 is 6 kDa. SDS-PAGE analysis showed that the ESAT6 protein was highly expressed in *E. coli* strain XL-1 Blue with the predicted molecular size after IPTG induction. ESAT6 GST was expressed in a soluble form and was purified easily on a Glutathione Sepharose column (Figure 2.4).

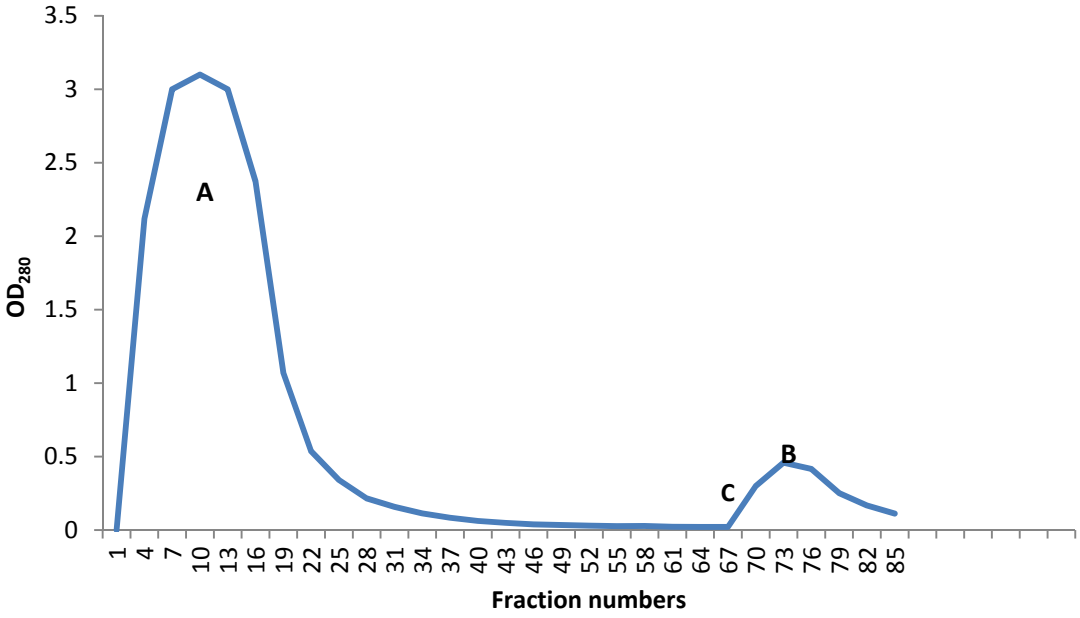

Figure 2.4: Affinity chromatography results illustrating the elution of ESAT6 GST from Glutathione Sepharose Affinity Column. Peak A (fractions 1-64), represented unbound material that was collected when the bacterial lysate was passed through the column. Peak B (fractions 69 – 78) that contained ESAT6 GST was eluted from the column with the addition of buffer containing 10 mM reduced Glutathione (indicated as point C on the graph).

Fractions measuring 4.5 ml each was collected with peristaltic pump speed set on 150 and the fraction collector on 150 drops per fraction. The OD_{280} of Peak B was 0.218. The 45 ml volume collected from Peak B yielded a concentration of 9.81 mg of ESAT6 GST (Figure 2.4).

2.3.5 Confirmation of ESAT6 GST protein expression on SDS-PAGE

Expression of ESAT6 GST in *E. coli* strain XL-1 Blue strain was performed using pGEX6P-1 expression vector. IPTG was used to induce protein expression.

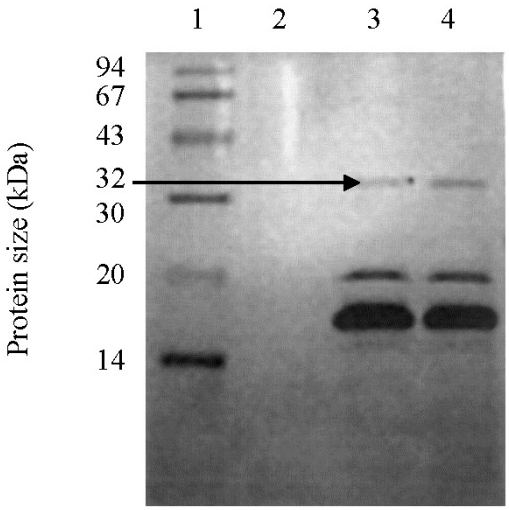

Figure 2.5: SDS PAGE confirming expression of recombinant ESAT6 GST protein in *E. coli* strain XL-1 Blue. SDS PAGE protein molecular weight standard was shown in lane 1. Protein bands visualised by Coomassie Blue staining indicate a 32 kDa band in lanes 3 and 4.

ESAT6 GST was expressed at 32 kDa on SDS-PAGE (Figure 2.5). The molecular weight of GST was 26 kDa and ESAT6 was 6 kDa. Therefore, a fusion protein band with an estimated molecular weight of 32 kDa was expected (Figure 2.5). SDS-PAGE analysis indicated that recombinant ESAT6 GST was expressed as a soluble fraction (Figure 2.5) and was purified with Glutathione Sepharose affinity chromatography. The GST tag on ESAT6 enabled the protein to bind to the resin in the column. The tagged protein was eluted by buffer containing 10 mM reduced Glutathione (Figure 2.4).

2.3.6 Enzymatic cleavage of GST from recombinant ESAT6 GST using Precission protease

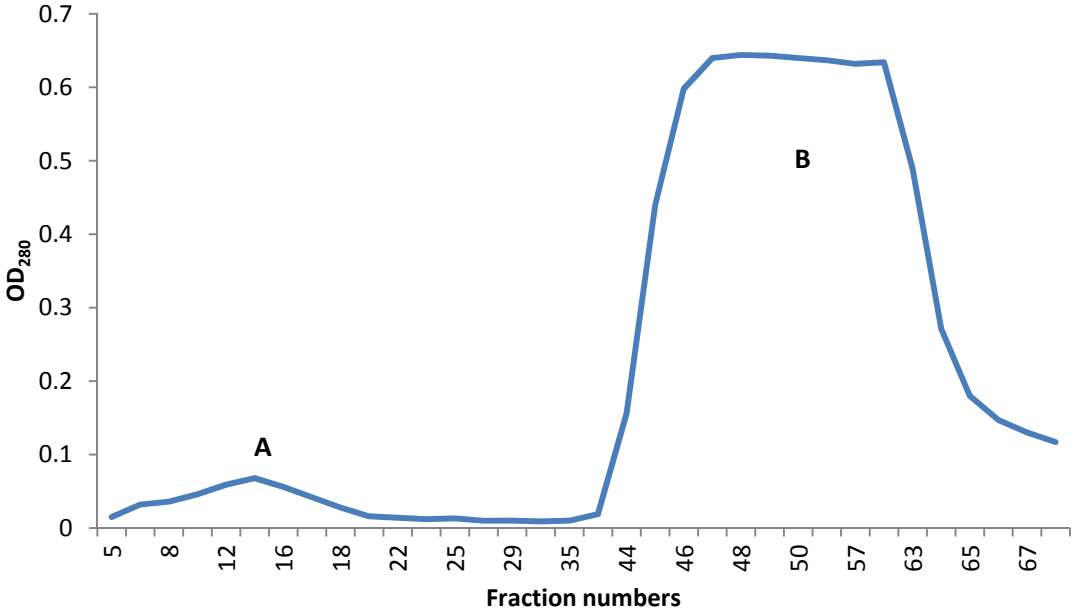

Figure 2.6: Glutathione Sepharose affinity Chromatography performed for the separation of ESAT6 cleaved from GST. Fractions 6-19 (peak A), contained ESAT6 0.044 mg/ml at OD_{280}. Peak B represented the GST eluted fractions using 50 mM Tris buffer containing 10 mM reduced glutathione pH 8.0.

Each fraction measured 4.5 ml with a flow rate of 150 drops at a speed of 150rpm. ESAT6 represented by fractions collected in Peak A (Figure 2.6), was concentrated in PEG 6000 (Appendix A17) to a final concentration of 0.434 mg/ml at OD_{280}. A 15% SDS-PAGE electrophoresis was carried out to resolve the bands from the concentrated protein and was used to determine its molecular weight (Figure 2.7).

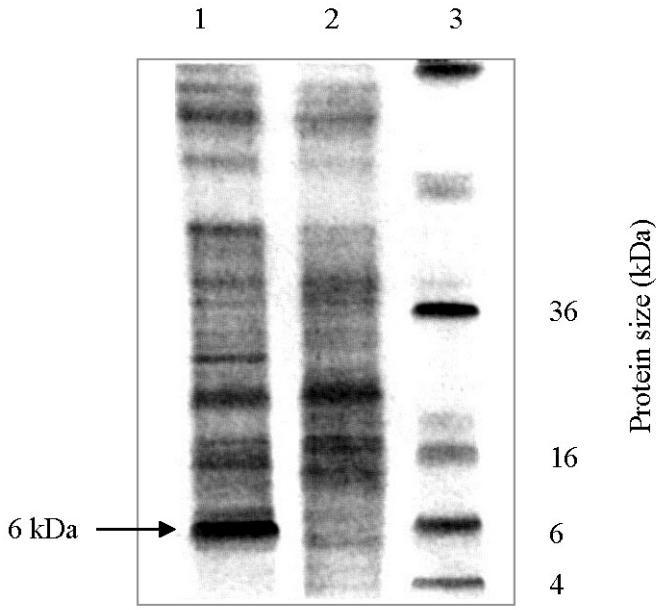

Figure 2.7: SDS PAGE indicating the expression of ESAT6 protein in E. *coli* strain XL-1 Blue. Lane 3 indicated the protein molecular weight standard. Lane 1 represented the IPTG-induced cell lysate expressing 6 kDa ESAT6 protein and Lane 2 showed the uninduced cell lysate.

2.3.7 Upscale of ESAT6 GST protein expression and purification

The peristaltic pump speed was set on 150 and the fraction collector on 150 drops per fraction for the Glutathione Sepharose chromatography of ESAT6 GST (Figure 2.8).

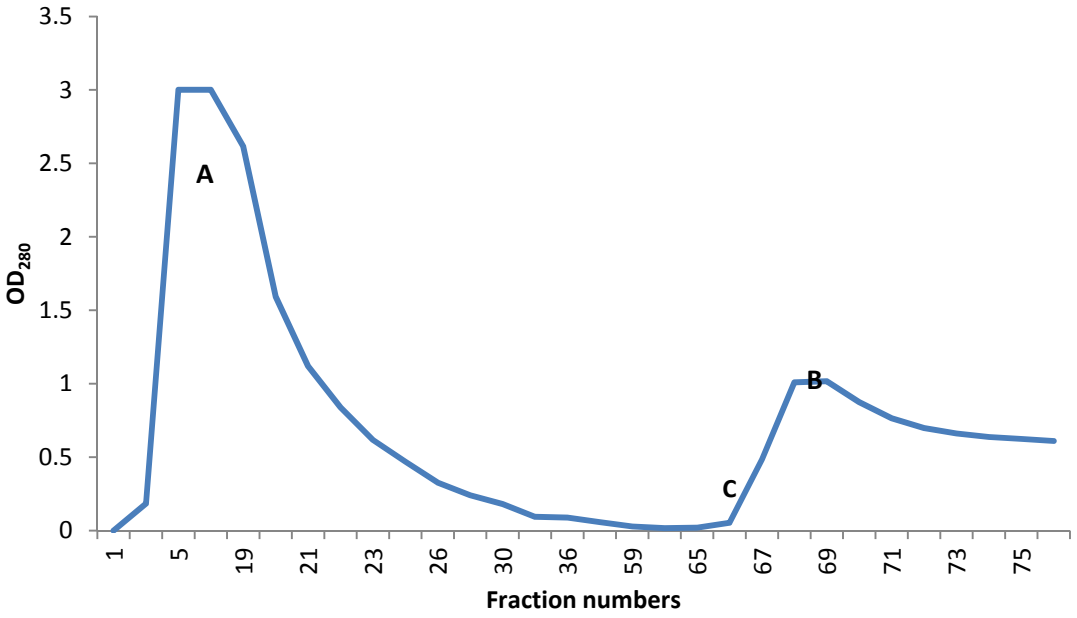

Figure 2.8: Glutathione sepharose affinity chromatography indicating elution of ESAT6 GST following the addition of 10mM glutathione reduced. Peak A (fractions 1 - 66) consisted of fractions that contained bacterial lysate without ESAT6 GST. Peak B (fractions 67 – 72) contained ESAT6 GST eluted from the column by the addition of 10mM reduced Glutathione buffer (at point C on the graph).

Each fraction measured 4.5 ml with a flow rate of 150 drops at a speed of 150rpm. From the 35 ml volume of filtered bacterial lysate that was passed through the chromatography column, fractions 67 to 72 yielded a concentration of 0.8 mg/ml of ESAT6 GST (Figure 2.8).

2.4. Discussion

It has been reported that secreted proteins of *M. tuberculosis*, are a rich source of immunogens (Brodin *et al*., 2004). These proteins have gained attention as virulence factors and are strong biomarker candidates for both vaccine and diagnostic tests (Mahmoudi *et al*., 2013). Furthermore, ESAT6 proteins have been recognised in over 70% of TB patients and can therefore be considered for use as a diagnostic reagent for *M. tuberculosis*. Therefore, the aims of the present study were to clone and express recombinant ESAT6 for use as an immunogen in the production of murine

MAbs against ESAT6 and to further use the recombinant protein in the development and optimization of an ELISA for the identification and characterization of the anti-ESAT6 MAbs.

In this study, genomic DNA from the H37Ra strain of *M. tuberculosis* was extracted using the silica method of DNA extraction and PCR amplified using ESAT6-specific primers with genomic DNA as a template. Studies have shown that the *PhoP* transcription factor is similar in both H37Ra and H37Rv. *PhoP* is responsible for the attenuation of H37Ra that involves the secretion of ESAT6 and is also responsible for the expression of genes involved in the biosynthesis of complex wall lipids (Malen *et al*. 2011). Results from using the H37Ra strain of TB from the present study correlated well with a similar study by Gao *et al*. (2015).

Farshadzadeh *et al*. (2010) used a commercial kit (Cinnagen, Iran) for the extraction of genomic DNA and his study together with those of Mahmoudi *et al*. (2013) and Gao *et al*. (2015), demonstrated that genomic DNA, PCR amplified under sets of different PCR conditions, successfully produced fragments 288 nucleotides in length (Farshadzadeh *et al*., 2010; Mahmoudi *et al*., 2013; Gao *et al*., 2015). Results from agarose gel electrophoresis in the present study, has confirmed the presence of the 288 base pair ESAT6 DNA. Futhermore, the protein sequencing results demonstrate that the recombinant ESAT6 protein was homologous to the amino acid sequence of H37Rv.

Results from the present study relates to the literature as it demonstrated the successful ligation of PCR product into cloning vector pGEM-T after purification using the Qiagen PCR product DNA extraction kit. Electrophoresis of the digested recombinant plasmid confirmed that the plasmids contained the ESAT6 DNA and sequencing results showed that the correct sequences of ESAT6 DNA were inserted into the cloning vector (Appendix A19). Literature has shown that electrophoresis of restriction digested recombinant plasmids contained ESAT6 when pTZ57R /T was used as the cloning vector and results were confirmed by sequencing (Mahmoudi *et al*., 2013). PCR product was ligated into pET102/DTOPO as cloning vector in a study by Farshadzadeh *et al*. (2010) and again electrophoresis showed the presence of ESAT6 DNA (Farshadzadeh *et al*., 2010). Results from the present study in relation to findings from previous studies demonstrates that there are no conflicting results and that several vectors that are available may be used for the successful cloning of ESAT6.

Expression of recombinant ESAT6 protein in *E. coli* strain XL-1 Blue, in the present study, was achieved with pGEX6P-1 expression vector. ESAT6 protein expression was induced with IPTG

and expressed at about 32 kDa, in soluble form, on polyacrylamide SDS-PAGE. This result was expected as the protein consists of 6 kDa ESAT6 and the 26 kDa GST tag. It has been shown that these results have related favourably to several studies in the construction of expression vector and the production of ESAT6 protein in the soluble fraction of the expression host. Previously, pET32a-ESAT6 plasmids were transformed into *E. coli* strain BL21 DE3. IPTG-induced protein expression was analysed by SDS-PAGE analysis and recombinant ESAT6 protein was found to be present in a soluble form (Mahmoudi *et al*., 2013). Similarly, Farshadzadeh *et al*. (2010) transformed pET102/D-TOPO-ESAT6 into *E. coli* strain BL21 DE3 and ESAT6 protein was expressed in a soluble form (Farshadzadeh *et al*., 2010).

Other expression vectors and prokaryotic expression systems, such as pQE30 and *E. coli* strain M15 have been successfully used in the cloning and expression of recombinant ESAT6 protein (Behr *et al*., 1999; Wang *et al*., 2005; Meher *et al*., 2006; Tebianian *et al*., 2009; Sarhan *et al*., 2011; Lu *et al*., 2012 and Dong *et al*., 2013). However, some systems have described the expression of ESAT6 as an insoluble protein requiring denaturation and protein refolding (Renshaw *et al*., 2002; Wang *et al*., 2005; Mukherjee *et al*., 2007).

The current project showed that recombinant ESAT6 GST protein was easily detected during protein purification, that the protein was expressed in a soluble form and easily purified from the cellular components of *E. coli* by glutathione sepharose affinity chromatography. The GST tagged to recombinant ESAT6 protein, facilitated one-step affinity purification as resins specifically bind these tags. Proteins tagged with 6xHistidine, on the other hand, are recovered from metal ion affinity chromatography using either Ni^{2+} or Co^{2+} -loaded nitrilotriacetic acid-agarose resins (Rosano and Ceccarelli. 2014).

Several studies have described the purification of 6xHis tagged-ESAT6 using Ni^{2+}-NTA agarose chromatography (Meher *et al*., 2006; Farshadzadeh *et al*., 2010; Mahmoudi *et al*., 2013). Although the results from the present study using the GST tag, meet expectations, more work seems to have been performed using His tagged-ESAT6 protein (Kimple *et al*., 2013). The reason for this may be that most companies provide expression vectors and expression and purification reagents for use with polyhistidine tags. More importantly, the small size and charge of polyhistidine tags rarely affect the function of the tagged protein while the mild eluent preserves the immunogenicity of the protein. Although a purity of >80% may be achieved using polyhistidine, insect and mammalian cells that have a higher percentage of histidine residues in their proteins than *E. coli*, result in higher background binding. Stringent wash conditions required

to reduce this problem, may result in the elution and loss of the protein of interest (Kimple *et al*., 2013).

Glutathione resin, although favorable, may only be regenerated for up to twenty times (Kimple *et al*., 2013). Mild 10mM glutathione used to elute protein from the resin, preserves protein function and antigenicity. Another advantage is that the 26 kDa GST may be easily cleaved from the protein of interest. Often, GST fusion proteins are expressed at high levels in *E. coli*. This may lead to the expression of the desired protein in inclusion bodies. This was however, not the case in the present study, as recombinant ESAT6 GST was expressed in a soluble form (Kimple *et al*., 2013).

The present work demonstrated the development and optimization of an ELISA with recombinant ESAT6 as the coating antigen, for use in the detection of anti-ESAT6 MAbs. Literature has cited several studies that have used recombinant ESAT6 in an ELISA for the diagnosis of TB. Talaat *et al*. (2010) used recombinant ESAT6 in an ELISA for the rapid serological diagnosis of TB. Results from the study yielded 97.6% sensitivity and 75% specificity for the diagnosis of *M. tuberculosis* infection. The study concluded that the low sensitivity and specificity of the ELISA may be overcome by using the assay in combination with radiological or microscopic analysis for negative results (Talaat *et al*., 2010).

Humoral immune responses against a combination of TB antigens were analysed, to improve test sensitivity in the diagnosis of smear negative and culture negative patients with active TB (Wu *et al*., 2010). The recombinant based ELISA showed that the sensitivity and specificity for the detection of anti-ESAT6/Cfp 10 antibodies were 60.4% and 73.8% respectively. Data from the study indicated that immune responses to TB antigens is heterogeneous. Therefore, a combination of antigens for culture or smear negative patients is required to improve both the sensitivity and specificity of the serodiagnostic ELISA (Wu *et al*., 2010).

Studies by both Talaat *et al*. (2010) and Wu *et al*. (2010) demonstrated the use of recombinant ESAT6 as a coating antigen in an ELISA for the detection of humoral immune responses in the diagnosis of TB. However, the present study did not use this approach in the diagnosis of TB. The aim of the present study was achieved with the use of ELISA plates coated with recombinant ESAT6 for the identification and characterization of hybridoma clones produced against recombinant ESAT6. MAbs produced against recombinant ESAT6 were then used as a coating antibody in an ELISA for the detection of ESAT6 with potential for use in the diagnosis of active

and latent TB. An ELISA that is used to test for antibodies against TB has been shown to produce results with low sensitivity and specificity as the humoral immune response is dampened in HIV patients co-infected with TB (Velayudhan *et al*., 2011).

Results from studies by Feng *et al*. (2011) and Leng *et al*. (2014) has supported the present study (Feng *et al*., 2011; Leng *et al*., 2014). It was shown that an anti-ESAT6-based ELISA, used for the detection of ESAT6 and diagnosis of *M. tuberculosis* produced a sensitivity of 92.4% and specificity of 100% (Leng *et al*., 2014).

There had been no unexpected findings in the cloning, expression and purification of recombinant ESAT6 from this study, as methods have been well documented in the literature (Behr *et al*., 1999; Wang *et al*., 2005; Meher *et al*., 2006; Tebianian *et al*., 2009; Farshadzadeh *et al*., 2010; Sarhan *et al*., 2011; Lu *et al*., 2012 and Dong *et al*., 2013).

Although the GST gene fusion system did not affect the validity of the findings in this study, it has potential limitations. Evidence indicates that GST fusion proteins are expressed at high levels in *E. coli* and this may lead to the formation of inclusion bodies. This would require additional steps to produce soluble protein, hence an increase in time. Cost is also increased for the cleavage of the 26 kDa GST using protease.

A recommendation for future work would be to clone, express and purify a combination of antigens that has the potential to increase the sensitivity of a diagnostic assay (Wu *et al*., 2010; Zhang *et al*., 2015).

In addition to the diagnostic potential of recombinant ESAT6, this protein in combination with other recombinant proteins may be used in the development of subunit vaccines. Experiments using animal models suggest that protective immunity may be accomplished following vaccination with purified antigens. This would overcome the existing problems of variable efficacy and dissemination of TB in immunocompromised patients experienced with the current BCG vaccine (Horwitz *et al*., 1995).

A study by Lu *et al*. (2012) describes the development of a new recombinant BCG vaccine co-expressing Ag85B-ESAT6-Rv2608. This approach involved the genetic modification of the BCG vaccine to improve the immunogenicity of the vaccine. The recombinant vaccine included some of the previously deleted genes and increased the expression of the immune-dominant genes of

TB. The advantage of this multi-genic vaccine was that it generated a broader immune response by targeting several TB antigens. Humoral responses and cellular immune responses were compared in a group of mice that were immunised with BCG (Danish strain) against those mice immunised with recombinant BCG (Ag85B-ESAT6-Rv2608).

Results demonstrated that mice immunised with the recombinant vaccine produced a higher antibody response than those immunised with BCG (Lu *et al*., 2012). It was also illustrated that there was a greater CD8 cell proliferation than CD4 cell proliferation in the recombinant BCG (rBCG) vaccinated group. CD8 cells are responsible for the lysis and clearing of infected TB cells and the CD4 cells activate macrophages. It was shown that immunogenicity in the rBCG group was stronger. It was further demonstrated that the rBCG elicited a stronger Th1 immune response characterised by strong cell mediated immunity and IgG2b antibody response than the BCG group. This experiment demonstrated that the rBCG (Ag85B-ESAT6-Rv2608) has the potential to replace the current BCG vaccine (Lu *et al*., 2012).

A challenge faced by researchers in accomplishing the SDG by the year 2030, is delayed diagnosis and treatment of TB, resulting in disease dissemination and decrease in the survival rate of patients (WHO, 2015). An approach in overcoming this challenge is advanced diagnostic techniques, focused on rapid results for the early detection of the disease. This study has therefore produced recombinant ESAT6 for the production of anti-ESAT6 MAbs that may be useful in an ELISA for rapid and early detection of TB. The need for a rapid and effective diagnostic test for countries with low to middle income groups is evident and much of the research that has been done to date focuses on virulence factors and diagnostic candidates such as ESAT6 (Mahmoudi *et al*., 2013).

The objectives in this study were successfully demonstrated with cloning, expression and purification of recombinant ESAT6 protein. Recombinant ESAT6 was applied as a coating antibody for the development and optimization of an ELISA. The specificity of the recombinant protein in the detection of commercial anti-ESAT6 antibody (ABCAM) showed that the ELISA developed in this study would successfully detect anti-ESAT6 MAbs with potential for use in the diagnosis of TB. Recombinant ESAT6 was also used for the immunisation of mice in the production of MAbs against ESAT6 using hybridoma technology (Kohler and Milstein, 1975).

Results from this study compared well with previous studies in the development of a reproducible, robust and cost-effective method of producing large quantities of recombinant ESAT6 protein as a reagent, with potential for use in the diagnosis of TB.

CHAPTER 3
THE PRODUCTION AND PURIFICATION OF MURINE MABS AGAINST RECOMBINANT ESAT6 FROM *M. TUBERCULOSIS*

3.1. Introduction

Tuberculosis (TB) remains of global concern with about a third of the world's population being infected by a disease exacerbated by drug resistance (WHO, 2015). It is difficult to diagnose the disease and it is estimated that 2 billion people live with LTBI that may potentially develop into active TB (Walzl *et al*., 2011). It is therefore critical to diagnose LTBI in order to administer preventative treatment that would control development of active disease and transmission and hence reduce the incidence of the disease (Harrington, 2015).

Farshadzadeh *et al.* (2010) cloned and expressed ESAT6 protein of *M. tuberculosis* in *Escherichia coli* as results from previous studies have indicated that RD1 gene based diagnosis was more accurate than TST in countries with low prevalence of TB (Dinnes *et al*., 2007). PPD produced a high positive rate while some active TB cases tested negative using this technique. Cloned and expressed ESAT6 from this study detected latent TB with more sensitivity and specificity than TST (Dinnes *et al*., 2007; Masood *et al*., 2014).

It is necessary to place greater emphasis on the development of a rapid and simple diagnostic test that may be used at a community level for both the active and latent forms of the disease (Leng *et al*., 2014). The selection of biomarkers are critical in the development of a dynamic diagnostic test. Zhang *et al*. (2015) demonstrated that a single TB biomarker generated a false positive rate of 30-40% when used in a diagnostic test, but a combination of TB markers improved the positive diagnostic rate. Furthermore, HIV co-infection results in loss of immune control with the immunosuppression or destruction of T and B cells making the diagnosis of TB by targeting antibodies, an unfavourable approach (Harrington, 2015). It was therefore shown that the production of monoclonal antibodies targeting significant TB antigens, offered a plausible approach toward TB diagnostics (Leng *et al*., 2014).

Anti-ESAT6 MAbs have demonstrated its efficacy in the IFNγ assay, immunohistochemistry, biosensing, DHR and the immunochromatographic assay. The suggestion MAbs may be regarded

as a powerful tool for the laboratory diagnosis of TB, formed the basis of the present study in the production and purification of murine MAbs against recombinant ESAT6 (Leng *et al*., 2014).

At present, the application of MAbs has expanded from diagnostics to disease prevention and treatment. Lanari *et al*. (2013), evaluated the use of Palivizumab, monoclonal antibodies toward Respiratory Syncytial virus (RSV), for the prevention of the nosocomial infection among infants. Results from the study indicated that Palivizumab was effective in reducing RSV related mortality and morbidity amongst infants. Evidence from this study supported the use of monoclonal antibodies toward the prevention and treatment of other infectious diseases (Lanari *et al*., 2013).

This chapter deals specifically with the production of *M. tuberculosis* anti-ESAT6 MAbs, using Hybridoma Technology to be used in a diagnostic ELISA. Success of the study objectives provides evidence that MAbs against other diagnostic TB antigens may be produced using this technology. These MAbs, when used in combination, have the potential of developing a much sought after, effective community based diagnostic test (Zhang *et al*., 2015).

3.2. Materials and Methods

3.2.1 Ethics Clearance

This study was carried out in strict accordance with the recommendations laid out in the University of Kwa-Zulu Natal Animal Ethics Guideline Application. The protocol for this work was approved by the Animal Ethics Research Committee (Appendix B1: Reference 077/11/Animal and Reference 029/12/Animal).

3.2.2 Immunisation of Balb/C mice with recombinant ESAT-GST

Balb/C mice were obtained from the National Bioproducts Institute animal facility based at the University of Kwa-Zulu Natal's (UKZN) Westville Campus. Ten female mice, 12 weeks old, were tail-bled and 200 µl sample was collected from each of the mice, into paediatric heparinised blood collection tubes (Lasec). These samples were referred to as pre-bleeds and were used as controls prior to immunisation of these mice. The immunisation schedule employed was adapted from the method described in Harlow and Lane (1988). Recombinant ESAT6 GST at 100 µg/ml was prepared in 0.9% physiological saline, pH 7.0. Five hundred microliters Complete Fruends Adjuvant (CFA) (Sigma) and 500 µl ESAT6 GSTrecombinant antigen were homogenised for primary immunisation of ten Balb/C mice. The mice were immunised with the 32 kDa ESAT6

GST protein in order to elicit a stronger immunological response. The injection was administered to the mice via the intraperitoneal route.

The same set of mice were immunised a month later, with Incomplete Freund's adjuvant (IFA; Sigma). A month after enhanced immunisation, 200 µl of blood was drawn from an incision in the tail vein of the pre-warmed mice. This sample was referred to as the test-bleed. The pre-bleed and test-bleed samples were screened by ELISA in order to determine which of the 10 mice had the most potent response to immunisation with the ESAT6 antigen. This was calculated by dividing the test-bleed sample value by the pre-bleed sample value read at a dual wavelength of $OD_{490/620}$. The mouse with the highest antibody titre was administered with a further immunisation (booster dose) with antigen at 100 µg/ml, prepared in 0.9% physiological saline, pH 7.0. The booster dose comprised homogenised FCA and ESAT6 GST, 100 µl, administered three days prior to the fusion procedure. The selected mouse was asphyxiated with CO_2 gas and the spleen removed for the next stage, the hybridoma fusion.

3.2.3 Conjugation of GST to HRP for the detection of anti-GST by ELISA

In parallel to the mouse immunisation schedule, recombinant GST with a molecular weight of 26 kDa was expressed in a pGEX vector (Sigma). GST was conjugated to enzyme-label HRP for use as a secondary antibody in an ELISA for the detection of antibodies to GST in an ELISA, using a modified Wilson and Nakane (1978) method. HRP was conjugated to GST at a molar ratio of 2:1. GST at a concentration of 6.78 mg/ml was dialysed overnight at 4°C in 0.2 M carbonate buffer at pH 9.5 (Appendix A8). Four milligram HRP (TypeV1, Sigma) was dissolved in 50 µl Milli-Q water. Fifty microliters of a 0.2 M sodium periodate ($NaIO_4$; Sigma Aldrich) solution was added rapidly to HRP. The mixture was oxidised for 20 min at room temperature. Excess $NaIO_4$ was removed from the activated mixture by desalting on a column of Sephadex G25 (GE Healthcare) equilibrated in 1 mM sodium acetate buffer, pH 4.0. The low pH of sodium acetate charged the amino groups of HRP thereby preventing them from reacting with the oxidised carbohydrate moieties of the protein. The fractions containing the activated HRP were collected and added immediately to GST at a molar ratio of 2:1. The mixture was incubated for 4 hrs at room temperature before an equal volume of stabilizing buffer, 50% glycerol in 0.05 M Tris-0.1 M NaCl buffer pH 8.0 was added. The GST HRP conjugate was stored at 4°C until further use.

3.2.4 Development and optimization of an ELISA for the detection of anti-GST antibodies

Using a method modified from Harlow and Lane (1988), a direct ELISA was developed and optimised for the detection and exclusion of antibodies produced to the GST portion of the ESAT6 GST immunogen. Recombinant GST was expressed in a pGEX vector (GE Healthcare Life Sciences). GST at a concentration of 6.78 mg/ml was serially diluted in a carbonate coating buffer at pH 9.5 in order to obtain a concentration range from 100 µg/ml to 0.78 µg/ml. One hundred microliters of each of the serial dilutions were immobilised on a 96-well flat bottomed micro-titre plate (Greiner). Each serial dilution was pipetted into wells A to H, of a single column and incubated overnight at room temperature. The plate was washed in TST buffer, pH 8.0 containing 0.05% Tween 20.

3.2.5 ELISA Development and optimization for identification of anti-ESAT6 MAbs

Using a protocol modified from Harlow and Lane (1988), recombinant ESAT6 was serially diluted in a 0.2 M carbonate coating buffer at pH 9.6, from 100 µg/ml to 0.78 µg/ml (Appendix A8). The eight dilutions were immobilised onto a column of eight wells each, on the 96 well microtiter plate and the plate was incubated overnight at room temperature. The plate was then washed in Tris Saline Tween buffer containing 0.15M sodium chloride, 0.05% Tween 20 and 0.005 M tris aminomethane, pH 8.0 (TST; Appendix A9). A 1:500 and 1:1000 dilution of mouse anti-ESAT6 IgG monoclonal antibody (ABCAM) was prepared using bosine (sheep and bovine serum) as a diluent (Appendix A18) and 50 µl, dispensed into the wells of the 96 well plate, for each of the antigen dilutions. Fifty microliters of bosine was dispensed in a separate well as a negative control. The plate was incubated for 30 minutes in a humidified chamber at 45°C, followed by a wash with TST. Sigma Goat anti-mouse IgG *Fab* horse radish peroxidase (HRP) conjugate was diluted in bosine at 1:3000 and dispensed into all the wells of the microtiter plate. The plate was incubated at 45°C for 30 minutes in a humidified chamber. After the last TST wash, colour was developed by the addition of 50 µl of chromogenic substrate to each well and the plate was incubated in the dark, at room temperature, for 30 minutes (Appendix B7). At the end of the incubation period, the reaction was stopped by the addition of one hundred microliters of sulphuric acid to all wells and the optical densities (OD) were measured using a dual wavelength of 490 and 620nm (490/620) with an automatic ELISA plate reader.

The positive / negative ratios were calculated by dividing the $OD_{490/620}$ value of the positive serum by the average $OD_{490/620}$ value of the negative bosine samples. The positive (anti-ESAT6 ABCAM) to negative (bosine) ratio was calculated at both the 1:500 and 1:1000 dilution of the

ABCAM anti-ESAT6 antibody, against each antigen dilution (100 µg/ml to 0.78 µg/ml). The results were plotted on a graph representing recombinant ESAT6 GST concentration (µg/ml) on the X-axis and the positive negative ratio on the Y-axis. The positive to negative ratios for both the 1:500 and 1:1000 dilutions were represented on the graph in order to extrapolate optimal parameters in the development of an ELISA for the detection of antibodies to ESAT6 (Modified method from Harlow and Lane, 1988).

3.2.6 ELISA performed for the detection of anti-ESAT6 and anti-GST serum antibodies from Balb/C mice

Blood samples taken from the 10 mice prior to immunisation (pre-bleeds) and following immunisation with antigen and enhanced immunisation a month later (test-bleeds), were screened by ELISA in order to select the mouse exhibiting the most potent immune response to ESAT6, for the fusion. The pre-bleed and test-bleed serum samples were diluted at a 1:10 v/v and a 1:20 v/v in conjugate diluent (CD; Appendix A21). The samples were tested by ELISA developed and optimised as described previously (Chapter 2, Section 2.3.8; Chapter 3, Section 3.2.3). The ELISA plates were coated with recombinant ESAT6 GST at 10 µg/ml and GST at 12.5 µg/ml. Fifty microliters of diluted serum samples were incubated on each of the ELISA plates for 30 min at 45°C in a humidified chamber. CD was used as a negative control. Anti ESAT6 IgG antibody (ABCAM) diluted 1:500 v/v and anti GST DE6 cells (diluted at 1:500 v/v in CD) were used as positive controls on the ESAT6 GST and GST plates respectively. After incubation, the plates were washed with TST. Sigma goat anti mouse IgG HRP (50 µl) and GST HRP (50 µl) at a 1:3000 v/v dilution in conjugate dilution, was added to the ESAT6 GST plate and the GST ELISA plate, respectively. The plates were incubated at 45°C for 30 min in a humidified chamber. The plates were washed with TST prior to incubation with 50 µl chromogenic substrate per well, for 30 min in the dark at room temperature. The criteria used for selection of the mouse to be sacrificed for the fusion was that the test-bleed produced a result of $OD_{490/620}$ = <0.5 on the GST ELISA plate and the strongest signal at $OD_{490/620}$ = >0.5 on the ESAT6 ELISA plate. The positive / negative ratio was calculated by dividing the test-bleed result by the pre-bleed result from each of the ELISA plates. The final selection of the fusion mouse was based on the mouse that produced the highest P/N ratio on the ESAT6 coated plate and at the same time produced a P/N ratio closest to zero on the GST coated plate.

3.2.7 Fusion of murine splenocytes to NS0-1 mouse myeloma cells using PEG

A 2% working stock of 8-azaguanine was prepared in Roswell Park Memorial Institute medium (RPMI- Sigma) containing 5% fetal bovine serum (FBS - Lonza), 10% horse serum (HS - Lonza) and 1% penicillin-streptomycin-amphotericin B (10 000 units/ml potassium penicillin, 10 000 µg/ml streptomycin sulphate, 25 µg/ml amphotericin B; Lonza).

A week prior to the fusion, the NSO-1 mouse myeloma cells that were required, were passaged in cell culture media containing 2% 8-azagaunine in order to render the HGPRT gene in the NSO-1 cells, non-functional and to ensure that the myeloma cells are sensitive to drug selection. In order for hypoxanthine aminopterin thymidine (HAT) drug selective media to achieve hybridoma selection, there had to be a mutation in the HGPRT gene to prevent the production of purines for DNA synthesis. A known inhibitor of purine nucleotide synthesis is 8-azaguanine, an analogue of guanine (Kornberg and Baker. 1992).

The fusion partner in this experiment was NS0-1 Balb/C mouse myeloma cell line obtained from the American Type Culture Collection (ATCC; Galfre and Milstein, 1981). One hundred millilitres of NS0-1 at a concentration of 1.2 x 10^5 cells/ml was cultured for the fusion. A sample of the NS0-1 was diluted 1:10 v/v by adding 50 µl of NS0-1 cell suspension into 250 µl of RPMI and 200 µl of Trypan Blue to perform a cell count.

In the step that followed, the spleen from the mouse that ESAT6 immunisation had induced the most potent antibody response, as identified by ELISA (Section 3.2.4), was selected for the fusion. The mouse was asphyxiated using CO_2 gas and immediately transported to the lab for the fusion procedure. The mouse was sterilised by being completely submerged in a beaker containing 70% ethanol for 1 minute. The excess alcohol was drained from the mouse and it was placed on its back on absorbent paper on the dissecting bench positioned immediately in front of the laminar flow unit. Using a pair of sterile scissors and forceps, a lateral incision of the skin was made from between the hind legs towards the head, thereby exposing the abdomen. A second pair of sterile scissors and forceps was used and the spleen was aseptically removed and placed in a small petri dish containing 5 ml of RPMI warmed to 37°C. The RPMI used throughout the procedure was maintained at 37°C in a water bath. The petri dish was then transferred within the laminar flow unit for processing.

The spleen was rinsed in warm RPMI and much of the connective tissue and fat was removed using the 21 gauge needles. The spleen was then transferred to a second small petri dish

containing 5 ml of warm RPMI where the bent needles were used to gently tease the splenocytes. Whilst one of the bent 21 gauge needles was used to secure the spleen against the bottom of the petri dish, the second bent needle was used to gently manipulate the spleen sac and create a tear that allowed the splenocytes to empty into the RPMI media. One needle kept the spleen sac in position while the second needle gently stroked the sac from end to end until it was empty. The empty spleen sac was discarded and the cells pipetted into a 15 ml centrifuge tube. The petri dish was rinsed with another 5 ml of RPMI to ensure that all the splenocytes were harvested (Harlow and Lane, 1988).

The 15 ml tube was left to stand in a vertical position to allow for the larger pieces of tissue to settle. The fluid from the 15 ml tube was pipetted into a fresh 15 ml tube, and the pieces of tissue that was left behind was discarded. The suspension of splenocytes in RPMI was centrifuged at 110 g for 5 min at room temperature, the supernatant discarded and the pellet was re-suspended in 10 ml of RPMI. NH_4Cl (0.85%) was prepared by dissolving 0.085 gram of NH_4Cl in 100 ml of sterile distilled water. The NH_4Cl was used to lyse red blood cells from the sample of the splenocyte suspension that was used to perform a cell count. The splenocyte count required, a 1:20 dilution of the splenocytes prepared by the addition of 750 µl of NH_4Cl and 200 µl of Trypan Blue to a 50 µl sample of splenocytes (Protocol adapted from Harlow and Lane, 1988).

Based on the cell concentrations, the splenocytes were fused to NS0-1 at a ratio of 5:1. All of the splenocytes were used and the concentration of the NS0-1 was adjusted to a 5^{th} of the splenocyte cell concentration (cells/ml). The cells were washed in 40 ml RPMI by centrifugation at 110 g for 5 min at room temperature. The supernatant was discarded and the end of the 50 ml tube was gently tapped in order to loosen the pellet for maximum exposure of the cells to the fusogen, polyethylene glycol (PEG), a critical step that enabled fusion of cell membranes. Three grams of PEG Serva 1550 (Roth, Germany) was autoclaved at 121°C for 15 min and 4.2 ml of RPMI at 37°C was added. The 2 ml PEG was added drop wise to the cell pellet, slow enough to keep the cells from blasting. The cells were incubated with PEG at 37°C for 1 min. Extreme care was taken not to exceed total exposure of the cells to PEG by more than a period of 4 min. During this process, PEG facilitated fusion of membranes of the freshly harvested spleen cells to the myeloma cells and this resulted in the immortalisation of the splenocyte-myeloma hybrids (Kohler and Milstein, 1975).

RPMI not exceeding 40 ml, was added drop wise to the cells in order to neutralise the PEG. The mixture was incubated for 20 min in a 37°C water bath. The cells were centrifuged for 8 min at

75 x g and the supernatant discarded. Hypoxanthine aminopterin thymidine 2% (HAT - Sigma) fusion media was prepared by adding 25 ml FBS, 50 ml HS, 4.5 ml PSF and 10 ml HAT, made up to a total of 500 ml in RPMI. The cell pellet was re-suspended in HAT media and the suspension incubated for between 1-2 hrs in a 37°C water bath. Two hundred and thirty microliters of cell suspension was pipetted into each well of eighteen, 96-well tissue culture plates. The plates were labelled alphabetically and incubated in a humidified, 5% CO_2 incubator for seven days. To ensure the sensitivity of the NS0-1 cells to HAT media, duplicate control wells were set up on the day of the fusion. The first set of NS0-1 cultures were fed with RP5/10 media (RPMI with 5% FBS and 10% HS) whilst the second set of NS0-1 control cultures were fed with HAT media. The growth of the control wells were monitored over a three day period.

A week after the fusion, the 96-well tissue culture plates were microscopically screened for hybrid cell growth. Larger clones that required a change in media were fed with 100 µl of HAT media. The fusion plates were left to incubate for a further 7 days at 37°C in a 5% CO_2 humidified incubator, to enable the clones to grow and produce detectable levels of antibody in culture fluid (Harlow and Lane, 1988).

3.2.8 Detection of hybridoma clones producing anti-ESAT6 antibodies by ELISA

Supernatant fluid from individual wells were screened using separate ELISA's testing for anti-ESAT6 and anti-GST antibody activity. Recombinant ESAT6 GST plates were incubated with 50 µl cell culture fluid and were incubated for 30 min at 45°C in a humidified chamber. Fifty microliters of anti-ESAT6 IgG (ABCAM) was used as a positive control and 50 µl bosine was used as a negative control sample. Following a wash with TST, 50µl Sigma goat anti-mouse HRP, diluted at a 1:3000 v/v was added to all wells and incubated at 45°C for 30 min in a humidified chamber. Following a wash step with TST, 50 µl chromogenic substrate was added and the plates were incubated at room temperature for 30 min, in the dark. One hundred microliters of 2 M H_2SO_4 was used to stop the assay and the results were evaluated at $OD_{490/620}$. Simultaneously, supernatant fluid from all fusion plates were screened on ELISA plates coated with recombinant GST, following the method described above. Anti-GST-DE6 culture fluid was used as the positive control and bosine was used as a negative control. GST-HRP conjugate was diluted at 1:3000 v/v. The assay conditions for the ESAT6 ELISA applied to the GST ELISA as well (Harlow and Lane, 1988).

Cultures of clones that were reactive on both the ESAT6 GST and GST plates were discontinued as it was indicative of the production of anti-GST antibodies. Clones reactive on the ESAT6 GST

plate only were identified as this suggested that antibodies produced by these clones were specific to recombinant ESAT6.

3.2.9 Distribution of hybridoma cells by limiting dilutions

At this step, cloning by limiting dilutions ensured that most of the wells contained at most, a single clone. Cells from individual mother clones were gently re-suspended in the culture plate and 1 ml of cell suspension harvested into a sterile 15 ml centrifuge tube (Cell Star). The number of cells in the 1 ml sample were counted on a Neubauer counting chamber, using Trypan Blue to exclude the dead cells. A 1:100 v/v dilution of the cells was prepared when 40µl of cells was added to 3960µl of RPMI that contained 5% FBS, 10% HS and 1% PSF (RP5/10). The cells were further diluted to produce the final concentration of cells for culture. The final cell concentration was dependant on the growth rate of the mother clone. For instance, a slow growing mother clone was prepared at a concentration of 200 cells per millilitre in a 10 ml volume. A clone that grew well was diluted to prepare a concentration of 50 – 100 cells per ml in a 10 ml volume. The 10 ml cell suspension was further diluted in RP5/10 at 1:2; 1:3; 1:4 and 1:6 v/v and each of these dilutions were pipetted onto 2, 96-well cell culture plates. These cell dilutions increased the probability of achieving a single cell per well. The cell culture plates were incubated at 37°C for 5-7 days in a humidified 5% CO_2 incubator. After a week the plates were microscopically viewed for the presence of clones. Two weeks later, an ELISA was performed to determine whether the clones produced antibodies specific to ESAT6. The clones that were reactive to recESAT6 were limit-diluted for a second time to further increase the probability of achieving monoclonality (Harlow and Lane, 1988).

Cultures of each of the stable hybridoma clones that were produced were expanded to increase cell numbers for injection into 10 female Balb/C mice at a minimum of $1x10^6$ cells per mouse. The injection of the clone into mice was carried ascertain whether the clone was able to induce ascitic fluid production in mice and whether the ascitic fluid that was produced contained antibodies to ESAT6. Cell cultures of the clones that produced anti-ESAT6 monoclonal antibodies in ascitic fluid were stored in liquid nitrogen vapour (LN_2). Ten percent dimethyl sulphoxide (DMSO; Merck) was used as the cryostorage preservative. The preservative contained 10% DMSO and 20% horse serum in RPMI (Harlow and Lane, 1988).

3.2.10 Distribution of hybridoma cells by Soft Agar Cloning

Soft agar cloning was a technique, alternate to limiting dilutions, that was used to establish stable hybridomas. A two percent Noble Purified agar (Difco) was prepared in distilled water

and autoclaved at 121°C for 15 min. The agar was not allowed to cool and immediately transferred to a 45°C water bath. Nutrient agar was prepared by the addition of 55 ml RPMI, 10 ml FBS and 10 ml HS, made up to 75 ml, and incubated at 45°C. A preparation of single strength nutrient agar medium was prepared with the addition of 25 ml of 2% Noble purified agar into 75 ml of nutrient mix. This produced a single strength nutrient agar medium at a concentration of 0.7%. The medium was gently mixed to avoid bubbles from forming and incubated at 45°C. The base nutrient layer was prepared by pipetting 7 ml nutrient agar medium into ten 60mm petri dishes. Once the agar had solidified, the petri dishes were stored in the 37°C incubator.

One millilitre aliquots of single strength nutrient agar were pipetted into each of ten 15 ml centrifuge tubes and incubated at 45°C. The polyclone was serially diluted in RP5/10 (not containing agar), ranging from a concentration of $1x10^6$ to 1x10. Five hundred microliters of each of the cell dilutions ($1x10^6$ to 1x10) was added to each of the 1 ml aliquots of nutrient agar mixture. Each dilution was prepared in duplicate and incubated at 45°C. The tubes were gently swirled to avoid air bubbles from forming and immediately poured over the base nutrient layer in the petri dish. The cell dilutions were plated out in duplicate petri dishes. The temperature of the cell / agar suspension did not fall less than 45°C thereby preventing the formation of clumps when the top layer of agar was poured. Strict temperature control prevented the temperature from rising above 45°C thus protecting the cells from heat shocked and loss of cell viability. The petri dishes were left open in the laminar flow for 15 to 30 minutes. This allowed for the polymerization of the top layer of agar.

The petri dishes were placed in a CO_2 incubator at 37°C for a period of 10 – 15 days to allow for the growth of colonies. Sterile tooth picks were used to transfer the pin-head sized colonies into 100 µl of RPMI on a 96-well cell culture plate. The clones were screened by ELISA for ESAT6 antibody production before being grown for injection in mice (Harlow and Lane, 1988).

3.2.11 Isotype identification of MAbs by ELISA

The subclasses of the MAbs in this study, were identified by isotyping which was carried out in the form of an ELISA. Characterization of the anti-ESAT6 monoclonal antibodies (MAbs) into isotypes was achieved using an Fc specific conjugate directed towards mouse IgG (Sigma) antibodies and mµ chain specific conjugate for mouse IgM (Sigma) antibodies. Both conjugates were produced in goat and diluted 1:3000 v/v with bosine.

ELISA plates were coated with recombinant ESAT6. Hybridoma cell culture fluid, was tested in duplicate, by incubating 50 µl of sample for 30 min at 45°C. Bosine was used as negative control and commercial anti-ESAT6 IgG (ABCAM) was included in the assay as a positive control. After a wash with TST, each sample was incubated with 50 µl Fc specific conjugate (Sigma), specific for mouse IgG antibodies and mµ chain specific conjugate (Sigma) specific for mouse IgM antibodies. The test was incubated for a 30 min at 45°C. After a wash with TST and the addition of 50 µl chromogenic substrate to all the wells, the plates were incubated in the dark for 30 min at room temperature. At the end of the incubation period 100 µl of 2M H_2SO_4 was added and the results were analysed at $OD_{490/620}$ (Harlow and Lane, 1988).

3.2.12 Isotype confirmation by Ouchterloney Double Diffusion

This gel diffusion technique was used to support results obtained from the ELISA described in 3.2.10 in subtyping MAbs produced to ESAT6 from *M. tuberculosis*. Immune precipitation was performed by reacting dilute ascitic fluid produced by mice injected with anti-ESAT6 hybridomas, against the antisera listed below.

Type 11 agarose (1.2%) was made up in 25 ml of PBS. The agar was microwaved and cooled to 55-60°C and 3.5 ml was pipetted onto each of 6 microscope slides. The slides were left at room temperature for about 15 min in order to solidify. The slides were then placed in a sealed container and left at 4°C, overnight, to set. The following day, each slide was inserted into a slide stencil and with the aid of a metal rod two sets of 6 wells each were punched (Harlow and Lane, 1988). Ten microliters of a panel of Sigma antigen subclasses was pipetted into the outer wells.

The panel of antigen subclasses included were as follows:

Well 1: Ascitic fluid
Well 2: Goat anti-mouse IgM Sigma M7019 (mµ chain)
Well 3: Goat anti-mouse IgG1 Sigma M8144 (heavy chain specific)
Well 4: Goat anti-mouse IgG2a Sigma M8269 (heavy chain specific)
Well 5: Goat anti-mouse IgG2b Sigma M8394 (heavy chain specific)
Well 6: Goat anti-mouse IgG3 Sigma M8519 (Heavy chain specific)

Ascitic fluid was diluted at 1:10 and 1:5 v/v in bosine and pipetted into the centre wells in the gel and the reaction was left to incubate in a humidified chamber at room temperature overnight. MAbs were subtyped by interpreting the lines of precipitation using a negatoscope.

3.2.13 Injection of hybridoma cells in mice for ascitices production

The Balb/C strain of mice was used in the production of ascitic fluid. The mice were primed with 0.5 ml of pristane (2,6,10,14-tetramethyldecanoic acid; Sigma) via the intraperitoneal route of injection, one week prior to injection of hybridoma cells into the mouse. Each hybridoma clone was injected into the peritoneum of 30 female Balb/C mice at a concentration of 1 – 2 million cells per millilitre. The ascites developed in the mouse peritoneum over a period of 10 – 14 days. The ascitic fluid was harvested two to three times, per mouse, using a 19 gauge needle. The ascitic fluid was collected into 5 ml ethylenediaminetetraacetic acid (EDTA) tubes. The procedure was carried out by skilled personnel according to strict animal ethics guidelines. The ascitic fluid was centrifuged at 10 000 g for 10 min at 4°C to remove insoluble material and was stored at -20°C until the ascitic fluid purification step (Harlow and Lane, 1988).

3.2.14 Purification of anti-ESAT6 IgG MAb using Prosep protein A affinity chromatography

Affinity chromatography, described as a powerful purification tool, was used to separate the monoclonal antibody from ascitic fluid. In this study, the Prosep protein A chromatography (Separations) was used to purify monoclonal antibodies of the IgG subtype. The protein A ligand immobilised on the chromatography column separated the IgG antibody by its attachment to the ligand. For the purification of the IgM subclass Cibacron Blue affinity chromatography (Separations) was used. Albumin bound to the column and allowed the PEG precipitated sample to flow through.

Ascitic fluid with antibodies to anti-ESAT6 DE2-1 was dialysed in 2 litres of borate buffer pH 8.5 overnight at 4°C (Appendix B2). Prior to antibody purification, the column was primed with five column volumes of borate buffer at pH 8.5, according to the manufacturer's instructions. Flushing agent (Afcon Industrial Equipment), equivalent to one fifth the volume of ascitic fluid, was added to the ascitic fluid and shaken vigorously for 2 to 5 min. The sample was centrifuged at room temperature for 10 min at 17,200 g. The supernatant was collected and centrifugation at 10°C for 10 min at 17,200 g. The clarified sample was loaded onto the column at 4°C. Borate buffer pH 8.5 was passed through the column and the unbound fractions were collected and detected at OD_{280}. When the fraction readings fell <0.1, the antibody was eluted off the column when citrate buffer pH 4.0 was loaded onto the column (Appendix B4). The protein concentration (mg/ml) was calculated from the fractions that were collected. Due to the acidity of the elution buffer, the fractions were dialysed immediately in 2 litres 0.05 M phosphate – 0.15 M NaCl buffer

(PBS; Appendix B3) pH 7.2. Overnight dialysis in PBS at 4°C was repeated. The column was stripped using citrate buffer pH 3.0 and equilibrated with borate buffer pH 8.5 once the fractions read OD_{280} <0.1 (Harlow and Lane. 1988).

3.2.15 Purification of anti-ESAT6 IgM MAbs using Cibacron Blue Affinity chromatography

Flushing agent was added to ascitic fluid in a ratio of 1:5 v/v and the mixture was vigorously shaken for two min. The lipids in the ascitic fluid was dissolved by the flushing agent and phase separated out by centrifugation at 17,200 g for 5 min at room temperature. While avoiding the Freon/lipid sediment, the supernatant was transferred to fresh tubes and centrifuged once more at 17,200 g for 10 min at room temperature. The supernatant was clarified by centrifugation at for 17,200 g 25 min at 10°C. One litre of PBS was de-aerated prior to use in order to prevent air bubbles from forming in the Cibacron Blue chromatography column. Ten percent PEG 6000 (Merck, Germany) was added to the clarified supernatant sample and the sample was vortexed for ten min taking care to avoid foam formation. The sample was incubated at room temperature for 10 min, and then vigorously shaken for 2 min before being centrifuged at 17,200 g for 10 min at room temperature. The supernatant was discarded and one tenth of the starting sample volume of PBS was used to dissolve the pellet. The same volume of PBS was again added and the sample centrifuged at 17,200 g for 10 min at room temperature. The fractionator was set at 150 drops at a speed of 150 at room temperature. The supernatant sample was collected and loaded onto the column after it had been primed with de-aerated PBS. PBS was again passed through the column and fractions containing antibody in PBS was collected and measured at OD_{280}. The column was regenerated using 6 M guanidine HCl (Appendix B5). Purified antibody fractions were pooled and dialysed for 48 hours in PBS at 4°C (Harlow and Lane, 1988).

3.2.16 Fractionation of serum protein by Serum protein electrophoresis to confirm presence of MAbs

Samples were processed according to manufacturer's instructions (Beckman Coulter Paragon). Sample concentrations of less than 8 mg/ml were used neat while sample concentrations of greater than 8 mg/ml were diluted at 1:5 v/v and 1:10 v/v in PBS. Samples were electrophoresed at 100 volts for 25 min before being stained with Paragon Blue stain (Linden and McKenna, 2016).

3.2.17 Conjugation of mouse anti-ESAT6 IgM and IgG to HRP for use as a secondary antibody in an ELISA

Anti-ESAT6 IgG and IgM MAbs were conjugated to HRP for application in an ELISA. This was significant in demonstrating the multi-epitopic nature of recombinant ESAT6. The molecular weights of HRP, an IgM antibody subtype and an IgG antibody subtype are 40 kDa, 100 kDa and 160 kDa respectively. HRP was conjugated to both IgM and IgG subtypes at a ratio of 2:1 w/w, despite the difference in molecular weight. The method described in this section was the same as the method that was employed for the preparation of GST HRP (Section 3.2.2).

Anti-ESAT6 DE2-1 (limit diluted from clone DE2), anti-ESAT6 KE10-1 (produced from soft agar clone KE10) was dialysed separately overnight at 4°C in 0.2 M carbonate buffer at pH 9.5. For each of the antibodies, 500 µl Milli-Q water was added to 2 mg HRP. The fractions containing the activated HRP was collected and added immediately to 4mg antibody. The mixture was incubated for four hrs at room temperature. Stabilizing buffer was made up of 50% glycerol in 0.05 M Tris-0.1 M NaCl pH 8.0. Equal volumes of antibody/HRP solution and stabilising buffer, were mixed and the conjugate was stored for use at 4°C (Wilson and Nakane, 1978).

In a sandwich type assay, anti-ESAT6 DE2-1 was coated on an ELISA plate at 10 µg/ml, followed by a wash step and an incubation with recombinant ESAT6 antigen at 320 µg/ml. After a wash step, the antibody-antigen complex was incubated with secondary antibody, anti-ESAT6 KE10-1 HRP. Anti-ESAT6 KE10-1 HRP was diluted 1:100 v/v in bosine. The plate was incubated, washed and chromogenic substrate was added for the development of colour. After incubation, the assay was stopped with 2 M H_2SO_4 (Appendix B6). Results were interpreted at $OD_{490/620}$. The incubation steps were 30 min at 45°C and TST was used for the wash steps.

3.3. Results

3.3.1 Development and optimization of an ELISA for the detection of anti-GST antibodies

P/N ratios of anti-GST antibody were calculated against serial dilutions of recombinant GST. A concentration range from 100 to 0.78 µg/ml was produced and was used to determine the optimal concentration at which to coat recombinant GST for an ELISA assay. Primary antibody GST DE6 was diluted at 1:500 v/v in bosine.

The optimum concentration at which to coat recombinant GST for an ELISA assay, utilised the positive/negative (P/N) ratio method of result analysis. Results obtained in Appendix B8 show that the P/N ratio is directly proportional to the GST antigen concentration. The values from Appendix B8 were used to plot a graph (Figure 3.1) that determined the optimal concentration of GST, as a coating antigen in an ELISA.

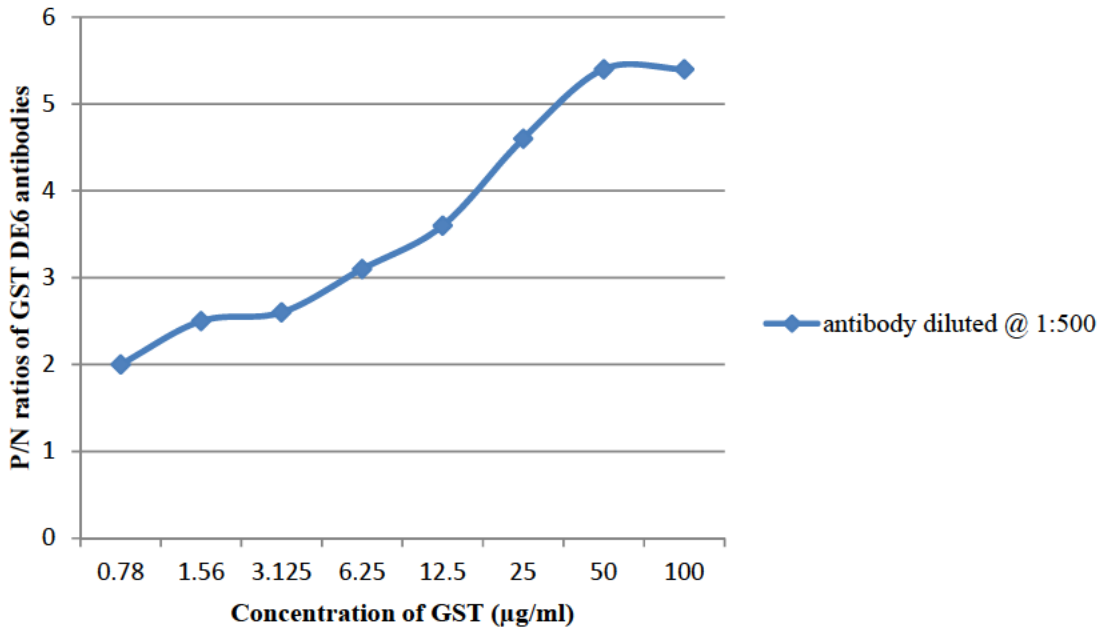

Figure 3.1: Coating curve used to establish the optimal concentration of GST required to coat an ELISA plate for the detection of antibodies to GST.

It is evident that the P/N ratios continue to rise with increasing GST antigen concentrations, up to a point and then the graph reaches a plateau. From Figure 3.1, the plateau is reached at a concentration >50 µg/ml of GST. The optimal antigen coating concentration is obtained from the graph before it reaches a plateau. Furthermore, the coating concentration should be obtained from within the range that depict an increase in P/N ratios. In order to avoid the use of GST in excess (towards the upper antigen concentration range of 25-50 µg/ml, an antigen coating concentration of 12.5 µg/ml was selected (Figure 3.1).

Appendix B9 reflects the optical densities at 1:1000 v/v and 1:3000 v/v dilutions of GST HRP in bosine, obtained at varying concentrations of recombinant GST. This data, when plotted in a graph (Figure 3.2), was used to determine the optimal dilution of GST HRP for use in an ELISA.

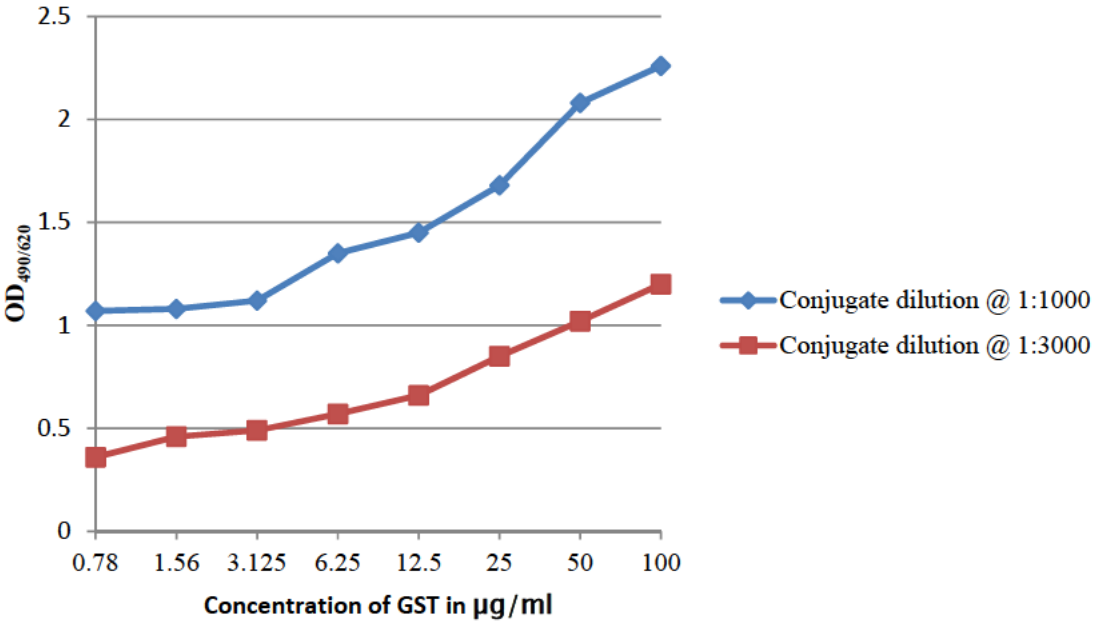

Figure 3.2: Coating curve used to establish the optimal dilution of GST HRP for the detection of antibodies to GST in an ELISA. Although the optical density of both GST HRP dilutions increase with an increase in the GST antigen concentration, it was evident that the 1:1000 v/v GST HRP dilution produced higher optical density values.

From Figure 3.2 it was shown that the 1:1000 v/v dilution of GST HRP produced favourable results for use in an ELISA. The significance of the results from Figures 11 and 12 was that an ELISA could be developed and optimised to detect antibodies to GST from both cell culture fluid and ascitic fluid.

3.3.2 The development and optimization of an ELISA to detect MAbs against ESAT6

The optimal concentration of ELISA coating antigen, recombinant ESAT6, was determined by generating a coating curve for antibodies to ESAT6 (Figure 3.12), using the P/N ratios calculated in Appendix B10 against the varying concentrations of recombinant ESAT6.

The data in Appendix B10 has been extrapolated at $OD_{490/620}$. It was found that the P/N ratios increase as the concentration of antigen increases and that the greater the antibody dilution, the lower the antibody P/N ratio (Appendix B10).

Varying concentrations of recombinant ESAT6 were coated on a microwell plate and the wells incubated with dilutions (1:500 and 1:1000) of the commercial ESAT6 antibody. Following an

incubation step with detection antibody, Sigma goat anti-mouse IgG *Fab* HRP conjugate, results were visualised by the addition of chromogenic substrate. The optimal antigen coating concentration was determined as the antigen concentration producing the highest PN ratio (Figure 3.3). From Figure 3.3 it can be concluded that optimum coating concentration of recombinant ESAT6, to be used in an ELISA, is 10 µg/ml.

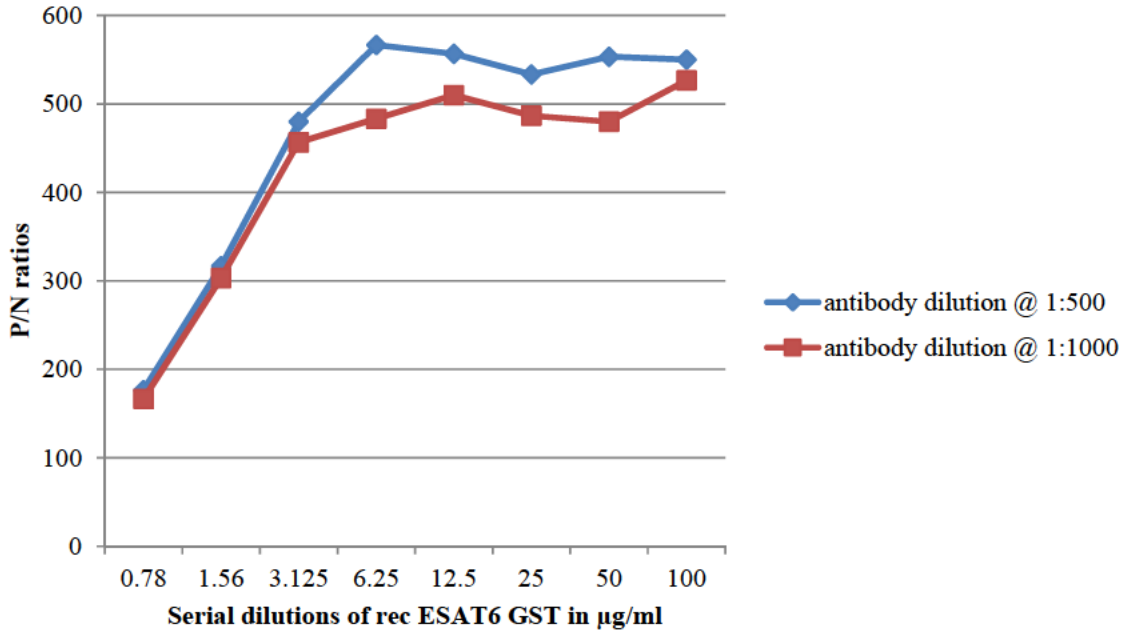

Figure 3.3: Coating curve used to establish the optimal concentration of recombinant ESAT6 antigen required to coat an ELISA plate for the detection of antibodies to recombinant ESAT6. The optimal antigen coating concentration was determined as the maximum P/N ratio.

This demonstrates successful optimization and development of an ELISA for the detection of antibodies to ESAT6 (Appendix B10, Figure 3.3) and verifies the success in cloning Figures 2.1, 2.2 and 2.3), expression (Figures 2.4 and 2.5) and purification of recombinant ESAT6 (Figure 2.8).

3.3.3 Monitoring of Balb/C mice immunised with recombinant ESAT6 GST

In accordance with animal ethics requirements, behavioural changes and changes in the eating and drinking habits of the mice were visually monitored throughout the immunisation process. No such adverse changes were noted and therefore none of the mice required euthanasia based on

these grounds. However, mice numbers 4 and 9 had died from natural causes and in the absence of test bleed samples for these mice, their data was not reflected in Table 3.1.

3.3.4 ELISA used for the detection of serum antibodies from mice immunised with recombinant ESAT6 GST

Table 3.1: P/N ratios of 1:10 v/v and 1:20 v/v dilutions of pre-bleed and test-bleed samples from immunised Balb/C mice tested for anti-ESAT6 antibodies.

Mouse number#	1	2	3	55	6	7	8	10
P/N ratios at 1:10	11.26	22.39	17.57	20.03	15.72	2.84	9.30	12.88
P/N ratio at 1:20	15	19.77	21.9	24.35	15.52	1.96	12.48	8.67

Results from Table 3.1 showed that mouse number 5 produced a higher P/N ratio when the average of the 1:10 and 1:20 v/v dilutions were calculated. This meant that mouse number 5 produced the most potent antibody response to ESAT6, following immunisation with recombinant ESAT6 GST.

Results from Table 3.1 could not be considered in isolation. All pre-bleed and test-bleed samples had to be screened by ELISA for antibodies to the GST portion of the immunogen. Table 3.2 summarised the data obtained from that experiment.

Results from Table 3.2 indicate that mouse number 3 produced a significant antibody response to GST and was excluded from the study. The remaining 7 mice had produced favourably low P/N ratios at both 1:10 v/v and 1:20 v/v anti-GST antibody dilutions. Data from tables 5 and 6 concluded that mouse number 5 was the best candidate as a spleen donor for the next step of the study, the hybridoma fusion. Mouse number 5 produced a high antibody response to recombinant ESAT6 and a low antibody response to GST following immunisation with recombinant ESAT6 GST (Table 3.2).

Table 3.2: P/N ratios of 1:10 v/v and 1:20 v/v dilutions of pre-bleed and test-bleed samples from immunised Balb/C mice tested for anti-GST antibodies.

Mouse number#	1	2	3	5	6	7	8	10
P/N ratio at 1:10	3.07	2.46	13.25	2.5	2.36	0.97	0.98	10.7
P/N ratio at 1:20	2.19	1.49	9.25	1.56	1.71	0.96	0.92	0.96

3.3.5 Fusion of splenocytes from immunised mouse with NS0-1 mouse myeloma cells for the production of hybridoma clones

The spleen produced 16.8 x 10^6 lymphocytes, resuspended in a 10 ml volume and 5.2 x 10^6 NS0-1 cells were harvested from cell culture, also resuspended in a 10 ml volume. Therefore, in order to fuse the splenocytes to NS0-1 cells at a ratio of 5:1 cells/ml, 6.6 ml of NS0-1 cell suspension was added to the 10 ml volume of splenocytes. The hybrid cells suspended in HAT media was plated onto eighteen, 96-well cell culture plates.

The control wells were microscopically examined 3 days after the fusion culture plates were incubated. Results from microscopic examination of the cultures revealed that the NS0-1 fed with RP5/10 were healthy and viable as opposed to the NS0-1 fed with HAT media. The inhibitor, aminopterin, in the HAT media killed the myeloma cells, passaged in 8-azaguanine and that lacked the HGPRT gene thus rendering them incapable of nucleotide synthesis and growth on their own.

A week after the fusion, the 96-well culture plates were microscopically screened for the presence of clones. The fusion had produced 98 hybridoma clones that were named according to the letter of the 96-well plate on which it was grown and its exact position on the 96-well plate. The sizes of the clones and their growth rates were monitored regularly. The clones that grew at a rapid rate and resulted in the media in the well turning yellow, before the clones were screened by ELISA, were fed by replacing 50 µl of spent media with fresh HAT media that was prepared on the day of the fusion. The 96-well fusion plates were left to incubate at 37°C in a humidified CO_2 incubator, until the day of the ELISA screen, two weeks after the fusion.

3.3.6 ELISA used to identify hybridoma clones producing antibodies to recombinant ESAT6

The fusion 96-well plates were screened by both ESAT6 and GST ELISA's. Results confirmed that thirty nine of the ninety eight clones that grew, either did not produce antibodies or were reactive on the GST ELISA plate and were therefore excluded from further testing. During mitosis and segregation into daughter cells, chromosomes carrying immunoglobulin heavy or light chain genes may be lost, resulting in loss of antibody production by the hybridoma. If the chromosome containing the gene used in drug selection, is lost, then the hybridoma will die. Due to the instability of some hybridoma clones, 3 such clones died during sub culturing. A further 6 clones stopped producing the antibody of interest when re-tested by ELISA. Based on growth rate of the

50 remaining clones ("mother clones") and their antibody strength when screened by ESAT6 ELISA at $OD_{490/620}$, eleven clones were selected for antibody characterization. The remaining 39 hybridoma clones were frozen away in liquid nitrogen vapour at concentrations of between 1-1.5 x 10^6 cells per ml suspended in RPMI media containing 10% dimethyl sulphoxide (DMSO) and 20% horse serum.

The specificity of the eleven hybridoma clones were determined by reacting the clones against non-specific antigens in an ELISA. These results are shown in Table 3.5.

Table 3.3: Hybridoma clones were screened by ELISA to confirm ESAT6 specificity and test for cross reactivity against non-specific antigens.

Clone	ELISA- plates coated with various antigens			
	ESAT6 GST	**GST**	**ESAT6**	**LDH-GST**
Positive control	3.57	2.95	3.45	2.89
Negative control	0.01	0.03	0.01	0.08
AE11	2.10	1.95	2.05	1.01
DE2	3.40	0.05	3.54	0.07
EG4	2.50	0.06	2.18	0.11
FE2	1.17	1.40	1.33	1.14
GH4	2.97	0.11	3.03	0.02
HA10	2.68	0.08	2.88	0.10
HG1	3.10	0.08	2.76	0.04
KE10	3.56	0.04	3.76	0.11
MG5	2.08	0.02	2.09	0.06
PB5	2.99	0.10	2.70	0.07
QF10	3.01	0.09	2.88	0.04

From the results obtained in Table 3.3, clones that read $OD_{490/620}$ >0.5 against all four antigens (ESAT6 GST, GST, ESAT6 and LDH-GST), were excluded from the study. Clones that showed this type of cross reactivity were AE11 and FE2. Results from the remaining nine clones were acceptable. Their GST and LDH-GST results were $OD_{490/620}$ = <0.5, with positive antibody results ($OD_{490/620}$ = >0.5) in the ESAT6 and ESAT6 GST assays (Table 3.3).

3.3.7 Anti-ESAT6 MAbs produced by distribution of cells by limiting dilutions

Anti-ESAT6 DE2, EG4, GH4, HA10, HG1, KE10, MG5, PB5 and QF10 were sub-cloned by limiting dilution and plated at 150 cells per ml (cpm) to bring the hybridomas to monoclonality. Of these 9 clones, anti-ESAT6 DE2 sub clones grew at a faster rate, producing a strong antibody

signal on ELISA. A single DE2 sub clone was selected for a second round of limiting dilutions. The more established sub clone was set at a lower cell count of 100cpm.

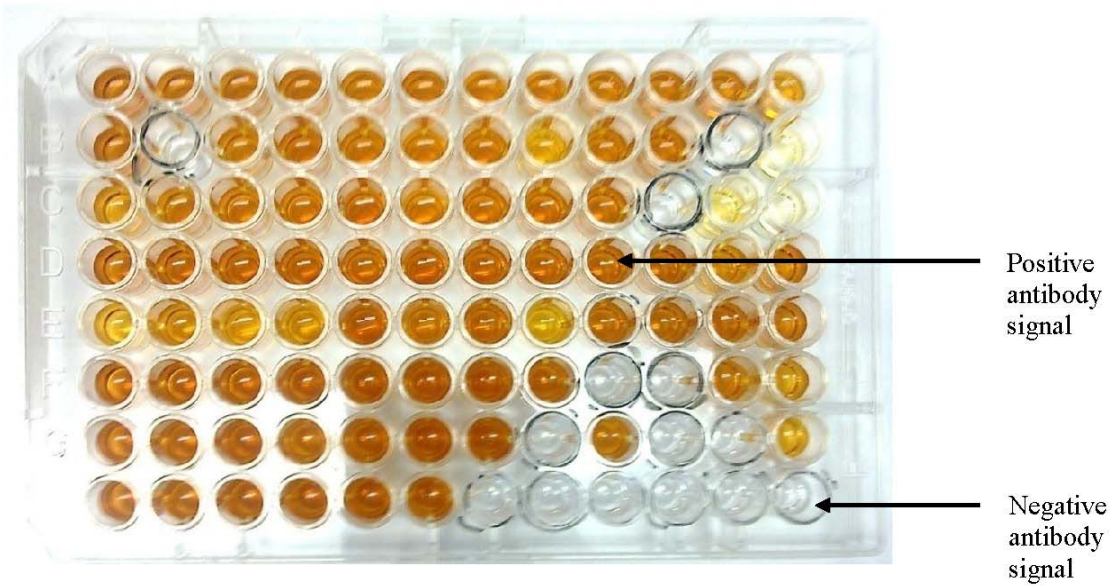

Figure 3.4: ELISA performed on limit dilutions for the detection of antibodies against recombinant ESAT6. The clear wells identified clones that did not produce antibody to ESAT6. The yellow-orange wells represented clones that produced antibodies to ESAT6.

The 96-well ELISA plate represented in Figure 3.4 above, was used to test the culture supernatant from the second round of limit dilution that produced monoclonal antibody ESAT6 DE2-1. Wells that appeared yellow-orange, read between 2.1 and 3.5 at $OD_{490/620}$. Microscopic examination of the cell cultures showed that the supernatant from the yellow-orange wells were produced by well-established clones from anti-ESAT6 DE2-1. There were no clones present in the wells that appeared clear (Figure 3.4) and this explained the negative ELISA result with $OD_{490/620}$ <0.5. Results from Figure 3.4 showed that after two rounds of limiting dilutions, all wells that produced colour and $OD_{490/620}$ >0.5 had clones present. Recombinant ESAT6 was used as the coating antigen, therefore the presence of clones that produced positive results by ELISA, confirmed that these clones produced anti-ESAT6 MAbs.

The culture of anti-ESAT6 DE2-1 hybridoma cells were expanded onto petri dishes. The cells were pooled and injected into 10 mice at 1.5 x 10^6 cells/ml for the production of ascites.

3.3.8 Anti-ESAT6 MAbs produced by distribution of cells on Soft Agar Cloning

In parallel to the limiting dilutions, anti-ESAT6 DE2, EG4, GH4, HA10, HG1, KE10, MG5, PB5 and QF10 were cultured on soft agar. With the exception of the anti-ESAT6 HA10 and QF10, the rest of the clones produced pin head sized colonies after 10 days of incubation at 37°C in a 5% CO_2 incubator, as shown in Figure 3.5.

Figure 3.5 showed the soft agar cloning of anti-ESAT6 KE10. Several single colonies were transferred onto a 96-well culture plate. Of these clones, KE10-1 and KE10-2 were selected for further characterization based on their growth rate and ELISA result.

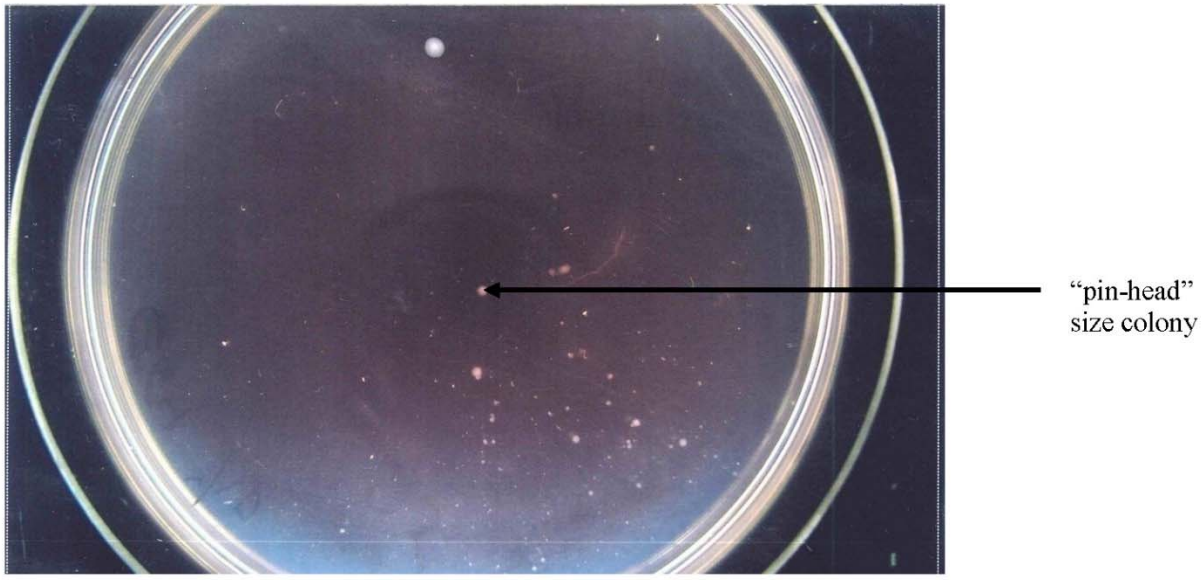

Figure 3.5: Soft agar cloning depicting pin head sized hybridoma clones. The single clones that were selected were cultured in 96-well plates and tested by ELISA for the presence of anti-ESAT6 MAbs.

Table 3.4: ELISA results at $OD_{490/620}$, of clones produced from Soft Agar Cloning.

Clone	Number of clones that grew	Number of clones that were picked	ESAT6 GST ELISA (highest signal)	LDH-GST ELISA (highest signal)
Positive control	-	-	3.20	2.91
Bosine/Negative control	-	-	0.02	0.03
DE2	6	4	0.60	0.023
EG4	11	7	0.03	0.019
GH4	4	4	0.04	0.031
HA10	0	0	-	0.001
HG1	8	8	0.05	0.025
KE10	9	7	2.95 and 3.1	0.016 and 0.03
MG5	10	6	0.4	0.016
PB5	8	5	0.03	0.028
QF10	0	0	-	0.029

Table 3.4 show soft agar clones (ESAT6 DE2, EG4, GH4, HA10, HG1, MG5, PB5 and QF10) that produced low ELISA signals in the ESAT6 GST assay and was therefore excluded from further characterization. Of the seven clones that were picked from KE10 soft agar, two clones, KE10-1 and KE10-2 produced strong signals from ESAT6 GST ELISA. All clones were tested for cross reactivity against recombinant LDH-GST. Anti-KE10-1 and anti-KE10-2 both tested negative for antibodies to LDH-GST.

3.3.9 Isotype of MAbs determined by ELISA

All tests that were conducted, from ELISA screening (Section 3.3.5), antibody specificity assays (Section 3.3.5), limit dilutions (Section 3.3.6), soft agar cloning (Section 3.3.7), anti-ESAT6 DE2-1, KE10-1 and KE10-2 MAbs produced a consistently good cell culture growth rate and demonstrated ESAT6 specificity. In completing the characterisation of these three mouse MAbs, it was important to determine their isotypes. For purposes and intent of this study, the significance in determining antibody isotypes was for protein purification purposes. Prosep Protein Affinity chromatography was used to purify the IgG antibody and the Cibacron Blue column was used to purify the IgM antibodies.

Results were interpreted as positive when $OD_{490/620} > 0.5$ and negative at $OD_{490/620} < 0.5$. Sigma goat anti-mouse FAB HRP detected antibody to both IgG and IgM, the Fc specific HRP detected IgG antibody subtypes and the mμ chain specific HRP detected IgM antibody subtypes. Results

from Table 3.5 concluded that both anti-ESAT6 KE10-1 and KE10-2 were of an IgM subtype and anti-ESAT6 DE2-1, an IgG.

Table 3.5: Isotype determination of anti-ESAT6 MAbs, using isotype-specific HRP in an ELISA

Sigma anti mouse Conjugate produced in goat	Fab HRP	Fc specific HRP	Mμ chain Specific HRP
Anti-ESAT6 KE10-1	>3.0	0.4	>3.0
Anti-ESAT6 KE10-2	>3.0	0.4	>3.0
Anti-ESAT6 DE2-1	>3.0	>3.0	0.2
ABCAM positive Control	>3.0	>3.0	0.2
CD Negative control	0.2	0.2	0.2

3.3.10 Isotype of MAbs confirmed by ODD

Immunodiffusion and precipitation was used to confirm antibody isotype results obtained using isotype specific HRP in an ELISA.

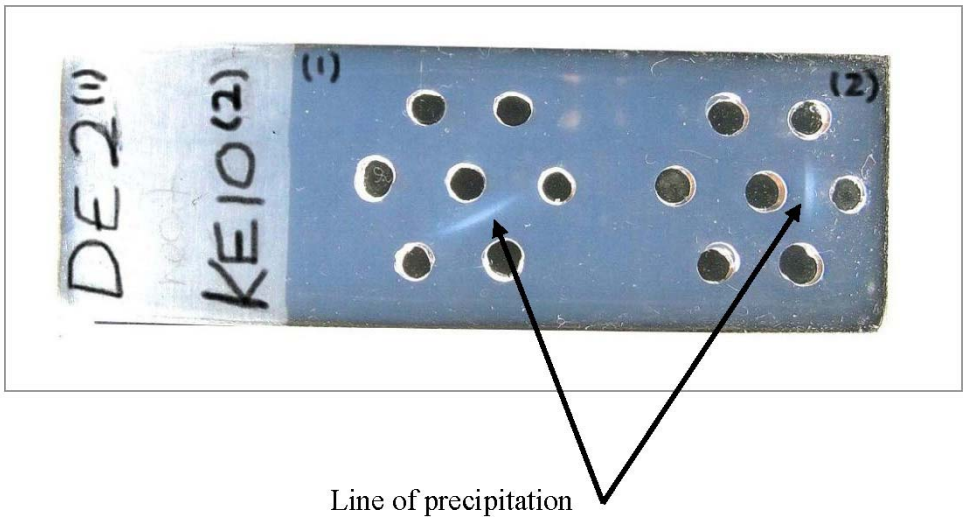

Figure 3.6: Gel immunodiffusion producing lines of precipitation, confirming the subtypes of anti-ESAT6 DE2 and anti-ESAT6 KE10. Antibody in each of the central wells were

allowed to diffuse toward anti-mouse isotype specific antisera in the surrounding wells, in an agarose gel. The presence of a precipitin line indicated a positive reaction.

Wells punched to the right of the slide in Figure 3.6, represented ascitic fluid results for anti ESAT6 KE10. The line of precipitin was formed between IgM (mμ chain) antisera and anti ESAT6 KE10 ascitic fluid. KE10 was therefore shown as IgM in nature.

Figure 3.5 depicts the class and subclass of each of the three monoclonals as determined by ODD. The set of wells on the left of the slide represented ascitic fluid results for anti-ESAT6 DE2. A single line of precipitin was formed between the IgG1 antisera and anti-ESAT6 DE2 ascitic fluid. Anti-ESAT6 DE2 was therefore of an IgG1 subtype.

Table 3.8 showed that, of the nine polyclonal antibodies that were produced from the fusion, two rounds of limit dilutions performed on seven of the clones (EG4, GH4, HA10, HG1, MG5, PB5 and QF10) produced weak ELISA results due to low antibody production by these clones. KE10 did not require limit dilutions as monoclones were produced by soft agar cloning. DE2 underwent 2 rounds of limit dilutions at 150 cpm and 100 cpm, and produced monoclonal antibody DE2-1. Soft agar cloning, produced no clones from HA10 and QF10. Soft agar colonies produced by DE2, EG4, GH4, HG1, MG5 and PB5, produced either low or negative results by ELISA. KE10 was the only clone that produced monoclonal antibodies, KE10-1 and KE10-2 from soft agar. ODD and antibody/HRP subtyping by ELISA produced results that were comparable. DE2-1 was subtyped as an IgG1 and both KE10-1 and KE10-2 was subtyped as IgM monoclonal antibodies.

Table 3.6: Cloning and characterization of hybridomas in the production of MAbs

Clone	Limiting Dilution	Soft agar Cloning	Ouchterlony Double diffusion	Antibody/HRP Subtyping by ELISA	Status
DE2	150 cpm 100 cpm	Sub-clones produced Low ELISA results $OD_{490/620}$ =0.6	IgG1	IgG	DE2-1 IgG1 monoclonal
EG4	150 cpm 100 cpm Weak ELISA	Negative ELISA	Ab reacted against more than one antisera	Not required	Not monoclonal
GH4	150 cpm 100 cpm Weak ELISA	Negative ELISA	Ab reacted against more than one antisera	Not required	Not monoclonal
HA10	150 cpm 100 cpm Weak ELISA	No Sub-clones produced	Ab reacted against more than one antisera	Not required	Not monoclonal
HG1	150 cpm 100 cpm Weak ELISA	Negative ELISA	Ab reacted against more than one antisera	Not required	Not monoclonal
KE10	Not required.	KE10-1 and KE10-2 Sub-clones produced	KE10-1 and KE10-2 Both IgM	KE10-1 KE10-2 Both IgM	KE10-1 and KE10-2 IgM monoclonal
MG5	150cpm 100cpm Weak ELISA	Subclones Low ELISA Result (0.4)	Ab reacted against more than one antisera	Not required	Not monoclonal
PB5	150cpm 100cpm Weak ELISA	Negative ELISA	Ab reacted against more than one antisera	Not required	Not monoclonal
QF10	150cpm 100cpm Weak ELISA	No Subclones	Ab reacted against more than one antisera	Not required	Not monoclonal

Prior to discontinuing clones QF10, PB5, MG5, HG1, HA10, GH4 and EG4 based on previous characterization studies (Table 3.6), the clones were retested for the production of antibodies to ESAT6. According to Figure 3.7, antibody levels for the above mentioned clones were confirmed as low at $OD_{490/620}$ = <0.5. MAbs KE10-1, KE10-2 and DE2-1 produced consistently high antibody levels throughout the study. Results from Figure 3.6 supported the results that were obtained from limiting dilutions and soft agar cloning.

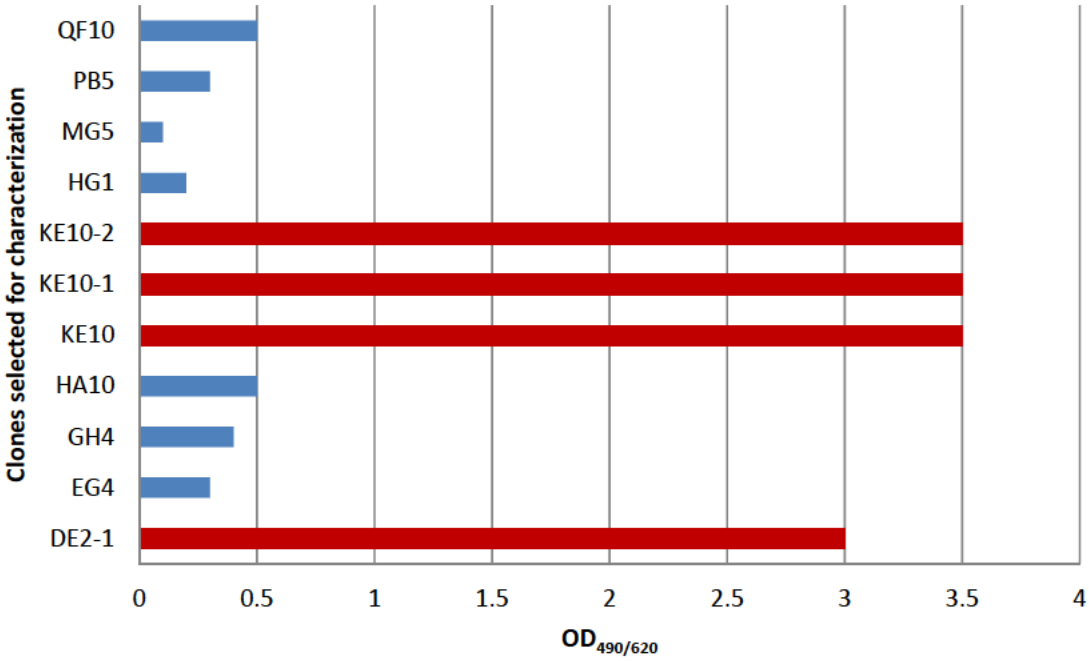

Figure 3.7: ELISA results of anti-ESAT6 hybridoma clones selected for characterization based on antibody strength measured at $OD_{490/620}$. The selection criteria were as follows:

- **Clones at $OD_{490/620}$ = >0.5, selected for further characterization**
- **Clones at $OD_{490/620}$ = <0.5 were discontinued.**

3.3.11 Genealogy of anti ESAT6 MAbs produced by hybridoma fusion

Anti-ESAT6 DE2 and KE10 were hybridomas that were produced from the splenocyte/NS0-1 fusion. Both hybridomas produced antibodies specific to recombinant ESAT6, when tested by ELISA. These clones were referred to "mother clones" that required clonal selection to produce MAbs. Limit dilutions and soft agar cloning produced MAbs anti-ESAT6 DE2-1, anti-ESAT6-KE10-1 and anti-ESAT6 KE10-2, as illustrated in Figure 3.8.

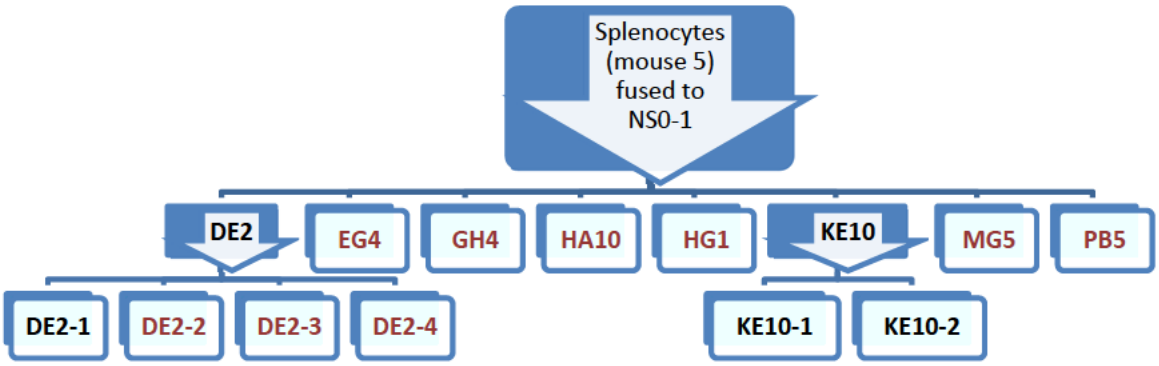

Figure 3.8: Genealogy used to trace the history of anti-ESAT6 clones. The black font traces cell lines that produced monoclonal antibodies.

3.3.12 Purification of anti-ESAT6 IgG MAb using Prosep protein A chromatography

MAb anti-ESAT6 DE2-1, was subtyped as an IgG1 and purified from ascitic fluid from a Prosep Protein A purification column (Figure 3.9). The first peak represented the flow through, while anti-ESAT6 DE2-1 at a concentration of 5.2 mg/ml, was eluted from the column at pH 4.0, as represented by the second peak (fractions 22-28) in Figure 3.9.

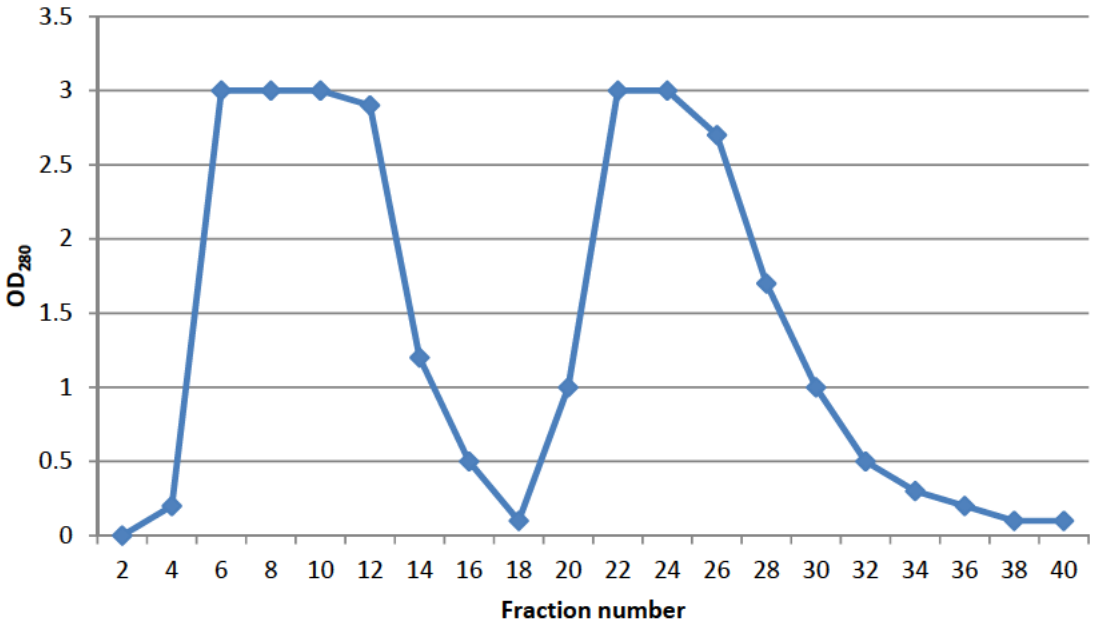

Figure 3.9: Binding of anti-ESAT6 DE2-1 on a Prosep Protein A chromatography column.

3.3.13 Purification of anti-ESAT6 IgM MAbs using Cibacron Blue chromatography

The first peak represented by fractions 4-10 in Figure 3.10, contained anti-ESAT6 KE10-1 MAb at a concentration of 6.4 mg/ml. The purified IgM MAb was used in an ELISA for the detection of ESAT6.

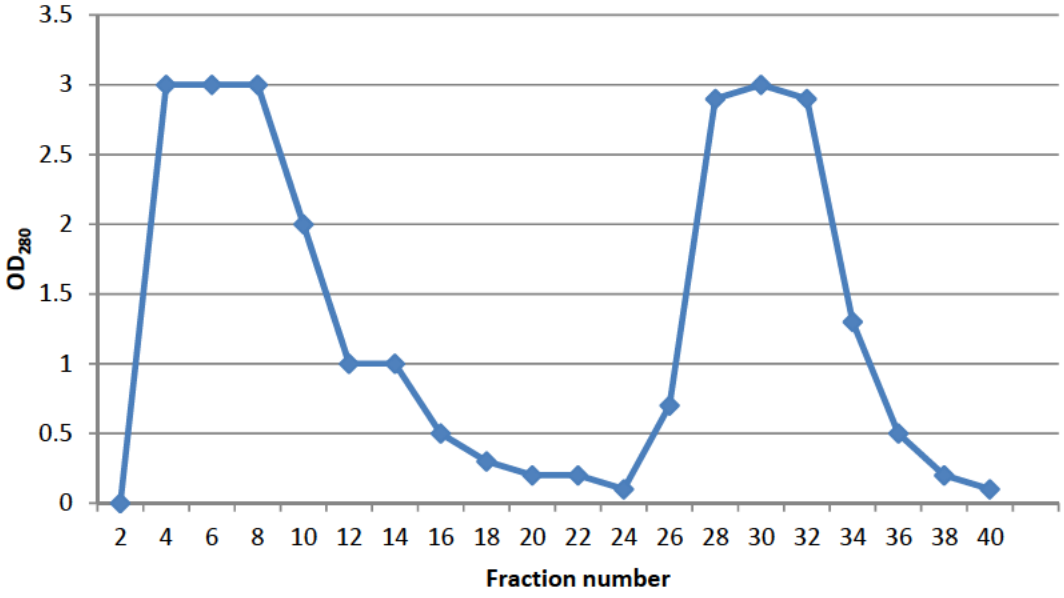

Figure 3.10: Albumin depletion from ascitic fluid using the Cibacron Blue chromatography column. The unbound fractions or flow through, represented by the first peak (fractions 4-12), contained the IgM antibody.

The first peak, represented by fractions 6-14, in Figure 3.11, contained anti-ESAT6 KE10-2 MAb at a concentration of 11.95 mg/ml. This MAb also had potential for use in ELISA for the detection of ESAT6.

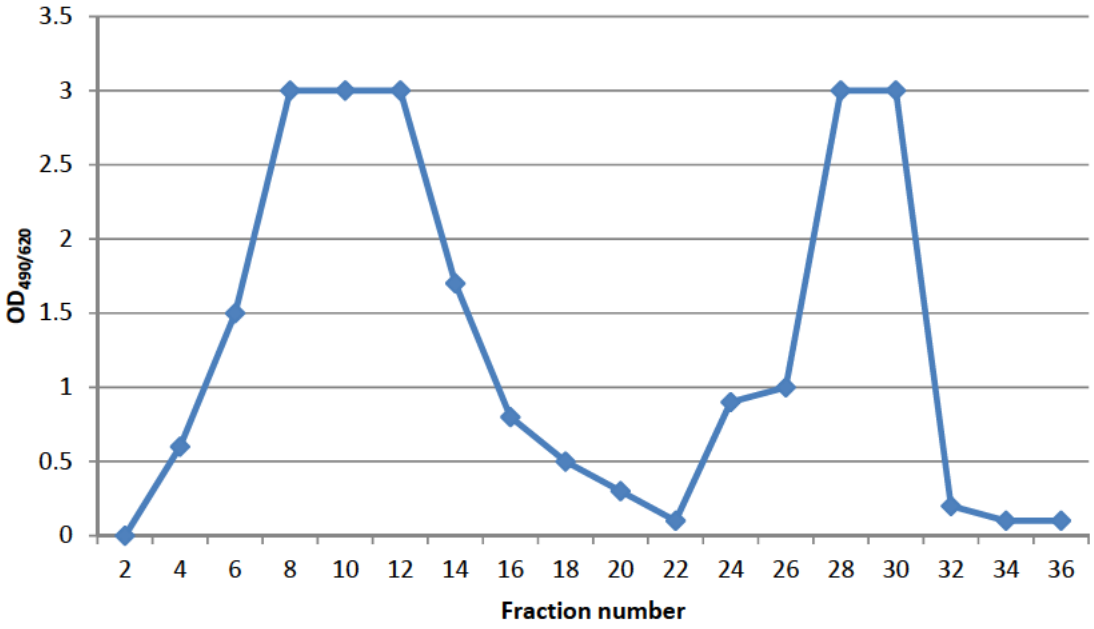

Figure 3.11: Albumin depletion from ascitic fluid using the Cibacron Blue chromatography column. The unbound fractions or flow-through, represented by the first peak (fractions 6-14), contained the IgM antibody.

3.3.14 Identification of anti-ESAT6 MAbs by serum protein electrophoresis

In Figure 3.11 it was shown that the IgG bands (Figure A) were positioned slightly higher than the IgM (Figure B) bands and this was confirmed with the presence of known positive IgG and IgM controls in lanes 1 and 7. Figure A confirmed that anti-ESAT6 DE2-1 was an IgG, by the representation of sample bands alongside the positive control band. Similarly, anti-ESAT6 KE10-1 bands were of the same size as the positive IgM control band.

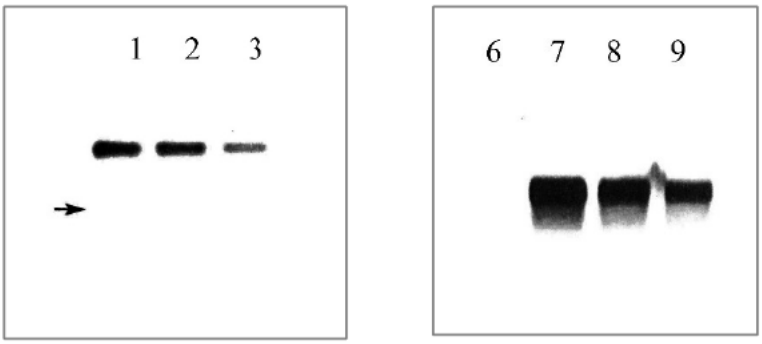

Figure A Figure B

Figure 3.12 (A and B): The γ globulin bands from purified ascitic fluid were demonstrated by serum protein electrophoresis. The band in lane 1 represented a control sample of the IgG subtype and the band in lane 7 represented an IgM subtype. Undiluted sample of anti-ESAT6 DE2-1 was shown in lanes 2 and lane 8 represented an undiluted sample of ant-ESAT6 KE10-1. Sample dilutions of anti-ESAT6 DE2-1 and anti-ESAT6 KE10-1 MAbs at a 1:5 v/v dilution was indicated by lanes 3 and 9 respectively.

3.3.15 Multi-epitopic nature of recombinant ESAT6 confirmed using anti-ESAT IgM HRP as a secondary antibody in an ELISA

When anti-ESAT6 DE2-1 was coated on a multiwall plate, incubated with recombinant ESAT6 and then incubated with anti-ESAT6 KE10-1 HRP as the secondary antibody, results were >3.00 at $OD_{490/620}$. The positive reaction concluded that both anti-ESAT6 DE2-1 and KE10-1 recognise different epitopes on the recombinant ESAT6 antigen.

3.3.16 Specificity of MAbs against recombinant ESAT6 confirmed by ELISA

MAbs anti-ESAT6 DE2-1, anti-ESAT6 KE10-1 and anti-ESAT6 KE10-2, were used in a non-competitive ELISA, in which their specificity to recESAT6 was confirmed. The first assay, in Table 3.7, showed that all three monoclones produced a positive result at $OD_{490/620}$ when incubated

with recombinant ESAT6 as the coating antigen, incubation with the monoclonal antibodies, followed by incubation with Sigma goat anti-mouse HRP.

From the second assay in Table 3.7, positive ELISA results at $OD_{490/620}$, obtained for anti-ESTA6 DE2-1, KE10-1 and KE10-2 indicated specificity of the MAbs for recombinant ESAT6. Goat anti-mouse HRP did not react with rabbit anti-ESAT6 antibodies (coating antibody), therefore the development of colour in the assay indicated that MAbs anti-ESAT6 DE2, KE10-1 and KE10-2, reacted specifically with recombinant ESAT6 antigen, in the antibody-antigen-antibody sandwich ELISA.

Table 3.7: Non-competitive ELISA designed to determine whether the monoclonal antibodies produced in this study, reacted specifically to recESAT6.

MAbs against ESAT6	Non-competitive immunoassay	
	recESAT6→monoclone→ Sigma goat anti-mouse HRP	**rabbitαESAT6→recESAT6→monoclone→ Sigma goat anti-mouse HRP**
DE2-1	>3.0	>3.0
KE10-1	>3.0	>3.0
KE10-2	>3.0	>3.0
	↓ **ELISA was specific for the detection of ESAT6 monoclonal antibodies**	↓ **Anti-mouse HRP did not react with rabbit antibodies. Colour change indicated that the antibody was specific for ESAT6 antigen**

Table 3.8: Specificity of MAbs ESAT6 DE2-1, KE10-1 and KE10-2 were tested by ELISA for reactivity against non-specific antigens.

MAbs	GST	HBs	VZ	Rabies	ESAT6
DE2	0.02	0.02	0.07	0.04	>3.0
DE2-1	0.02	0.02	0.2	0.03	>3.0
KE10	0.03	0.02	0.2	0.2	>3.0
KE10-1	0.02	0.02	0.02	0.03	>3.0
KE10-2	0.05	0.02	0.03	0.03	>3.0
Pos control	0.67	2.252	0.862	0.902	>3.0
Neg control	0.01	0.02	0.005	0.02	0.11

Results demonstrated that anti-ESAT6 DE2-1, KE10-1 and KE10-2 monoclonal antibodies and their mother clones anti-ESAT6 DE2 and KE10 did not react with GST, Hepatitis B surface (HBs)

antigen, Varicella zoster (VZ) antigen and Rabies antigens (Table 3.8). The antibodies reacted specifically with recombinant ESAT6. A cut off value of $OD_{490/620} = 0.5$ was used to interpret the results. Values <0.5 were negative reactions and values >0.5 were interpreted as positive at $OD_{490/620} = 0.5$.

3.4. Discussion

The United Nations SDGs set a 2030 target date for the eradication of TB, focusing on early detection and diagnosis which is key in the control of the disease. In line with this goal, this chapter focused on the production of anti-ESAT6 monoclonal antibodies from *M. tuberculosis*, to be used in an inexpensive, rapid diagnostic test (Harrington, 2015).

In a study by Feng *et al* (2011), monoclonal antibodies against ESAT6 were developed using the hybridoma technology of Kohler and Milstein (1975). As ESAT6 is relatively small, a glycine peptide chain linker was used to join the protein to CFP10. CFP10 forms a heterodimeric complex with ESAT6, located in the RD1 region and together with ESAT6, is responsible for virulence of the bacterium. The resulting 27 kDa fusion protein was used to immunise Balb/C mice in order to induce a stronger immunological response. Six MAbs against ESAT6 were identified and validated using ELISA, immunoblotting and immunoprecipitation. The ESAT6 antibodies produced were tested on clinical samples and results showed that they could be used for the clinical detection of TB. ESAT6 had a positive detection rate of 95.4% and a negative detection rate of 100% (Feng *et al*,. 2011).

The challenge with ESAT-GST as an immunogen was that the mice produced antibodies not only to ESAT6 but to the GST portion of the immunogen as well. Hybridomas that produced antibodies to GST were excluded from the present study. Although GST is larger in size than the recombinant ESAT6 portion of the immunogen, only about 30% of the hybridomas produced antibodies to GST. Apart from enhancing the immunogenicity of the protein, the GST tag was also used to facilitate downstream processes such as protein purification and antibody detection (Pillai *et al*,. 1995).

Studies showed that immunisation strategies for the production of anti-ESAT6 MAbs, varied amongst investigators with success from different immunisation regimes being obtained. For example, Leng *et al.* (2014) injected 6 week old Balb/C mice with 0.2 ml of antigen, once in two weeks, repeated four times. Six weeks later lymphoblasts from the spleen were used in a successful fusion. In the immunisation method employed by Feng *et al* (2011), the primary immunisation consisted of equal volumes of Freund's complete adjuvant and antigen. Repeat immunisations of antigen in Freund's incomplete adjuvant was administered thrice, at three week intervals. The mouse with the highest antibody titre was given a 100µg booster immunisation,

without adjuvant, 3 days prior to the fusion. In the production of monoclonal antibodies to the RpfB domain of tuberculosis, Fan *et al.* (2010) immunised Balb/C mice three times, at two week intervals before the fusion process (Fan *et al*,. 2010; Feng *et al*,. 2011; Leng *et al*,. 2014).

The mouse immunisation strategy adopted in this study was modified from Cold Spring Harbour protocols (Greenfield, 2014). Pre-bleed samples were collected before primary, intraperitoneal immunisation with recombinant ESAT6 emulsified in Freund's complete adjuvant (CFA). CFA, an immune booster, contained heat killed Tubercle bacilli that stimulated a vigorous immune response in the mice. Four weeks later, the mice were administered with an immunisation booster, using Incomplete Freund's adjuvant (IFA). The positive to negative ratio method of analysis was applied and the best immune responding mouse was given a recombinant ESAT6 GST immunisation booster emulsified in IFA. The GST fusion-protein enhanced the immunogenicity of the 6 kDa ESAT6 protein. GST produced in *E. coli* as a recombinant protein was required for the protein purification process. Studies showed that the immune response of the immunised mouse, was key in influencing the random process of cell fusion, cloning efficiency and the probability of obtaining specific antibody secreting hybridomas (Frobert and Grassi, 1998).

In order to select the specific antibody secreting hybridomas produced by the fusion, an ELISA was developed and optimised. The ELISA, an effective tool in detecting an antigen or antibody from a mixture, was developed by immobilizing either the antigen or antibody in micro-well plates. ELISA's have been a reliable technique in many areas of medical research (Engvall *et al*. 1971). Although the development and optimization of the assay was a well-defined technique, several factors require analysis (Harlow and Lane,. 1988).

Each monoclonal antibody in this study, was evaluated separately as not all monoclonal antibodies may be used successfully in an ELISA. It may happen that during adsorption of the antigen to the micro-well plate that the three dimensional structure of the antigen may be altered resulting in the target epitope being masked. If antibody was directed towards a peptide that was not exposed, then the antibody would not be successfully detected by ELISA. Also, when two antibodies are used in a sandwich ELISA, then it is important that both these antibodies target different epitopes on the antigen or to an epitope that appears several times on the antigen. When the capture antibody immobilises the antigen by interacting with an epitope, then the signal or detection antibody should preferably interact with a different epitope to prevent steric hindrance from the first antibody or the plate (Harlow and Lane,. 1988).

It was shown in a study by Harboe *et al.* (1998) that the ESAT6 antigen was multi-epitopic. A B-cell epitope had been confirmed at amino acid sequence 3-15 and 40-62 of ESAT6. When synthetic peptides produced to both these regions were used to immunise mice, monoclonal antibodies produced, recognised native ESAT6 (Harboe *et al*., 1998).

Dai *et al* (2012), described the significance of multi-epitopic recombinant antigens in the development of ELISA based diagnostic kits. It was therefore important, in meeting with this study's objectives, to determine whether anti-ESAT6 had more than one epitope. Purified anti ESAT6 KE10-1 monoclonal antibody was conjugated to HRP at a molar ratio of 1:2. In a direct sandwich ELISA, purified anti-ESAT6 DE2-1 was coated on an ELISA plate and the plate incubated with recombinant ESAT6 followed by incubation with anti-ESAT6 KE10-1 HRP. The development of colour following the addition of chromogenic substrate indicated that both anti-ESAT6 DE2-1 and anti-ESAT6 KE10-1 recognised different epitopes on the recombinant ESAT6 protein (Dai *et al*,. 2012).

It was equally important to ensure that the coating and detection antibodies to ESAT6, were raised in different animals in order to prevent the detection antibody and the capture antibody from reacting with one another. The coating antibody in this study, anti-ESAT6 DE2-1, was raised in mouse and the detection antibody, in goat (Sigma goat anti-mouse HRP; Harlow and Lane, 1988).

In the present study, goat anti-mouse HRP did not react with rabbit anti-ESAT6 antibodies (coating antibody). Therefore the development of colour in the assay indicated that MAbs anti-ESAT6 DE2, KE10-1 and KE10-2, reacted specifically with recombinant ESAT6 antigen, in the antibody-antigen-antibody sandwich ELISA. It was concluded that the MAbs produced in this study and Sigma rabbit αESAT6 antibodies recognised different epitopes on recombinant ESAT6.

Hybridoma technology may sometimes produce "sticky antibodies" that bind to plastic, glass or polypropylene micro-well plates coated with non-specific antigens (Sun *et al*., 2001) resulting in false positive results. In the present study, an ELISA was performed to test anti-ESAT6 monoclones produced in this study, against antigens of different specificity to ensure that the monoclones produced were not "sticky" or non-specific and reacted only to recombinant ESAT6 antigen. The results were favourable in that anti-ESAT6 DE2-1, KE10-1 and KE10-2 reacted specifically against recombinant ESAT6 and not against GST, HBs, VZ and Rabies antigens (O'Kennedy *et al*., 1990).

During the development and optimization of the ESAT6 ELISA in this study, standard curves were prepared to ensure that enzyme conjugate, HRP, was used at an optimal concentration, as the amount of enzyme conjugate that bound was directly proportional to the amount of signal that was generated. Too little enzyme conjugate binding would have resulted in a low ELISA signal, whereas the use of too much enzyme conjugate would have produced a high background colour in the assay. This would have been detected with a negative control value of >0.5 at $OD_{490/620}$. These guidelines, ensured that the study's objective was met in developing and optimizing an ELISA for the detection of ESAT6 antigen (Harlow and Lane,. 1988).

One of the factors that could result in a sub-optimal yield of antibody producing hybridomas is the ratio of antibody specific splenocytes (as compared to naive B-cells) to myeloma cells, at the time of the fusion. The mouse spleen has on average 2 x 10^8 splenocytes and it has been reported that only about 55% of these cells were the CD19 B-cell fraction (Pellegrini *et al*. 2007). The fusion ratio of splenocytes to myeloma cells is important in generating hybridomas and preventing an overgrowth of myeloma cells. In this study the ratio of splenocytes to myeloma cells were used at a ratio of 5:1. The use of a B-cell enriched sample of splenocytes as opposed to a mixture of spleen cell, would determine a more accurate number of NS0-1 cells for the fusion and this would enhance the efficiency of the fusion (Kohler and Milstein, 1975).

The use of feeder cells (human lung fibroblasts and peritoneal macrophages) in a fusion promotes secretion of the necessary growth factors to help the hybridomas, growing in very low cell numbers, to maintain good health until the hybridomas expand to larger numbers, to be able to independently secrete sufficient growth factors (Alves *et al*. 2012). In the present study, "conditioned" media was used to support hybridoma growth. The "conditioned" media was the supernatant fluid containing the necessary growth factors from fresh cultures of NS0-1 cells (Harlow and Lane, 1988).

Fusion efficiency may also be greatly reduced if the serum from the NS0-1 cultures have been inadequately removed prior to the addition of PEG (Greenfield, 2014). In addition, if the supernatant is not completely removed from the NS0-1 and splenocytes after they have been centrifuged, prior to the addition of the PEG, then the PEG becomes too dilute, again affecting the fusion rate. If the cell pellet is inadequately disrupted, after centrifugation, prior to the addition of PEG, then not all cells become exposed to PEG. Overexposure of cells to PEG also results in cell death and a low fusion rate (Kohler and Milstein, 1975).

Reasons for the production of a few numbers of clones may also be due to a low viability and low cloning efficiency of the hybridomas. Hybridoma viability and cloning efficiency is the result of a low viability of the myeloma cells stemming from poor cell growth when thawed from liquid nitrogen or the other extreme where the myeloma cells are too dense on the day of the fusion (Kohler and Milstein, 1975).

The viability of the spleen cells required for the fusion is an important factor. An old or unhealthy immunised mouse and a lengthy period between the harvesting of the spleen and the fusion process could also adversely affect hybridoma viability and cloning efficiency. The fusion process is to be swiftly carried out and it is recommended that the fusion be carried out within the hour of harvesting the spleen. Freshly fused cells have fragile cell membranes and should be handled gently between the fusion process and plating. Vigorous pipetting of the fused cells and cells that are exposed to rapid changes in temperature may result in the plasma membrane of the cells rupturing, resulting in cell death. All of these factors were taken into account during the fusion process in this study (Harlow and Lane, 1988).

Another challenge, related to no or too few positive hybridomas, even though a normal number of hybridomas were generated from the fusion may be the result of too low dose or immunogenicity of the antigen. Usually, 20-100µg of antigen elicits a good immune response. However, nanogram quantities of antigen have also been successfully used. If the immunisation schedule is not optimised by for example too short an interval between injections, then the result may be too few specific antibody forming cells at the time of the fusion and the antibodies that are produced are of a low affinity (Gustafsson. 1990).

All of the above factors were monitored and ensured a successful fusion in the present study. Results do not reflect a low fusion rate, low viability or cloning efficiency or that there were too few positive hybridomas. However, CD19 B-cell enrichment prior to the fusion has the potential to enhance anti-ESAT6 yielding hybridomas and should be considered for future work.

Although there were several plating strategies used to identify hybridomas that secrete specific monoclonal antibodies, there were no rules to dictate which strategy was correct. In the present study, the single cell per well, distribution of cells by limit dilution and the plating in soft agar strategy, were employed. Limiting Dilution required a preparation of a series of increasing dilutions of polyclonal antibodies for culture onto ninety six-well plates in order to bring these clones to monoclonality. The process was repeated to ensure the probability that the clones that

grew originated from single cells. Limiting dilution ensured that spontaneous variants or non-producing hybridomas from undesired fusions did not overgrow the "desired" or specific antibody producing hybridomas. The production of anti-ESAT6 DE2-1 following limit dilutions in this study, showed stability and a consistently strong antibody signal when tested against ESAT6 antigen, by ELISA (Puck and Marcus, 1955; Rota *et* al,. 2012).

The soft agar technique using purified agar (Oxoid), although less labour intensive and more economical, proved to be a greater challenge in that a fewer number of clones were produced by this technique. This result was expected as hybridomas do not have a high plating efficiency in soft agar. Isolated colonies were cultured in 96-well cell culture plates before being screened by ELISA for anti-ESAT6 activity. Monoclonal antibodies anti-ESAT6 KE10-1 anti-ESAT6 KE10-2 were cultured from soft agar and found to be of the IgM subtype (Hamburger *et al*,. 1978; Hamburger, 1979).

Subtyping of MAbs that were produced was required to determine the affinity of reagents such as Protein A and relevant for the conjugation of the monoclonal antibody to HRP. In the past, the Ouchterlony double diffusion assay was the most common form of antibody subtyping. This has been superseded by other assays such as the ELISA. In the ELISA based method, the antigen-antibody complex was screened by an HRP class-specific secondary antibody. Both of the above mentioned techniques have been employed in the present study and confirmed that anti-ESAT6 DE2-1 was an IgG and both anti-ESAT6 KE10-1 and KE10-2 were of the IgM subtype.

Studies have indicated that the variation in the number of hybridoma cells injected per mouse had no effect in the production of ascites. In the present study an injection of 1-3 x 10^6 cells per mouse was sufficient to induce ascites in pristine primed mice. Feng *et al* (2011) had injected cells at 1x 10^6 while Leng *et al* (2014) injected cells at 5 x 10^6 hybridoma cells per mouse with effective ascites production in mice (Feng *et al*,. 2011; Leng *et al*,. 2014).

Purification of the ascites was performed to concentrate and enrich monoclonal antibodies from and to remove non-specific proteins (Harlow and Lane,. 1988). Since its inception, affinity chromatography was said to be the most selective type of chromatography in the biotechnology industry and this method separated proteins on the basis of reversible interaction between a protein and specific ligand coupled covalently to a chromatography matrix (Campbell *et al*., 1951). Chromatography is a critical step in producing monoclonal antibodies in the biopharmaceutical industry (Liu *et al*,. 2010).

It is believed that Protein A chromatography is the work-horse of commercial monoclonal antibody production. Protein A, a 42 kDa cell wall component produced by several strains of *Staphylococcus aureus* binds specifically to the Fc region of IgG immunoglobulins through four high-affinity binding sites. Protein A sepharose affinity chromatography is highly selective. It has a high flow rate and cost effective binding capacity producing a pure and stable antibody. It also removes components from the ascitic fluid that may result in antibody degradation. The Protein A resin was prepared by immobilizing recombinant Protein A to a hydrogel within a proprietary porous ceramic bead (PALL – Protein A ceramic Hyper D F resin). Prior to passing the ascitic fluid through the column, the sample was clarified using flushing agent with a high binding capacity for lipids and floating fats and minimal cross reactivity with proteins (Liu *et al*,. 2010).

Protein A and protein G bind IgM immunoglobulins very poorly and is therefore not suitable for these antibody subtypes. This may be because the binding sites on the Fc region is sterically hindered by the pentameric structure of the IgM molecule. Albumin depletion chromatography using Cibacron Blue Dye F3GA as the ligand, was therefore used to purify IgM molecules from ascitic fluid (PALL – Blue Trisacryl M chromatography resin; Liu *et al*,. 2010).

The anti-ESAT6 monoclonal antibodies were purified by affinity chromatography and the purified antibody was used in the ELISA assays and for conjugation to HRP. In order to avoid the possibility of destroying the monoclonal antibody under acidic elution conditions during protein A purification, care was taken to avoid excessive exposure of the antibody to the acidic buffer by immediate dialysis of the antibody in PBS pH 7.2 following purification (Harlow and Lane,. 1988).

Antibody binding capacity varies between antibody species. Mouse IgG, requires 1 ml of hydrated protein A that will bind about 5mg of mouse antibody as compared to 1 ml of hydrated protein A that binds 8mg of human IgG antibody. The pH of the buffers used in affinity chromatography were critical as incorrect use would have resulted in the antibody being lost in the flow through. The column fractions were retained until after serum protein electrophoresis was performed. Significantly low antibody yields would have posed as a warning that possibly, non-producing hybridomas were overgrowing the antibody-producing hybridomas. This would have been corrected by re-cloning and re-screening of the hybridomas (Harlow and Lane,. 1988).

In the present study, monoclonal antibodies anti-ESAT6 DE2-1, anti-ESAT6 KE10-1 and anti-ESAT6 KE10-2, to recombinant ESAT6, were produced and characterised. The monoclonal antibodies showed no cross-reactivity with non-specific antigens and demonstrated the efficacy of conventional Hybridoma techniques. Studies have described alternative methods in ensuring the specificity of monoclonal antibodies. Jeong *et al*. (2012) has described a platform for the generation of exceptionally specific MAbs. Microarray-analysis of antibody specificity was successful using a pooling strategy. Similarly, Hybridoma technology can similarly employ an ELISA based pooling strategy to test individual hybridomas against multiple antigens in a single test. This method deserves consideration as it would be cost effective and less time-consuming.

Future work should include titration of the monoclonal antibodies produced. Ideally, the MAb that is required for immunocytochemistry should be used at a high dilution such that low-affinity, non-specific reactions are prevented (Ivell *et al*. 2014). Titration of the anti-ESAT6 MAbs against ABCAM anti-ESAT6 gold standard would have further examined the strength of these MAbs. The monoclonal antibodies produced in the present study were of IgG and IgM subtypes, showed stability in cell culture, specificity for ESAT6 and had the ability to induce ascites for the *in vivo* production of monoclonal antibodies. The monoclonal antibodies produced, were used in the optimization and development of an ELISA for the detection of recombinant ESAT6 antigen. The present study was promising as previous studies have shown that monoclonal antibodies to ESAT6, when used in an ELISA, showed potential as a tool for the diagnosis of TB (Ebrahimi *et al*,. 2010; Feng *et al*., 2011).

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

The current study has investigated the cloning and expression of recombinant ESAT6 for use as an immunogen in the production of murine MAbs against *M. tuberculosis*-associated ESAT6. The recombinant protein was then used in the development of an ELISA for the detection and characterization of anti-ESAT6 MAbs produced using Hybridoma technology. It was then demonstrated that the anti-ESAT6 DE2-1, KE10-1 and KE10-2 MAbs produced, were successfully used as coating antibodies in the development and optimization of an ELISA for the rapid detection of recombinant ESAT6 (Figure 4.1).

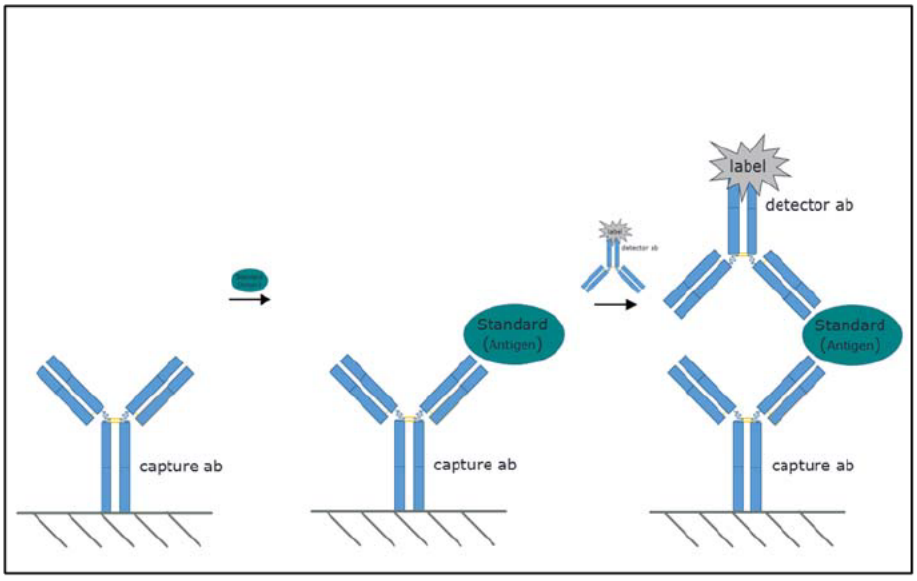

Figure 4.1: Sandwich ELISA illustrating anti-ESAT6 KE10-1 as the capture antibody, followed by the addition of antigen, ESAT6, to which the enzyme labelled detector antibody, anti-ESAT6 KE10-2 HRP is attached.

ELISA and lateral flow tests have been shown to provide rapid results, are simple to use and do not require skilled personnel or expensive equipment in the diagnosis of TB (Shen *et al*., 2011; Bekmurzayeva *et al*., 2013). MAbs to TB-specific markers are reagents amenable to use in ELISAs and lateral flow tests that effectively meet the criteria for rapid antigen detection tests. These assays may be used to distinguish between *M. tuberculosis* and MOTT and may differentiate between active and latent TB (Shen *et al*., 2011; Bekmurzayeva *et al*., 2013). Evidence has suggested that a substantial risk towards the control of TB was the difficulty

experienced in the detection of the latent form of the disease. However, a continuum of host-pathogen responses result in a spectrum of immune responses that make it difficult to define active and latent TB. Discrimination between the two is significant towards patient management as it is estimated that one third of the global population live with latent TB infection with the potential of developing into active disease (Walzl *et al*., 2011; Harrington, 2015).

A report by the Treatment Action Group in 2015 restricted the use of IGRASs and TST, tests currently available for the detection of LTBI (Harrington, 2015). The WHO supported this view, more especially for the diagnosis of LTBI in India, China and South Africa as it was shown that both IGRA and TST were not specific to *M. tuberculosis* and these resulted in many cases being undetected (WHO, 2015).

LAM has been a favourable biomarker because it is detectable in urine and has been successfully used for the rapid detection of TB. The major drawback is that the test is only sensitive for HIV positive patients presenting with low CD4 counts (Minion *et al*., 2011). LAM used in a lateral flow test for the diagnosis of active TB seemed unlikely to improve the rate of same-day treatment (Peter *et* al., 2015). The Rv1681 protein, also detected in urine, has been shown to be more sensitive than LAM, in the diagnosis of TB. However, this protein has low sensitivity when used in an ELISA (Pollock *et al*., 2013).

The present work has been motivated by drawbacks experienced with current TB detection methods that include, delays in the turn-around time for test results, affordability, the need for skilled personnel and inability to detect LTBI (Dorman, 2010; Find, 2012; Dayal., *et al*., 2008; Ben-Selma *et al*., 2011; Harrington, 2015; Zhang *et al*., 2015). In addition, current assays based on the detection of antibody e.g. the detection of serum antibodies to the 38 kDa, 16 kDa and ESAT6 proteins (Standard Diagnostics, South Korea), showed inconsistent results and poor sensitivity and specificity, especially in high HIV burdened areas such as Africa. (WHO, 2011b).

The rationale for the present study was to produce a reagent for rapid antigen detection in the diagnosis of TB. This study has been motivated by previous studies have demonstrated the high specificity and sensitivity of anti-ESAT6 MAbs when used in an ELISA for the detection of ESAT6 from sputum culture supernatant and pleural effusion (Leng *et al*., 2014). Feng *et al*. (2011) developed an ELISA using anti-ESAT6 MAbs for the rapid detection of TB where biotin-streptavidin enzyme labelled conjugate was used to increase the sensitivity of an ELISA for the detection of low levels of target protein (Feng *et al*., 2011).

Previously, a setback in using rapid detection methods was that the clinical samples such as urine and sputum required bacterial culture in a Biosafety level (BSL)-3 facility prior to testing (Namba, 2010). Namba's disclosure provided evidence that rapid assays may be carried out on non-cultured samples, providing safe, non-invasive and accurate results. Although the sample requires pre-treatment for bacterial inactivation prior to testing, the invention reduced the risk of infection to laboratory staff, negated the use of expensive equipment, skilled personnel and reduced the turnaround time for results (Namba, 2010).

One of the prominent biomarkers that have been investigated in the diagnosis of TB, is dormancy antigen ESAT6. The expression of ESAT6 in both active and inactive states of disease accentuates its role in the diagnosis of active and latent infection from individuals that have been BCG-vaccinated (Velayudhan *et al*., 2011; Bekmurzayeva *et al*., 2013; Tucci *et al*., 2014). ESAT6 was shown to detect latent TB with more sensitivity and specificity than TST (Farshadzadeh *et* al., 2010). The apoptopic ability of this antigen results in disease dissemination, making it the ideal diagnostic target (Ahmad, 2011). The extracellular location of ESAT6 together with the presence of B-cell epitopes on the surface of this antigen makes it the ideal target for anti-ESAT6 MAbs in the diagnosis of TB (Harboe *et al*., 1998; Jiang *et al*., 2013).

Furthermore, the multi-epitopic nature of this antigen is significant for the development of an ELISA for the diagnosis of TB (Dai *et al*., 2012). This secretory protein is present in sample matrices such as sputum, blood and pleural fluid. Based on these findings, ESAT6 was selected, in the present study, as a biomarker for the development of a rapid test for the early diagnosis of TB (Mukundan *et al*., 2012).

Some of these characteristics, however, are not unique to ESAT6. There is evidence that the 38 kDa protein also has the ability to induce apoptosis in infected macrophages resulting in the release of the bacilli from the host reservoir leading to disease progression (Lim *et al*., 2015). CFP10, Ag85 and the 16 kDa protein on the other hand do not share the apoptopic abilities of both the 38 kDa and ESAT6 proteins (Ahmad, 2011). CFP10, Ag85, LAM, TB7.7, 16 kDa and 38 kDa proteins are secreted into sample matrices and are responsible for the pathogenicity of *M. tuberculosis*. Some of these biomarkers have been extensively researched by diagnostic test manufacturers and have been used in IGRAs, IHC, Biosensing, DHR, ELISA and lateral flow assays (Farshadzadeh *et al*., 2011; Mukundan *et al*., 2012; Jiang *et al*., 2013).

Researchers have further investigated a combination of TB7.7 and ESAT6 in IGRAs, for the detection of LTBI (Wang *et al*., 2013). These results have therefore encouraged the development of a rapid test that would be able to detect a combination of biomarkers for the diagnosis of TB (Zhang *et al*., 2015). The 16 kDa protein has been described as the most immunogenic, latency protein. This protein may therefore be considered as a potential candidate in combination with ESAT6, for the early detection of TB. The development of an ELISA or lateral flow test for the detection of a combination of TB7.7, 16 kDa and ESAT6 may offer the sensitivity and specificity required for the rapid and early detection of TB (Belay *et al*., 2015).

It has further been demonstrated that recombinant TB-specific antigens have potential for the development of rapid tests like the ELISA and lateral flow test. Hence recombinant DNA technology was used in the present study to clone and express recombinant ESAT6 protein (Leng *et al*., 2014). In this work, ESAT6, ligated into pGEX6P-1 expression vector and transformed into *E. coli* strain XL-1 Blue, produced the recombinant protein in the soluble form. It has been shown that protein in the soluble form is important for downstream processes such as protein purification by affinity chromatography (Jiang *et al*., 2013). There is further evidence that non-peptide fusion partners such as GST, MBP and Protein A, enhances protein solubility(Rosanno and Ceccarelli, 2014). Therefore recombinant ESAT6 was tagged to GST in the current study.

In a similar study, Farshadzadeh *et al*. (2010) successfully produced ESAT6 in a soluble form using expression vector TOPO10, transformed into *E. coli* strain BL21 and purified using Nickel NTA affinity chromatography (Farshadzadeh *et al*., 2010). Other studies compared poorly when the use of expression vectors pQE30 and pET22b and *E. coli* strains TG1 and BL21, produced ESAT6 as an insoluble protein (Wang *et al*., 2005; Meher *et al*., 2006; Mukherjee *et al*., 2007).

In addition to promoting purification by affinity chromatography, the GST tag enhanced immunogenicity of ESAT6 in the immunisation of Balb/C mice. The immunogenicity of ESAT6/GST was also demonstrated in a study by Ebrahimi *et al*. (2010), in which the Balb/C mice immunisation schedule entailed 100µl ESAT6/GST as the primary injection and for enhanced immunisation a month later (Ebrahimi *et al*.,2010).

The best immune responding mouse, in this study, then received a final antigen boost three days before the hybridoma fusion. Studies have shown that different immunisation schedules have also been successful. Six week old Balb/C mice that were immunised with 200µl of antigen once in two weeks, repeated four times, also produced a successful hybridoma fusion (Feng *et al*., 2011).

In the same study, ESAT6 was joined to CFP10 by a glycine peptide chain linker. The resultant 27 kDa fusion protein was used as an immunogen that successfully produced six MAbs. These MAbs were validated by ELISA with a 95.4% positive detection rate and 100% negative detection rate and therefore had the potential for use as a diagnostic tool for TB (Feng *et al*., 2011).

Limitations experienced in the present study have not compromised the validity of results obtained. However, due to time and the labour intensive techniques involved in hybridoma production, it was not practical to produce MAbs towards more than one biomarker at a time. As previous studies have indicated that TB-specific biomarkers, when used in combination, enhance the sensitivity of a diagnostic assay it would have been favourable to produce MAbs against other biomarkers such as TB7.7 (Zhang *et al*., 2015).

Due to the scope and the financial constraints of the study, the specificity of the ELISA using anti-ESAT6 MAbs as the coating antibody, could not be evaluated. Future work would therefore require that patient samples be tested for the presence of ESAT6 antigen (Feng *et al*., 2011In addution, these MAbs when conjugated to colloidal gold, may have potential for use in a lateral flow test, ideal for use at the POC (Mdluli *et al*., 2014).

A key factor in the production of MAbs is the selection of an appropriate myeloma fusion partner. The present study has produced three murine MAbs using mouse myeloma cell line NS0-1, as studies have indicated that mouse myeloma cell lines are favoured over rats and goats although their fusion partners are also commercially available (Pytela *et al*., 2012). The production of murine MAbs have also been successful with the use of SP2/0 mouse myeloma cells (Leng *et al*., 2014). However, for antigens that have been poorly immunogenic in mice, rabbit myeloma cell line 240E-1 has been successful in producing MAbs in rabbits. Furthermore, there is evidence that MAbs produced in rabbits have a higher affinity than those MAbs that have been produced in mice. It is therefore proposed that future work include the immunisation of rabbit with recombinant ESAT6 in order to evaluate immunogenicity (Pytela *et al*., 2012).

Success of the present study, further supports the production of human MAbs by fusing anti-ESAT6 antibodies to human myeloma cell line, Karpas 707H (Karpas *et al*., 2001). Human MAbs have the potential for use in passive immune therapy (Casadevall, 2002). This is of particular benefit in TB cases with HIV co-infection where active immunisation is not an option. This was demonstrated in mouse models where TB immunotherapy was used in combination with other treatments even after host infection (Balu *et al*., 2011).

Passive immunisation with human MAbs towards TB is beneficial for immunocompromised individuals that are unable to develop antibodies to TB bacilli. The success of such an experiment would enable the immortalization of specific antibody producing B-cells that are produced *in vivo* by a TB infected individual. These hybridomas may prove to have tremendous advantages in immunotherapy, an alternate method of treating TB by helping the host eradicate the disease. Immunotherapy will effectively reduce the length of current TB treatments, reduce drug resistance and prevent reactivation of the disease (Bhattacharya *et al*. 2013).

Anti-ESAT6 MAbs have shown potential for use in immunotherapy that would effectively increase the survival rate of patients and have been shown to play a significant role in immunodiagnosis of the disease (Baumann, 2012; Wang *et al*., 2014; Lessem, 2015).

It is proposed that future work focus on evaluating the sensitivity of the ELISA by conjugation of anti-ESAT6 DE2-1 to biotin-streptavidin, for use as a detection antibody as this method has been successfully demonstrated (Feng *et al*., 2011). Anti-ESAT6 DE2-1 also has the potential for conjugation to colloidal gold in the development of a lateral flow test for use at the POC (Mdluli *et al*., 2014). Tests used at a community level, by first-contact healthcare workers would enable rapid and early diagnosis of TB, thereby facilitating prompt administration of anti-TB drugs in the control of the disease (Mdluli *et al*., 2014). However, literature has emphasised that there is no stand-alone test for early and rapid detection of TB and new tests are to be linked to the existing diagnostic algorithm (WHO, 2015).

In conclusion, the findings of this study has contributed to the current field of knowledge in the cloning and expression of soluble recombinant ESAT6, a significant biomarker responsible for immunodominance and virulence of TB. The current study further demonstrated that murine MAbs produced against recombinant ESAT6, may be used in a rapid test such as an ELISA for the detection of *M. tuberculosis*-specific ESAT6. Anti-ESAT6 DE2-1, KE10-1 and KE10-2 MAbs produced, have the potential as reagents for the rapid and early diagnosis of TB and is therefore aligned with the SDG for early detection, treatment and eventual eradication of the disease.

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APPENDIX A

A1. The silica method of DNA extraction from bacterial cultures

Preparation of reagent L1:

Materials

12 gram	guanidinium thiocyanate (GuSCN - Sigma)
10 ml	0.1 M Tris buffer, pH 6.4 (Merck)
0.26 gram	Triton X100 (Sigma)
2.2 ml	0.2 M EDTA (Merck Millipore)

Method

The reagents were dissolved in 90 ml distilled water and 1.5 ml aliquots of reagent L1 were stored at 4°C.

Preparation of Reagent L2:

Materials

12 gram	GuSCN
10 ml	0.1 M Tris buffer, pH 6.4

Method for preparation of silica particles

Sixty grams silica (Sigma Aldrich) was suspended in 500 ml distilled water. The solution was stirred at room temperature for 25 hours. The supernatant was discarded leaving behind about a 70 ml volume that was topped up to 500 ml with distilled water and shaken to resuspend. The solution was stirred for 5 hours at room temperature. The supernatant was discarded, leaving behind a 60 ml volume. HCL 600 µl of 32% (w/v) was added and vortexed. One millilitre aliquots of silica was autoclaved at 121°C for 20 minutes.

A2. Preparation of buffers

DNA electrophoresis buffer: 50 X 2 M Tris, 17.5 M glacial acetic acid 100% and 0.5 M EDTA (TAE) pH 8.0

Materials

242.0 gram	2 M Tris (Merck)
18.6 gram	0.5 M EDTA (Merck)
57.1 ml	17.5 M Glacial acetic acid (Merck)

Method

The materials above were added together, pH adjusted to 8.0 and made up to a final volume of 1 litre.

Preparation of 1 X Tris acetate EDTA (working stock)

A 1/50 dilution of 50 X TAE was prepared by adding 6 ml of 50 X TAE to 294 ml distilled water.

A3. Transformation of competent *E. coli* strain XL-1 Blue with T-vector/ESAT6

Preparation of TSB

TSB Bacterial Transformation Buffer	
PEG3350 (Serva)	2 gram
LB media	16.6 ml
Sterile 1 M MgCl2 (Merck)	0.2 ml
Sterile 1 M MgSO4 (Merck)	0.2 ml
Autoclave	35 minutes at 121°C
Cool	
DMSO (Merck)	1 ml added aseptically
Total volume	20 ml
Mix & store in fridge	

A4. Method of large scale recombinant ESAT6 GST Fusion Protein Expression

Tris saline with 0.02% NaN3
(4 litres are required for 1 clone)

0.1 M NaCl (58.44 g/mol)
0.05 M Tris (121.14 g/mol)
0.02% sodium azide (Merck -65.01 g/mol)

NaCl	23.36 gram
Tris	24.16 gram
Sodium azide	0.8 gram
Final Volume	4 L

The pH was adjusted to 8.0 using 1 M HCL

A5. Purification of recombinant ESAT6 GST by sepharose 4B Glutathione affinity chromatography

Preparation of Tris saline with reduced Glutathione Elution Buffer
One hundred and fifty millilitres of Tris saline with 0.02% NaN3 was added to 10 mM reduced Glutathione (Sigma Aldrich) at 0.4608 gram/mol. The mixture was stored at 4°C.

A6. Preparation of SDS PAGE electrophoresis buffer

A 1x buffer was prepared from 5 x stock (materials listed below) i.e. 40 ml buffer stock + 160 ml water.

5 x SDS / TRIS / Glycine Electrophoresis Buffer
Materials
15 gram TRIS hydroxymethyl

72 gram Glycine (Merck)

5 gram SDS

Method

The above materials were mixed. The pH adjusted to 8.4 and made up to 1 L.

A7. Preparation of Precission Protease Cleavage Buffer

Materials

Reagent	Gram / 2L
50 mM TRIS HCL mw= 121.14	12.14
150 mM NaCl mw = 58.44	17.52
1 mM EDTA mw= 372.27	0.744
1 mM DTT mw = 154.2	0.308

Method

Reagents were weighed out into a beaker. Two hundred microliters of Triton X-100 was added (100 µl in 1L) equivalent to a final concentration of 0.01%. One and a half liters of water was added to the beaker which was placed on a magnetic stirrer. The pH was adjusted to 7.0 before being topped up with water to a total volume of to a 2 L. The buffer was chilled at 4°C before use.

A8. Preparation of a 0.2 M carbonate solution pH 9.5

23.4 gram NaHCO3 (molecular weight 84.01 - Merck)

12.7 gram Na2CO3 (molecular weight 105.99 - Merck)

Dissolve in 1800 ml of distilled water

pH adjusted to 9.5

Volume adjusted to 2 L with Distilled water

Preparation of a 1 mM sodium acetate solution pH 4.0

0.0360 gram Sodium Acetate Trihydrate (molecular weight 136.08 - Merck)
Dissolve in 80 ml distilled water
pH adjusted to 4.0
Volume adjusted to 100 ml

Preparation of 0.2 M sodium periodate – (BDH Chemicals)

Molecular weight = 213.9 = 1 M (g/l)
0.2 M = 42.78 g/l
= 0.043 g/ml

Preparation of conjugate stabilising buffer

Fifty milliliters of glycerol was mixed with 49 ml 0.05 M Tris-0.1 M NaCl buffer pH 8. One millilitre of bovine serum albumin was added and mixed well.

A9. Preparation of TST

Materials for 10 x concentrate

4 L	Reverse Osmosis (RO) water
351 gram	Sodium chloride (Merck)
24 gram	Tris (Merck)
20 ml	
Tween 20 (Merck)	

Method

Sodium chloride, tris and tween 20 were weighed out and dissolved in 4 litres of reverse osmosis (RO) water. Nine thousand and two hundred microliters of Hydrochloric acid was added and the concentrate was diluted by topping up to 40 L with RO water. The pH was adjusted to 8.0.

A10. Preparation of media

Lysogeny broth (LB) for transformation of XL-1 Blue *E. coli* with T-vector (Blue / white screening)

250 ml Lysogeny agar plates:

Materials

2.5 gram	tryptone (Oxoid)
1.25 gram	yeast extract (Oxoid)
1.25 gram	NaCl (Merck)
5 gram	bacterial agar (Oxoid)
200 ml	RNAse free Water
500 µl	Ampicillin (Sigma)
1250 µl	IPTG (Promega)
400 µl	X-gal (Sigma)

Method

The dry ingredients were dissolved in 200 ml of distilled water at pH 7.0. The volume was topped up to 250 ml autoclaved. When the media cooled to 45°C, ampicillin, IPTG and X-gal were added. Five hundred microliters of 50 mg/ml ampicillin stock was added to 250 ml media (1/500 dilution). One thousand, two hundred and fifty microliters of 0.1 M IPTG stock was added to 250 ml media (1/200 dilution of IPTG ≡ 0.5 mM). Four hundred microliters of 50 mg/ml X-gal stock was added to 250 ml media (1/625 dilution of X- gal ≡ 80 µg/ml). Culture plates were poured, set and stored at 4°C.

Preparation of working stocks:

0.1 M IPTG

Six hundred milligram of IPTG was dissolved in 25 ml water. The solution was filter sterilised using a 0.2 µ filter and stored at 4°C.

50 mg/ml X-gal

Two hundred milligram of X-gal was dissolved in 4 ml *NN*-dimethylformamide. The container was covered in foil and stored at -20°C.

50 mg/ml Ampicillin

Five hundred milligram of ampicillin was dissolved in 10 ml water. The solution was filter sterilised using a 0.2 µ filter and stored at -20°C.

A11. Transformation of competent *E. coli* strain XL-1 Blue with T-vector/ESAT6

Luria-Bertani media

1.0 gram	Tryptone (Oxoid)
0.5 gram	Yeast extract (Oxoid)
1.0 gram	NaCl (Merck)
80 ml	Milli-Q water

Tryptone, yeast extract and NaCl was dissolved in the quantities above, the pH adjusted to 7.0 and the media made up to a final volume of 100 ml.

Five millilitres of LB media with 5 mg/ml tetracycline, was inoculated with 80 µl *E. coli* strain XL-1 Blue strain and grown overnight at 37°C. The following day a 1/500 dilution (40 µL of overnight culture of *E. coli* strain XL-1 Blue was added to 20 ml LB media) of the overnight culture in LB media was made in a 250 ml conical flask and grown to an early log phase (A_{600} = 0.3 – 0.6). This took between 3 to 4 hours at 37°C. The cell culture was poured into a sterile 50 ml conical tube that had been pre-cooled on ice and pelleted at 1600 x g for 30 minutes at 4°C. The cells were resuspended in cold transformation storage buffer (TSB) and incubated on ice for a further 10 minutes.

One millilitre of competent *E. coli* strain XL-1 Blue cells was aliquoted into 1.5 ml Eppendorf tubes. One hundred microliters of pUC19, 0.1ng was added to the Positive Transformation tube. To the Positive Ligation tube, 0.1 ml of 0.1 ng T-vector was added. The Negative control tube contained XL-1 Blue cells only. One hundred microliters of MR1, MR2 and MR3 were added to the respective eppendorfs and incubated on ice for 30 minutes.

Description of transformation reaction mix

Transformation tube	XL-1 Blue cells (µl)	Ligation mix (µl)
Negative Transformation	100	0
Positive Transformation	100	4 µl pUC19 vector
Positive Ligation	100	5 µl T-vector
MR1	100	5
MR2	100	5
MR3	100	5

Nine hundred microliters of cold TSB was added to all eppendorfs and incubated for 1 hour in a 37°C water bath to allow for the expression of the antibiotic resistance genes. Transformation mixtures were plated out onto LB plates containing ampicillin; IPTG and X-gal, in order to select for transformants. LB media was prepared by adding ampicillin at 50 mg/ml, isopropyl β-D-thiogalactopyranoside 0.5 mM and 5-bromo-4 chloro-3-indolyl-β-D-galactoside (X-gal) at 80 µg/ml and made up to a final volume of 250 ml (Appendix A10.1). One hundred microliters of each of the controls were plated out. On plates with MR1, MR2 and MR3, 200 µl; 300 µl and 400 µl respectively were plated out. The plates were dried, inverted and incubated at 37°C overnight.

A12. Method of large scale recombinant ESAT6 GST Fusion Protein Expression

Recipe for 1 L of LB media

Tryptone	10 gram
Yeast extract	5 gram
NaCl	10 gram
Milli-Q water	950 ml
pH	7.0
Final Volume	1 liter

Four litres of LB media were prepared in Erlenmeyer flasks.

Media was adjusted to pH 7.0 using 5 M NaOH and 1 M HCl. Media 100 ml from each 1 L flask was aliquoted into each of 4 x 250 ml flasks. Four, 900 ml and 4 x 100 ml aliquots of media were autoclaved at 121°C for 20 minutes. In the afternoon, 100 µl of ampicillin (50 mg/ml) was added to the 250 ml flasks with 100 ml LB media. The LB media was inoculated with 100 µl of recombinant ESAT6 clone 1 glycerol stock and grown on a shaker at 40%, overnight at 37°C.

A13. Restriction Digests

Sample	DNA added (µl)	RE added (µl)	Buffer added (µl)	H2O added (µl)	Total (µl)	T°C	Time
Clone 6	7.02	2+2	4	24.98	40	37	2 hrs
Clone 6	7.02	2+2	4	24.98	40	37	2 hrs
Clone 6	7.02	2+2	4	24.98	40	37	2 hrs
Clone 6	7.02	2+2	4	24.98	40	37	2 hrs
Total x4	28.08	8+8	16	99.92	160		

RE = Restriction enzyme

A14. Spectrophotometric analysis of purified ESAT6 DNA following transformation of competent *E. coli* strain XL-1 Blue with pGEX6P-1

Spectrophotometric analysis of purified DNA

Sample	Mean OD_{260}nm	DNA Concentration (ng/ml)	DNA volume (µl)
1	0.025	62.5	16
2	0.024	60	16.67
3	0.026	65	15.38
4	0.021	52.5	19.05
5	0.013	32.5	30.77
6	0.023	57.5	17.39
7	0.015	37.5	26.67
8	0.031	76.25	13.11
9	0.021	51.25	19.51
10	0.015	36.25	27.59
11	0.026	65	15.38
12	0.022	55	18.18
13	0.020	50	20
14	0.033	82.5	12.12
15	0.028	70	14.29
16	0.034	85	11.76
17	0.020	50	20
18	0.023	57.5	17.39
19	0.033	82.5	12.12

20	0.034	83.75	11.94
21	0.028	68.75	14.55
22	0.026	65	15.38
23	0.024	60	16.67
24	0.030	75	13.33

Volumes of reagents used for the Restriction digestion of colonies 1 – 24

Sample	*DNA (µl)	*Bam* H1 + *Eco* R1 (µl)	Buffer (µl)	H2O (µl)	Total (µl)
1	16	2+2	4	16	40
2	16.67	2+2	4	15.33	40
3	15.38	2+2	4	16.62	40
4	19.05	2+2	4	12.95	40
5	30.77	2+2	4	1.23	40
6	17.39	2+2	4	14.61	40
7	26.67	2+2	4	5.33	40
8	13.11	2+2	4	18.89	40
9	19.51	2+2	4	12.49	40
10	27.59	2+2	4	4.41	40
11	15.38	2+2	4	16.62	40
12	18.18	2+2	4	13.82	40
13	20	2+2	4	12	40
14	12.12	2+2	4	19.88	40
15	14.29	2+2	4	17.71	40
16	11.76	2+2	4	20.24	40
17	20	2+2	4	12	40
18	17.39	2+2	4	14.61	40
19	12.12	2+2	4	19.88	40
20	11.94	2+2	4	20.06	40
21	14.55	2+2	4	17.45	40
22	15.38	2+2	4	16.62	40
23	16.67	2+2	4	15.33	40
24	13.33	2+2	4	18.67	40

A15. Preparation of SDS-PAGE gel

The Bio Rad model 360 mini vertical slab cell was assembled and clamped into position. The bottom of the apparatus was sealed by being placed over a layer of 6% molten agarose and allowing the agar to polymerise.

Bis – Acrylamide (36.5: 1 cross-linker ratio)

Materials

0.8 gram *N, N'* – methylene – Bis – acrylamide (Sigma)

29.2 gram Acrylamide (Sigma)

Method

The above materials were dissolved in 60 ml distilled water at 37°C. Once dissolved, the solution was made up to 100 ml, filter sterilised and stored at 4°C in a dark bottle.

Preparation of 15% Running gel

Materials

1.9 ml distilled water

2.5 ml TRIS buffer pH 8.8

5 ml Bis Acrylamide (Sigma) 36.5:1 cross-linker ratio detailed below

0.1 ml 10% SDS

0.5 ml 1.5% APS (Roche)

10 µl TEMED (Sigma) was used to catalyse polymerization and was added when gel was ready to use.

Method

A 1.5% ammonium persulphate (APS) was prepared by dissolving 0.1 gram APS in 5 ml of water. With the use of a syringe and blunt ended needle, the running gel was set in the gel apparatus and was left to polymerise for 10 – 20 minutes at room temperature.

Preparation of 5 ml of 5% stacking gel

Materials

5.6 ml distilled water

2.5 ml 0.5 M TRIS buffer pH 6.8

1.25 ml Bis acrylamide

0.1 ml 10% SDS

0.5 ml 1.5% APS

10 µl TEMED was added when running gel was set

Method

The excess water over the top of the running gel was removed using blotting paper. When the running gel had set, then the TEMED was added to the stacking gel which was poured above the polymerised running gel. The stacking gel had polymerised in 20- 30 minutes. Left over agar from both running and stacking agar was left behind in the beaker as an indicator of when polymerization was complete.

Reduction and alkylation of protein.

Reducing agent (reduces disulphide bonds):

A 1/10 v/v 0.1 M dithiothreitol (DTT) / dithioerythritol (DTE) was diluted in distilled water i.e. 0.771 gram was dissolved in 50 ml distilled water and incubated at 37°C for 30 minutes.

Alkylating agent

Added 1/10 v/v 0.22M Iodoacetamide (IA)

2.03 grams of IA was dissolved in distilled water, made up to 50 ml and stored in the dark.

Method

Five microliters of 0.1 M DTT was added to 50 µl of protein and incubated for 30 minutes in a 37°C water bath. IA 5 µl was added and vortexed.

Sample preparation

Materials

60 µl reduced and alkylated sample

6 µl 10% SDS

5 µl pryonin G tracking dye

Few granules sucrose

Method

Water was boiled in a beaker in the microwave. The materials above were added together and immersed in the boiled water for 3 minutes.

Destaining solution (2.5 L)

Materials

250 ml	Glacial acetic acid
625 ml	Isopropanol
1625 ml	distilled water

Method

The above materials were mixed.

Coomassie Blue

One gram of Coomassie Brilliant Blue was stirred for 30 minutes in 325 ml distilled water. Fifty millilitres of glacial acetic acid and 125 ml Isopropanol were added and mixed well.

A16. Removal of GST tag by enzymatic cleavage using PreScission Protease

A 45 ml volume of recombinant ESAT6 GST was dialysed in 2 L Cleavage Buffer for 48 hours at 4°C.

Cleavage of GST tag in solution

Twenty millilitre of eluate was extensively dialyzed against cleavage buffer to ensure the removal of reduced glutathione from the sample. One microliter of Protease (GE Healthcare) was added for every 100 µg of fusion protein in the eluate. The sample was placed on a rocker at 4°C for 4 hours. Once the digestion was complete, the sample was applied to a washed and equilibrated Glutathione Sepharose column to remove the GST portion of the fusion protein and the precision protease from the protein of interest. The unbound material was collected and dialysed for 48 hours in TRIS saline at pH 8.0 with 0.02% NaN_3.

A17. Concentration of proteins in PEG 6000

Materials

anti-ESAT6 MAb

PEG 6000 (Merck)

Dialysis tubing with a 3,500 molecular weight cut off
TRIS saline + 0.02% NaN3 (method for preparation described above)

Method
PEG 6000 is highly hygroscopic enabling moisture to flow out of the protein and into the PEG powder, thereby concentrating the protein. The dialyzed protein was concentrated in PEG 6000. The protein was placed in narrow bore dialysis tubing with a 3,500 molecular weight cut off. The tubing with protein was placed on a bed of PEG 6000 and covered with a layer of PEG 6000 and left at room temperature. The volume was monitored until the required volume was reached. The protein concentration was determined by spectrophotometric analysis. The spectrophotometer was blanked against TRIS saline + 0.02% NaN3and the 3 ml protein sample at was read at A_280nm.

A18. Preparation of Bosine 2%

Materials (for 100 bottles):

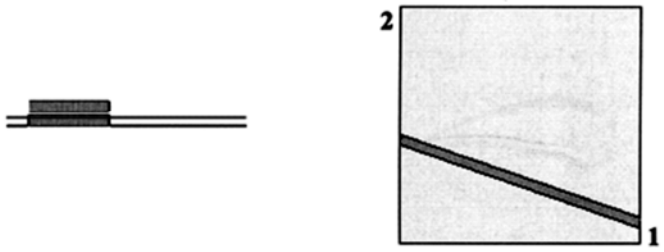
117.66 ml	Sheep serum
117.66 ml	Bovine serum
564 ml	10X Tris saline, pH 8.0

The mixture is filtered through a 0.45 µ and 0.22 µ filter. Volumes of 6.6 – 6.8 ml was dispensed into glass bottles and freeze dried. The lyophilised product is reconstituted for use by the addition of distilled water 50 ml.

A19. *ESAT6* gene sequence result

Sequence 1: gi|90811459|Mycobacterium tuberculosis isolate 7 Esat6 gene, complete cds
Length = 288 (1 .. 288)

Sequence 2: lcl|65536
Length = 840 (1 .. 840)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

```
 Score =  554 bits (288),  Expect = 7e-155
 Identities = 288/288 (100%), Gaps = 0/288 (0%)
 Strand=Plus/Minus

Query  1    ATGACAGAGCAGCAGTGGAATTTCGCGGGTATCGAGGCCGCGGCAAGCGCAATCCAGGGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  366  ATGACAGAGCAGCAGTGGAATTTCGCGGGTATCGAGGCCGCGGCAAGCGCAATCCAGGGA  307

Query  61   AATGTCACGTCCATTCATTCCCTCCTTGACGAGGGGAAGCAGTCCCTGACCAAGCTCGCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  306  AATGTCACGTCCATTCATTCCCTCCTTGACGAGGGGAAGCAGTCCCTGACCAAGCTCGCA  247

Query  121  GCGGCCTGGGGCGGTAGCGGTTCGGAGGCGTACCAGGGTGTCCAGCAAAAATGGGACGCC  180
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Sbjct  246  GCGGCCTGGGGCGGTAGCGGTTCGGAGGCGTACCAGGGTGTCCAGCAAAAATGGGACGCC  187

Query  181  ACGGCTACCGAGCTGAACAACGCGCTGCAGAACCTGGCGCGGACGATCAGCGAAGCCGGT  240
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Sbjct  186  ACGGCTACCGAGCTGAACAACGCGCTGCAGAACCTGGCGCGGACGATCAGCGAAGCCGGT  127
Query  241  CAGGCAATGGCTTCGACCGAAGGCAACGTCACTGGGATGTTCGCATAG  288
            ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126  CAGGCAATGGCTTCGACCGAAGGCAACGTCACTGGGATGTTCGCATAG  79
```

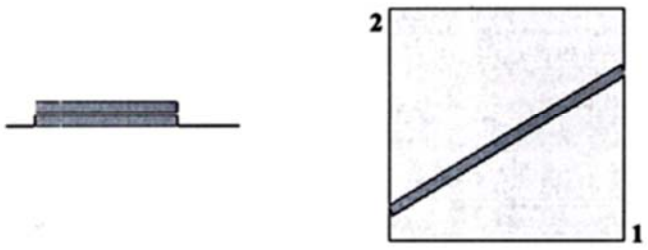
http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi?0

A20. *ESAT6* gene sequence results

Sequence 1: gi|90811459|Mycobacterium tuberculosis isolate 7 Esat6 gene, complete cds
Length = 288 (1 .. 288)

Sequence 2: lcl|65536
Length = 473 (1 .. 473)

Clone 1



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

```
 Score =  554 bits (288),  Expect = 7e-155
 Identities = 288/288 (100%), Gaps = 0/288 (0%)
 Strand=Plus/Plus

Query  1    ATGACAGAGCAGCAGTGGAATTTCGCGGGTATCGAGGCCGCGGCAAGCGCAATCCAGGGA  60
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Sbjct  62   ATGACAGAGCAGCAGTGGAATTTCGCGGGTATCGAGGCCGCGGCAAGCGCAATCCAGGGA  121

Query  61   AATGTCACGTCCATTCATTCCCTCCTTGACGAGGGGAAGCAGTCCCTGACCAAGCTCGCA  120
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Sbjct  122  AATGTCACGTCCATTCATTCCCTCCTTGACGAGGGGAAGCAGTCCCTGACCAAGCTCGCA  181

Query  121  GCGGCCTGGGGCGGTAGCGGTTCGGAGGCGTACCAGGGTGTCCAGCAAAAATGGGACGCC  180
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Query  241  CAGGCAATGGCTTCGACCGAAGGCAACGTCACTGGGATGTTCGCATAG  288
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Sbjct  302  CAGGCAATGGCTTCGACCGAAGGCAACGTCACTGGGATGTTCGCATAG  349
```

http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi?0

A21. Preparation of conjugate diluent

Materials for 600 bottles

192.0 ml	Sheep serum
192.0 ml	Bovine serum
192.0 ml	Human serum
326.4 ml	Tris saline 10x concentrated

Method

The materials above were measured into a 5 L plastic beaker and mixed on a magnetic stirrer for 30 minutes. The conjugate diluent was filtered through a 0.45 and 0.2 µ filter and 1.4 ml volumes were dispensed into 10 ml glass vials. The vials were freeze dried and capped and stored at 4°C. The lyophilised product is reconstituted for use with the addition of 6 ml distilled water.

APPENDIX B

1. Animal Ethics Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

Research office
Animal Ethics Research Committee

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email: Animalethics@ukzn.ac.za

01 June 2011

Reference: 077/11/Animal

Mrs N Pillay
Biochemistry, Genetics & Microbiology Department
UKZN
WESTVILLE CAMPUS

Dear Mrs Pillay

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2011 on the following project:

Production of murine monoclonal antibodies to ESAT46 of Mycobacterium tuberculosis

[redacted] Coetzer
Chairperson: Animal Ethics Sub committee

Cc Registrar, Prof. J Meyerowitz
Research Office, Mr N Moodley
Head of School, Prof. B Pillay
Supervisor, Prof. B Pillay
Biomedical Resource Unit, Dr S Singh

1910 - 2010
100 YEARS OF ACADEMIC EXCELLENCE IN KWAZULU-NATAL

Founding Campuses:
- Edgewood
- Howard College
- Medical School
- Pietermaritzburg
- Westville

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

Research office
Animal Ethics Research Committee

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email: animalethics@ukzn.ac.za

14 March 2012

Reference: 029/12/Animal

Mrs Nethi Pillay
School of Life Sciences
Westville Campus

Dear Mrs Pillay

Renewal: Ethical Approval of Research Project on Animals

I have pleasure in informing you that on recommendation of the review panel, the Animal Ethics Sub-committee of the University Research and Ethics Committee has granted ethical approval for 2012 on the following project:

"Production of murine monoclonal antibodies to ESAT6 of Mycobacterium tubercolosis."

[redacted]air)
COMMITTEE

Cc Registrar, Prof. J Meyerowitz
Research Office, Mr Nelson Moodley
Head of School & Supervisor, Prof. B Pillay
BRU, Dr S Singh

1910 - 2010
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- Westville

B2. Preparation of 0.1 M Borate – 0.15 M NaCl buffer, pH 8.5

61.8 gram	Boric acid (Merck)
87.7 gram	NaCl (Merck)

Boric acid and NaCl dissolved in distilled water and the pH adjusted to 8.5 using 5 M NaOH. The solution was made up to a final volume of 10 liters.

B3. Preparation of 0.05 M phosphate – 0.15 M NaCl buffer (PBS), pH 7.2

246 gram	Disodium hydrogen phosphate $12H_2O$ (Merck)
38.8 gram	Potassium dihydrogen phosphate (Merck)
175.4 gram	Sodium chloride (Merck)
4 gram	Sodium azide (Merck)

The chemicals were dissolved in 20 liters of distilled water and the pH adjusted to 7.2 using 5 M NaOH.

B4. Preparation of 0.1 M Sodium citrate

58.8 gram	Trisodium citrate dihydrate (Merck)

Trisodium citrate dihydrate was dissolved in 2 liters of distilled water and using concentrated HCl, the pH may be adjusted to 3, 4 or 5.

B5. Preparation of 6 M Guanidine hydrochloride

229 gram	Guanidine HCl (Sigma)

The chemical was dissolved in 400 ml distilled water.

B6. Preparation of 2M H_2SO_4

98.08 gram 1 M H_2SO_4

Add 196.16 ml H_2SO_4 slowly down the side of a beaker containing 500 ml of distilled water. The beaker may be kept on ice because of the heat that is generated. The volume was then made up to 1 L by adding distilled water.

B7. Preparation of OPD Substrate

6 gram OPD.2HCl

200 ml Distilled water

O- Phenylenediamine Dihydrochloride (OPD; Sigma) is light sensitive and was therefore prepared in a dark glass beaker. The OPD was brought to room temperature before use. Two hundred millilitres of distilled water was added to 6 gram of OPD.2HCL and the solution was mixed for 30 minutes on a magnetic stirrer. One millilitre volumes of OPD was dispensed into 30 ml amber glass vials and freeze-dried. Once freeze-dried, the vials were capped, labelled and stored at 2-8°C.

B8. P/N ratios of anti-GST antibody were calculated against serial dilutions of recombinant GST

Dilutions of GST (µg/ml)	P/N ratios of anti-GST DE6 diluted at 1:500 v/v
0.78	2.0
1.56	2.5
3.125	2.6
6.25	3.1
12.5	3.6
25	4.6
50	5.4
100	5.4

B9. Optimization of GST HRP dilution based on varying GST concentrations in an ELISA.

GST concentration µg/ml	Optical densities at varying GST HRP Dilutions	
	1:1000	**1:3000**
0.78	1.07	0.36
1.56	1.08	0.46
3.125	1.12	0.49
6.25	1.35	0.57
12.5	1.45	0.66
25	1.68	0.85
50	2.08	1.02
100	2.26	1.2

B10. Positive/negative ratios of anti-ESAT6 antibodies against serial dilutions of recombinant ESAT6.

Concentration of recombinant ESAT6 (µg/ml)	P/N ratios of anti-ESAT6 antibody diluted 1:500	P/N ratios of anti-ESAT6 antibody diluted 1:1000
0.78	176.66	166.67
1.56	316.67	303.33
3.125	480	456.67
6.25	566.67	483.33
12.5	556.67	510
25	533.33	486.67
50	553.33	480
100	550	526.67

B11. Description of the cloning strategy