STANDARDISATION AND COMMERCIALISATION OF A FILARIAL ANTIBODY TEST FOR USE IN THE GLOBAL LYMPHATIC FILARIASIS ELIMINATION PROGRAMME

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In partial fulfilment of the requirements for the
Degree of Doctor of Philosophy
in the College of Public Health, Medical
and Veterinary Sciences,
James Cook University, Queensland, Australia

STATEMENT OF SOURCES DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for any degree in another university or institution of tertiary education. All the information from the works of others, published or unpublished has been acknowledged in the text and in the reference list.

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PREFACE

This translational research is a collaborative work of the College of Public Health, Medical & Veterenary Sciences, in the Division of Tropical Health and Medicine, James Cook University (JCU) and Cellabs Pty Ltd.

This research was made possible by the support and generosity of Dr Anthony Smithyman, managing director of Cellabs Pty. Ltd., for providing me with a research laboratory to carry out all experiments, including equipment, accessories, computers and financial assistance for all other associated costs for my research work. Cellabs has approved the use of proprietary methods for development of the Filariasis Bm14 Antibody CELISA.

This research was also made possible by the full scholarship granted by the College of Public Health, Medical & Veterinary Sciences, in the Division of Tropical Health, under the following candidature committee:

Head of Academic Group: Dr Sue Devine
Research Student Monitor/Chair: A/Prof Jeffrey Warner
Advisory Panel: A/Prof. Wayne Melrose, A/Prof. Dr Patricia Graves, Prof Rick Speare
Associate Dean of Research Education: A/Prof Jeffrey Warner

The research-grade Bm14 ELISA was the original work of Professor Gary Weil and his group at the School of Medicine, Washington University in St. Louis, MO, USA. This research-grade ELISA was brought to Cellabs Pty Ltd for standardisation and commercialisation. The design and development work commenced in 2002, headed by Dr G-Halli Rajasekariah, Research & Development, Cellabs Pty Ltd. I worked as the assistant technical officer. From the development of the kit described in Chapter 2 through to the release of the prototype kit in 2007, I performed and analysed a majority of the experiments under the guidance of Dr Rajasekariah. The optimisation and validation of the kit from 2008 through to the release of Mark II in 2010 was my original work. All the data presented in Chapter 2 were selected experiments that I had performed and analysed. All the other experiments including data analysis for the rest of the chapters were my original work except for Chapter 7, which were the work of external evaluators of the Bm14 Antibody CELISA, acknowledged accordingly.

The recombinant Bm14 antigen was licensed to Cellabs Pty Ltd by the Barnes-Jewish Hospital, St. Louis, Missouri, USA in an agreement made in 2008 for the development of an ELISA for the detection of antibodies for lymphatic filariasis. The Bm14 recombinant antigen was used for researching the assay performance in a rapid test, dipstick format that is complimentary to the ELISA, covered in Chapter 4. Additional work on optimisation and evaluation is required for commercialisation. The current assay is research use only and is not included for commercialisation use under the Cellabs Bm14 recombinant protein license agreement at the time this thesis was completed.

DEDICATION

I would like to dedicate this thesis to my daughter Maya and my late father, James.

To Maya, you have always been my light during those nights I stayed up to finish my writing. Thank you for being a good baby. To my father who taught me perseverance, each time I felt like giving up, I have always thought about you - this is for you.

ACKNOWLEDGEMENT

This thesis has been made possible through the contributions of many other people. I would like to thank my supervisor Associate Professor Wayne Melrose for his guidance and unwavering support. I consider myself fortunate to have a dedicated supervisor who believed that this story needed to be told. After retirement, Wayne chose to take an adjunct position at the University and continued to support me with his words of wisdom and encouragement that fostered my will to get to the finish line. For this, I am grateful. I would like to thank my advisors. Dr G-H Rajasekeriah has been my mentor from the beginning. He believed in me and without his encouragement, I would never have embarked on this challenging post-graduate study. I am grateful for his guidance, teachings, leadership and patience throughout the years of assay development and thesis writing. The guidance and feedback of Dr Patricia Graves during the thesis writing has been extremely helpful. Being an off-campus student, discussion with advisors can be difficult but Tricia has been quick to reply to my emails and arrange interstate phone calls. Her advice and feedback have given me the confidence to get through to the end. I would also thank Professor Richard Speare for his kind support at the beginning and halfway through this research. Dr Anthony Smithyman has been instrumental in this research work. He has always found the time out of his busy schedule to sit down with me each time I needed consultation. His wisdom and expertise in the field of tropical and neglected diseases and immunodiagnostics has been monumental in the progress of this research. For this, I am grateful.

And last but not the least, I would like to thank my colleague, Neil Marshall for all his help in running countless ELISAs, repeatedly recovering corrupted thesis files, proofreading and listening to my complaints and worries. My dearest family, I thank you for the love and support throughout these challenging times of further education. To my friends, Rebecca and Shaun, you don’t know how much your support, understanding and encouragement meant to me, I thank you both for always believing in me.

ABSTRACT

This thesis addresses an important research question of how to take a diagnostic test from the research and development laboratory to a commercially-available product that can be used reliably in a global disease control programme. Laboratory procedures, regulatory requirements and administrative steps required to standardise a filarial antibody test are covered. The question is answered by following the progress of a test developed for use in the Global Program to Eliminate Lymphatic Filariasis. The “lessons learnt” in this translational research apply to the development and commercialisation of any similar product.

The need for reliable diagnostic tools for defining end-points in transmission assessment surveys and post-intervention surveillance becomes imperative as the lymphatic filariasis elimination efforts are moving swiftly to achieve the set objectives by 2020. A variety of diagnostic methods are used, including the detection of microfilariae and antigen detection assays. These tests have served very well in the mapping and preventive chemotherapy phases of the elimination programme, but for the final stages of the programme, there is a strong belief that they may not be adequate to confirm that the transmission of infection has ceased. After a few rounds of preventive chemotherapy, these tests no longer have the same sensitivity due to low mf and antigen prevalence.

Exposure to infective larvae can take months or years before an adult worm becomes established, a patent infection develops and filarial antigen and mf can be detected. What is required is a robust test that can detect immunological changes in any individual after the successful implementation of preventive chemotherapy. The absence of filarial antibody is a key point for concluding that transmission has ceased, marking the elimination of lymphatic filariasis.

Assays for filarial antibodies have been available in the 1970s, but they are based upon the use of crude, whole-worm antigens. These consequently cross-reacted with other parasites, resulting with great concern for the lack of specificity. The “research-grade” methods are varied from one laboratory to the other, making comparisons between institutions virtually impossible. Also, research laboratories do not have the

expertise and capability of producing industry-standard commercial kits required for a global programme.

This thesis describes certain specific methods and techniques used in optimisation and standardisation of an ELISA based on a recombinant antigen Bm14. The Bm14 ELISA is specific to brugian and bancroftian filariasis and, therefore, ideal for use in lymphatic filariasis elimination programmes. The Bm14 ELISA aims to address the general agreement that any antibody test for the global LF elimination programme has to meet the following requirements:

- must be based upon recombinant antigens;
- needs to be reactive with all three lymphatic filariasis species;
- must have excellent sensitivity and specificity;
- must be produced as a standardised commercially-produced kit;
- produced under high-quality control standards with on-going quality assurance;
- can be produced in large-scale to satisfy demand, but at an affordable price;
- has excellent transport and storage stability under tropical conditions and;
- preferably produced in an ELISA and rapid test format.

The standardisation process for Bm14 ELISA includes evaluation of raw materials, assay optimisation and validation. The raw materials and reagents for the assay were evaluated for best results by experimentation. Optimisation was achieved by experimenting on the important concepts of ELISA kinetics for each critical assay component. Validation of the final protocols and assay performance of the optimised ELISA was completed to demonstrate that the test will perform as intended. The standardised test has a sensitivity and specificity of 100% and 98% respectively. No cross-reactivity with strongyloidiasis, schistosomiasis, dengue, malaria, Chagas disease and toxoplasmosis was found but it was cross-reactive with onchocerciasis, therefore, cannot be used in other African countries where the disease overlaps. A shelf-life of 12 months was validated. Repeatability and reproducibility calculated by percentage coefficient of variation were 4.2% and 3.3% respectively and both were within the 10% acceptable performance criteria.

To address the need for a convenient field-based test, the ELISA construct was converted to a rapid diagnostic test (RDT) in a dipstick format. Evaluation of the Bm14 dipstick found 100% concordance with ELISA results.

To commercialise the assays, conformity to International Standards of in vitro medical devices manufacturing (ISO13485) (ISO, 2012) is required by the Australian Therapeutic Goods Administration (TGA). A series of documents structured to follow a quality management system (QMS) was prepared. Documents include scientific methods and procedures, kit inserts and performance results to support the design and development of the commercial product. The output aims to provide a set of guidelines on the commercialisation process that can be used as a reference for future developers of innovative diagnostic tests.

Antibody subclass IgG4 is the widely accepted marker for lymphatic filariasis active infection however, studies also show that IgG1 may represent a response to incoming infective larvae exposure. The standardised Bm14 Antibody CELISA was used in an experiment to investigate the role of IgG1 in antibody detection. Serum samples from a high-transmission area of Papua New Guinea were used in an age-profile study. It was found that both subclasses were equally present across the age groups, regardless of antigen status although, levels of IgG4 were higher than IgG1. Follow-up work is needed to ascertain whether a subclass switching occurs much earlier by investigating the antibody response of infants in a low-transmission setting.

The standardisation of the Bm14 Antibody CELISA and information from this study will fill the critical gap in one of the challenges of the lymphatic filariasis elimination programme. There is currently the need for a reliable recombinant-based antibody test for stopping preventive chemotherapy namely:

(1) end-point determination of preventive chemotherapy and;

(2) post-preventive chemotherapy surveillance: for the detection of hot-spots and verification of transmission interruption.

This study will provide industry commercialisation information for diagnostic test research, a gap that needs to be addressed to show how to convert a research-grade test to a standardised, commercially available test that can be utilised for both clinical and research applications. This study provides information for:

(3) industry-based standardisation and optimisation methods for diagnostic tests to ensure product performance meet the intended purpose and;

(4) quality-based development and commercialisation procedures for a diagnostic test that meets international standards ISO13485.

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LIST OF ABBREVIATIONS

Ab	antibody
ADL	adenolymphangitis
ADLA	acute dermatolymphangioadenitis
Ag	antigen
ANOVA	analysis of variation
ARTG	Australian Register of Therapeutic Goods
AU	assessment units
Au	gold
Avg.	average
Bm	*Brugia malayi*
BmR1	*Brugia malayi* recombinant antigen clone 1
Bm14	*Brugia malayi* recombinant antigen clone 14
BNI	Bernard Nocht Institute
BSA	bovine serum albumin
CA	conformity assessment
CDC	Centers for Disease Control and Prevention
CELISA	Cellabs ELISA range of products
CE Marking	Conformite Europeane manufacturing regulations certification
CFA	circulating filarial antigen
COV	cut-off value
CTS	child transmission survey
CV	coefficient of variation
^{o}C	degrees Celsius
DEC	diethylcarbamazine
DNA	deoxyribonucleic acid
D	disease
d	disease absent
DF	dilution factor
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EU	evaluation Unit
FAMW	product code for kit component - microwell plates

FCS	foetal bovine serum
GAELF	Global Alliance for the Elimination of Lymphatic Filariasis
GHTF	Global Harmonization Task Force
GMDN	Global Medical Device Nomenclature
GMP	Good Manufacturing Practices
GPELF	Global Programme to Eliminate Lymphatic Filariasis
GSK	GlaxoSmithKline
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IC	internal positive control
ICT	immunochromatographic test
IFU	instructions for use
Ig	immunoglobulin
IL	interleukin
ILM	Institute of Louis Malarde
IMDR	International Medical Device Regulators Forum
ISO	International Standards
ISO13485	International Standards for IVD Manufacturing
IU	implementation unit
IVD	in-vitro medical device
JCU	James Cook University
KF3	Bm14 Filariasis Antibody CELISA (product code)
Kit-ve	kit negative control
Kit+ve	kit positive control
LDT	laboratory-designed tests
LF	lymphatic filariasis
L_3	infective third stage larvae
Mab(s)	monoclonal antibody(antibodies)
MDA	mass drug administration
mf	microfilariae
mg	milligram
mL	milliliter
NC	negative control
NCS	newborn calf serum
nm	nanometer
NPV	negative predictive value

NSW	New South Wales
NTD	Neglected Tropical Disease
OD	optical density
Og4C3	*Onchocerca gibsoni* clone 4C3
P	prevalence
PBS	phosphate buffered saline
PBS/T	phosphate buffered saline + Tween 20
PC	positive control
PCT	Preventive Chemotherapy
PICT	Pacific Countries and Territories
PNG	Papua New Guinea
PO	product code for kit conjugate
POC	point of care
PPV	positive predictive value
PS	polystyrene
PV	polyvinyl
QC	quality control
QMS	Quality Management Systems
rAg	recombinant antigen
RDT	rapid diagnostic test
Ref-ve	reference negative control
Ref+ve	reference positive control
R&D	research and development
RO	reverse osmosis
RZ	Reinheitszahl value
SD	standard deviation
S/N	signal-to-noise ratio
T	positive test
t	negative test
TAS	Transmission Assessment Survey
t-crit	T-test critical value
TGA	Therapeutics Goods Administration
TGO	Therapeutics Goods Order
TH	T helper cell
TMB	3,3’,5,5’-tetramethylbenzidine
t-stat	T-test statistic value

ug	microgram
uL	microliter
USA	United States of America
UoC	University of Copenhagen
Wb	*Wuchereria bancrofti*
WHA	World Health Assembly
WHO	World Health Organization
WU	Washington University, St Louis MO

CHAPTER 1

INTRODUCTION TO THE THESIS AND REVIEW OF THE RELEVANT LITERATURE

1.1 INTRODUCTION TO LYMPHATIC FILARIASIS

1.1.1 Historical Perspective

Lymphatic filariasis (LF) is one of the oldest diseases known to man. Many early records of ancient civilisation describe and depict disease, characteristic of filariasis infection such as lymphangitis and elephantiasis, the grotesque enlargement of limbs, scrotum and breasts. These early descriptions may not be reliable as they also refer to symptoms of other similar diseases such as leprosy, tumors, tuberculosis and even genetic deformities (Nelson, 1996; Cox, 2002). From 2000 BC in Ancient Egypt, the swelling of legs was depicted in a statue of Pharoah Mentuhotep II, suggesting he may have suffered from elephantiasis. Today, filariasis is still endemic around the Nile Delta in contrast to the middle and upper Egypt where filariasis transmission is absent (Harb et al., 1993). A mummified body of priest Natsef Amun was found to have filarial worms during an autopsy by a group of British experts in 1973 (David & Tapp, 1992), a discovery underpinning the existence of elephantiasis in Ancient Egypt (Cox, 2002). Greek philosopher Lucretius (99-55 BC) describes elephantiasis in communities near the Nile. The 10th century Roman and the Nok culture of West Africa (AD 500) show elephantiasis-like drawings and statues (Cox, 2002).

Although the disease referred to in these early records may not be exactly filariasis, it was clear that the early physicians have made some differentiation from other diseases. A book by Miller & Nesbit (2014) discusses in detail how elephantiasis and leprosy have been described similarly and also differentiated using a variety of names in records by physicians in Ancient Egypt. Greek, Roman and Arabic physicians like Avicenna also have differentiated elephantiasis from other diseases (Kiple, 1993). Elephantiasis was first referred to as *elephantiasis arabum* and leprosy as

elephantiasis graecorum (Chandy et al., 2011). A common observation included in the early writings, is the existence of this human condition near stagnant waters. It holds true for filariasis transmitted by Culex mosquitos that like to breed in stagnant waters (Melrose, 2002). A medical book estimated to have been written in 70 AD by an Indian physician named Sushruta Samhita describes a disease he referred to as *slipida* meaning “elephant leg”. And like many early accounts, also noted the occurrence of slipada in patients living close to stagnant waters (Rajan, 2000).

(a) (b)

Figure 1.1: Ancient depiction of filariasis disease. A statue of (a) Pharoah Mentuhotep II from 2000BC Egypt depicting swollen legs characteristic of elephantiasis and; (b) Natsef Amun, an Egyptian priest from the same century whose mummified body contained filarial worms.
Photo source: http://parasitology.kasralainy.edu.eg/draft-plan-status

Between 1588 and 1592, a Dutch explorer by the name Jan Huygen Linschoten travelled to Goa in the Indian Peninsula. He wrote about “elephant-legs”, “St. Thomas leg”, *pericaes* (perunkal = Tamil for big leg) and “cocheen leg” described by previous explorers. Laurence (1970) extensively covers the story of “the curse of St. Thomas”. Linschoten’s accounts of elephantiasis in Malabar would be the first definitive record in the 16th century. Many other records from parts of Africa and Asia followed after (Cox, 2002; Chandy et al., 2011).

1.1.2 The Great Discoveries of the 19th Century

The 19th-century colonialism of tropical islands and coastal areas of the tropics and sub-tropics introduced the existence of tropical diseases to western physicians in early colonial period (Rajan, 2000). In his 1849 book, William Prout, describes chyluria, as milky due to the passing of lymph (Chandy et al., 2011). In 1863, a French physician, Jean-Nicolas Demarquay operated on a 19-year old Cuban patient when he discovered mf in a milky fluid from tappings of the swollen scrotal sac (hydrocele). He described "worm-like" creatures that by his sketches were undoubtedly filariasis (Nelson, 1996; Rajan 2002). Three years later, Otto Henry Wucherer discovered mf in urine while working in Bahia Brazil in 1868, which he distinguished from *Schistosoma*. In 1872, Timothy Lewis a Welsh physician made some remarkable observations of the chronic nature of the disease in India. He wrote about "filariae" being linked to lymphatic obstruction and chyluria and called it *Filaria homonis sanguinis* (Chernin, 1983).

Later in 1876, Joseph Bancroft, an Australian physician based in Brisbane, Queensland, discovered the adult worm. He wrote: "about the thickness of human hair, and is from three to four inches long". It was later named "*Filaria Bancrofti*" after him, as proposed by Britain's ranking helminthologist, Spencer Cobbold (1877). It was re-named *Wuchereria bancrofti* in 1921 by Seurat (Xie et al., 1994) as recognition of the discovery of the species by both, Otto Henry Wucherer and Joseph Bancroft.

Patrick Manson (1844-1922) a Scottish physician was the most prominent figure in modern filariasis history, penned as the "Father of Modern Tropical Medicine". Manson, while working as a medical officer in Amoy, South China, discovered the transmission of filariasis by a blood sucking arthropod, and later, the discovery of the nocturnal periodicity of the mosquito (Chernin, 1983). He published the life cycle and clinical features of the disease in 1878. Manson incorrectly hypothesised that the transmission was through contaminated water where mosquitos have deposited the worms. The theory was dismissed years later in 1890 with the discovery of the microfilariae (mf) in the proboscis of a mosquito by George Carmichael Low. He also identified the transmission of infective larvae (L_3) by the mosquito during a blood meal (Chandy et al., 2011). Manson's work was also the driving force to the discovery of malaria transmission by Ronald Ross. These new developments were a medical landmark, which led to tropical medicine as a separate branch of discipline, the

founding of London School of Tropical Medicine in 1898 and Royal Society of Tropical Medicine and Hygiene in 1907, saw Manson elected as its first residing President (Nelson, 1996; Eldridge, 1992).

1.1.3 Filariasis in Early Colonial Settlement in Australia

Filariasis in Australia was thought to be non-existent before colonial settlement. The indigenous peoples who inhabited the land more than 40,000 years before the colonial settlement had no recorded history of having the disease. The first report of the disease was the account of T.B Wilson of the inhabitants of Torres Strait Islands in 1822, as discussed by Boreham and Marks (1986) who wrote a comprehensive description of human filariasis in Australia. In an attempt to present the events to decipher the origins of filariasis in Australia, they also discussed the three groups of immigrants, all whom hail from an area where filariasis exists, a key to the nocturnal periodicity of the recorded filariasis in the 19th century settlement. The first immigrants were the Indian servants of early British settlers who once lived in the Indian subcontinent but due to the slave labour controls implemented in 1833, the number of Indian servants who immigrated were limited. The second immigrants were the Chinese workers or coolie labour from Amoy in 1848 who were destined to New South Wales rather than Queensland where filariasis were most noted. However, Chinese immigrants to Queensland and further north of the continent continued to arrive in 1851 through to the early 1900s where they worked as miners, gardeners and domestic help. The third group of immigrants into Queensland were the so-called Kanakas, sugar cane workers from the New Hebrides and the Solomons in the mid-1960s.

Joseph Bancroft and J.H.L. Cumpston believed that the origins of *Wuchereria bancrofti* in Queensland was from the Chinese of Amoy (Boreham and Marks, 1986). Heydon (1931) also shares this idea as the Kanaka labour did not begin until 1863, and confined in sugar fields of North Queensland. These so-called Kanakas had little contact with the population in Brisbane where filariasis was established among the “white settlers” by 1875 (Heydon, 1931). Sir Raphael Cilento and A.G. Salter Anderson on the other hand, believe that the Kanakas were the origin (Boreham and Marks, 1986). There was however, a decline in filariasis in Queensland from 1910 (Mackerras, 1958) which coincided with the end of the Kanaka labour in the early 1900s (Heydon, 1927).

1.1.4 The Lymphatic Filarial Parasite and Vector

1.1.4.1 The LF Parasites and Species

Filariasis is an infection caused by the parasitic roundworms belonging to phylum Nematoda, family Onchocercidae and superfamily Filariodea. Adult filariae are white, threadlike and unsegmented; the female worm is approximately 8 - 10 cm long and 200mm diameter, bigger than the male counterpart that measures approximately 4 cm long and 50 mm diameter. *Wuchereria bancrofti* mf measures 290um, *Brugia malayi* measures 222um and *Brugia timori,* 310um approximately (Scott, 2000).

There are eight filarial parasites commonly affecting man, these are *Wuchereria bancrofti; Brugia malayi; Brugia timori; Loa loa; Onchocerca volvulus; Mansonella perstans; Mansonella ozzardi and; Mansonella streptocerca* (Nutman, 1991). The three important species that affect the lymphatics of man are *W. brancrofti, B. malayi* and *B. timori* (WHO, 2010). *M. perstans* and *M. ozzardi* inhabit body cavities and inhabiting cutaneous and sub-cutaneous is the *Onchocerca volvulus that* can also co-exist with LF infections in Central, West and East Africa (Hotez, 2008; Hopkins et al., 2002). Other species that have similarities to filariasis are *Dracunculus* (guinea worm), *Loa loa* found in cutaneous tissues, the pinworms and ascarids responsible for soil-transmitted helminthic infections, hookworms and Rhabditina.

Named by Cobbold (1877), *W. bancrofti* was thought to be the only species of genus *Wuchereria* found exclusively in man (Van den Berghe et al., 1963; Bockarie et al., 2009). Although another species described by Palmieri, Purnomo & Marwoto (1980) was found in the lymph nodes and testes of leaf monkeys limited to South Kalimantan in Indonesia. *Wuchereria kalimantani* is closely related to the human *Wuchereria* parasite by molecular phylogenetic studies (Xie et al.,1994).

Wuchereria and *Brugia* were separated as two distinct genera by Buckley in 1959 (Melrose, 2002). *Brugia* contains ten species namely: *B. pahangi, B. malayi*, *B. beaveri, B. buckleyi, B. patei*, *B. timori*, *B. tupaiae, B. lepori, B.*

ceylonensis, B. guyanensis. B. malayi is the only *Brugian* parasite infecting both man and animals while the rest are parasites of animals (Cheng, 1964).

1.1.4.2 Life Cycle

The human stage of *W. bancrofti* life cycle begins once mf from the proboscis of a vector mosquito taking a bloodmeal is deposited on the skin, and enters the human host through the wound (Bartholomay & Christensen, 2002). This mode of transmission is quite inefficient, unlike the *Anopheles* malaria vector that injects parasites directly into the bloodstream through the puncture in the skin. In the human host, mf penetrates through connective tissues and migrate to the local lymphatic vessels (Maizels et al., 2001). After 10-12 days from entry, the L_3 moults into the L_4 stage (Hotez, 2008) and develops to adult worms within 2 - 12 months of the second moulting (Cheng, 1964), surviving in the lymph system of the human body. These developmental processes are transmission stages are crucial for their survival (Kazura, 2002). Their average life span is 4 - 6 years (WHO, 2013a) but may survive as long as 18 years (Vanamail et al., 1990). Both adult female and the male must be present together in the same 'nest' to produce progeny. With a stable female reproductive life of 5 - 6 years (Kazura, 2002; Paily, Hoti & Das, 2010), the female releases thousands of sheathed microfilariae into the lymph circulation of the human host following sexual reproduction. It occurs within a circadian periodicity, a mechanism that facilitates their survival where peak production of mf corresponds with the peak feeding times for the vector. Periodicity is observed depending on the geographical location of the vector mosquito (Cross, 1996; Rebollo & Bockarie, 2013).

The mosquito stage life cycle of the worm begins once a vector mosquito ingests mf through a blood meal from an infected human host. Tiny mf, microscopic slender worms from the blood meal migrate out from the midgut to thoracic muscles (Cross, 1996). They transform to an inactive sausage stage L_1 that rapidly elongates to a pre-infective larvae stage (L_2). Exsheathment is known to be a prerequisite to stage development but where this takes place has not been very clear (Scott, 2000). Bartholomay & Christensen (2002) rationalise that exsheathment takes place during the crossing to the haemolymph, during the process of mid-gut penetration. Mf does not reproduce in the vector

(Melrose, 2002) but develop into L_3 after the second moulting of the sheath. The L_3 migrate to the proboscis where during a blood meal, they are deposited onto the skin of the next human host to complete the life cycle.

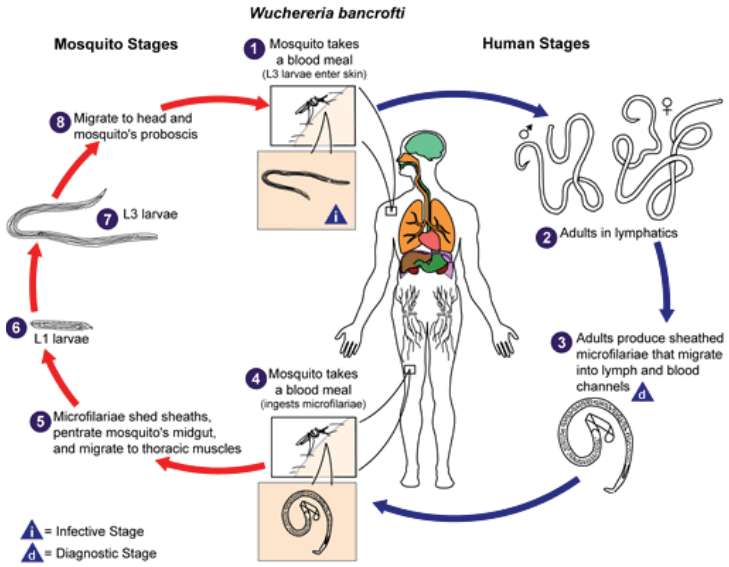

Figure 1.2: The life cycle of *W. bancrofti* showing both the vector mosquito stage and the human host stages. Photo reference: http://www.cdc.gov/parasites/lymphaticfilariasis/biology_w_bancrofti.html

1.1.4.3 Global Distribution and Burden of Disease

The WHO Progress Report 2000 – 2009 (WHO, 2010) estimates a total of 120 million people infected in 81 countries considered to be endemic for LF. Of the global estimates, bancroftian filariasis is responsible for 90% of human infections in Asia, Latin America and Caribbean nations, sub-Saharan Africa and in Egypt (Dennis et al., 1992). Brugian filariasis, being less host-specific (Cross, 1996), accounts for the rest of the infections worldwide. 9% is attributed by *B. malayi* in Asia and the Pacific, and 1% to *B. timori* that is limited to some islands in Indonesia (WHO, 2010; Das & Shenoy, 2008). Some 1.34 billion who live in LF endemic areas are at risk of infection, 65% residing in the South-East Asia Region. There are 53 countries that have implemented preventive

chemotherapy (PCT), with 37 countries close to completing. There are currently 27 countries out of the 81 endemic countries that have actively introduced a morbidity management programme.

The current data from the World Health Organisation (WHO) Global Health Observatory (WHO, 2015) LF global distribution estimates 98% of those who are infected live in the African and South-East Asian region where 94% of the at-risk population also live. The South-East Asian region has the highest infected population at 57% in 9 endemic countries. The African region accounts for 38% who live in the 35 listed endemic countries. The remaining 6% accounts for 4 countries in the American region, 3 in the Eastern Mediterranean region and 22 in the Western Pacific region. As at 2015, 72 out of the 73 endemic countries have initiated mapping, 60 of 73 have implemented PCT and 15 of these have already met the targets to stop PCT.

Recent estimates of the burden of disease by Ramaiah & Ottesen (2014) after thirteen years of the Global Programme to Eliminate Lymphatic Filariasis (GPELF) shows a global decline of 59% from a baseline of 3.55%. Although Africa remains high at 5.51%, the estimates show a reduction of 36.45 million cases of microfilaremia, 19.43 million cases of hydrocele and 16.68 million cases of lymphoedema.

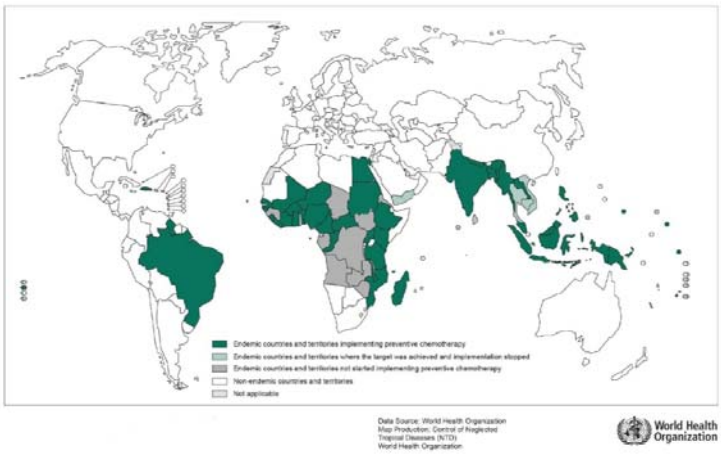

Figure 1.3: Global distribution of LF in 2014. The dark green shade represent endemic countries implementing PCT. Light green shade have met their targets and grey areas are still to complete PCT. Image source: http://www.who.int/gho/neglected_diseases/lymphatic_filariasis/en/

1.1.4.4 Important Vectors of LF

Although there are over 70 species and subspecies of mosquitos that were identified as vectors for LF, the four important genera that transmit LF in humans are: *Culex, Anopheles, Aedes* and *Mansonia* (Scott, 2000, p.6). Species in each genera differ in their biological characteristics that determine how they transmit LF in different geographic locations. These diverse biological characteristics are important in employing the most effective control strategies (WHO, 2013a).

The Practical Entomology Handbook for WHO (2013) describes the biological characteristics information on the vectors involved in the transmission of LF. In the Oriental region, the more important subgenus *Mansonioides* transmits *B. malayi* but also transmits the periodic and sub-periodic *W. bancrofti*. *Mansonia mosquitos* are largely found in marshy areas and vegetation and are mainly night biters.

The genus *Culex, a vector for W. bancrofti*, is probably the most adaptable vector due to its widespread distribution and existence in a range of temperate latitudes. *C. quinquefasciatus* and *C. pipiens* are the most common. *Culex* species are known to overlap and interbreed, mainly surviving with urbanisation in a variety of polluted and stagnant waters. It can be found in coastal and rural areas of East Africa (Kelly-Hope et al., 2013) and Micronesia in the Pacific where the parasite is periodic (Burkot et al., 2006). *Culex* contributes about 57% disability-adjusted life year (DALY) in total out of the four main vectors (Das & Shenoy, 2008).

Genus *Anopheles* is a vector for all three LF parasites with a flight range of 1000-3000m, allowing them to travel far and wide to seek a host and reproduce. A night biter, it is responsible for the periodic transmission of *W. bancrofti* in Africa and New Guinea and both *W. bancrofti* and *B. malayi* in South Asia. *An. gambiae*, *An. arabiensis* and *An. merus* transmit human LF with *An. barbirostris* the only known vector for *B. timori*. In West Africa, the *Anopheles* observes the phenomenon called “facilitation” whereby high mf load facilitates efficiency in transmission, a vector control mechanism (de Souza et al., 2012).

Genus *Aedes*, a vector of both *W. bancrofti* and *B. malayi* are mainly day biters living and breeding in axils of plants and tree holes. *Ae. polynesiensis* and *Ae. samoanus* are mainly vectors in Samoa and French Polynesia and, the nocturnal *Ae. poecilius* in the Philippines. *Ae. polynesiensis*, the most important vector in the Pacific observes “limitation” behaviour where the increased production of mf that develop to L_3 stage is dependent on a decreasing mf density (Pichon, 2002; Burkot et al., 2006).

1.1.5 Diagnosis

LF is a complex disease where clinical symptoms are not always present. Detection of mf, antigen, antibody and DNA from samples of blood, serum and other body fluids and tissues are used for diagnosis. This section will briefly cover the range of tests that are used for diagnostics and the value and drawbacks of each. The need for improved, reliable diagnostic tools for the global elimination programme will be discussed in a later section.

1.1.5.1 Methods for Microfilaria Detection

Microfilariae detection by microscopy is the traditional diagnostic method for LF. Direct observation and concentration methods from blood samples are commonly used techniques (Melrose, 2002). Blood smears can be prepared easily and stained by Giemsa. Although other stains such as modified hematoxylin stain (Hira, 1976; Awogun, 1978), Romanowsky stain or acridine orange using ultraviolet microscopy are used (Goldsmid & Rogers,1976).

Mf detection is said to be insensitive due to the low sample volumes used. The 20-60uL samples of fingerprick blood used for direct observation of thin or thick blood smears (Nicolas, 1997; Melrose et al., 2000) may not be enough to detect those with low parasite densities (Melrose, Durrheim & Burgess, 2004). Denham (1975) notes that higher sample volumes improve accuracy and capillary blood is said to have more mf than venous blood (Eberhard et al., 1988 cited from Melrose, 2002). The use of venous blood to improve accuracy is also recommended by CDC DPx (2013). Although high volume sampling improves the method, Denham et al., (1971) and Panicker et al., (1991) agrees that loss of mf during blood smear processing is a potential for unreliable

results. Shouthgate & Hamilton, (1974) suggests that sensitivity can be improved by concentration methods.

Concentration methods include the widely used Knott's test (1935). The detection limit is improved by using a sample between 1 - 10mL of blood (Southgate & Hamilton, 1974) diluted in 1% formalin solution. Formalin is used to lyse blood and preserve mf before concentrating and staining before microscopic examination. To improve mf visualisation, a method by Melrose et al. (2000), was devised by adding Triton X-100 to the 1% formalin solution to dissolve and clarify precipitated plasma gamma globulins commonly present in samples from tropical populations. Other methods were developed such as the microhaematocrit centrifugation buffy coat capillary tube method, also known as the quantitative buffy coat anlalysis (QBCA) as described by Levine & Patton (1989) and also a variation by Mylonakis et al., (2004), and adaptation of the counting chamber for quantification by Bell (1967), Chularerk & Desowitz, (1970) and Denham et al. (1971).

A major drawback of mf detection is its impractical application in field settings (Southgate & Hamilton, 1974; Weil, 2005; Weil & Ramzy, 2007; Nuchpryoon, 2009). Where parasite is nocturnally periodic, collection is optimal two hours either side of midnight (Melrose, 2002). This night collection is difficult and presents the main drawback for mf detection method (Nicolas, 1997; Melrose, 2002, Melrose et al., 2004). Not only is this method inconvenient for the community and health workers, the conditions are poor with limited resources such as lack of electricity, cold chain transport and, trained health workers (G.H. Rajasekariah, personal communication, October 13, 2014).

Ultrasonography is the use of frequency for detecting adult worm movements in nests, the "filarial dance sign". It may not be a useful tool for PCT programmes but it has an important application in individual health management. Studies by Mand et al. (2003, 2011) found ultrasonography to be useful in differentiating the types of scrotal enlargement or "filaricele" that is important in making health management decisions for suffers of advanced stages of LF.

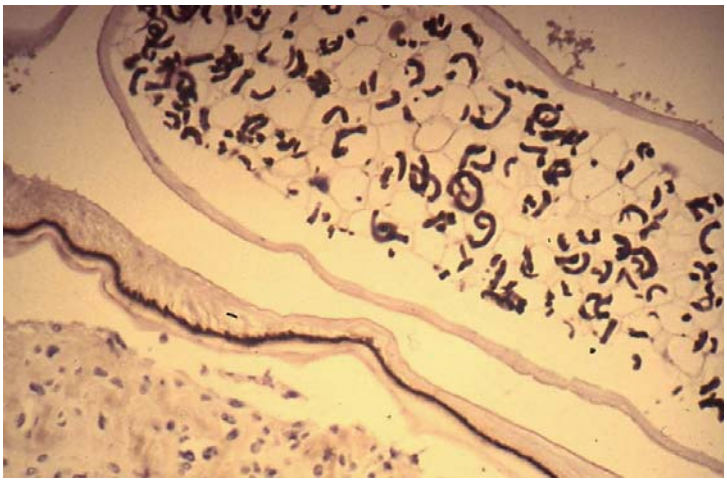

Figure 1.4: Cross-section of a female parasite. Microfilariae are seen inside the lumen. Photo source: Dr Graham Burgess, College of Public Health, Medicine and Veterinary Sciences, JCU, QLD, Australia

1.1.5.2 Antigen Detection

Reliable antigen detection was made possible by the development of monoclonal antibodies (MAbs) AD12 by Weil & Liftis (1987) and Og4C3 by More & Copeman (1990). Both these MAbs were further developed into two antigen diagnostic tests for *W. bancrofti* antigen used in LF research since the introduction (Nicolas, 1997). Adult worms and microfilariae antigen is released into the bloodstream, unaffected by the variation of filarial periodicity (Lalitha et al., 1998), making it an ideal marker for active infection.

The availability of antigen tests is a breakthrough that addresses the problems of night blood sample collection (Simonsen & Dunyo, 1999). Antigen testing using dried blood spots offers more convenience by replacing venous blood collection, eliminating the need for major logistical coordination (Uzicanin et al., 2011). On the contrary, the value of antigen testing diminishes after a few rounds of PCT because antigen levels fall below the detection limits of the assays. As observed from the work of Rocha et al., (1996) using the Og4C3

ELISA, the antigen detection is limited to high antigen levels and not suitable for mf loads less than 1 mf per mL of blood.

Antigen detection methods were developed in the 1980s but the first antigen test made commercially available was the Tropbio Og4C3 ELISA (Melrose, 2002). Based on MAb Og4C3 developed against antigens of *Onchocerca gibsoni,* the ELISA is specific to *W. bancrofti* antigens from both mf and adult worms (Nicolas, 1997). It is cross-reactive with *Onchocerca volvulus, Difilaria immitis, Ancylostoma caninum and Toxocara canis* but does not bind to phosphocholine (PC) (More & Copeman, 1990), that is generally present in helminth antigens (Weil, 2009). The assay was well received by early research evaluations showing excellent sensitivity and reliability (Weil, Lammie & Weiss, 1997; Nicolas, 1997; Simonsen & Dunyo, 1999).

In the late 1990s, another revolutionary development was that of the immunochromatography test (ICT) rapid card test (ICT Diagnostics, Brookvale, Australia). Now called BinaxNOW Filaria (Alere, USA), the card test was based on the MAb AD12, ideal for field testing. Originally designed for use with serum samples, the test was modified at the end of the 1990s for use with whole blood (Nguyen et al., 1999), making it even more convenient for field use. Fingerprick blood can be used directly on the card, taking 10 minutes to get the results. In early 2000, the test had some performance issues (Weil, 2005) with false positive readings if not read within the recommended timeframe of 10 minutes (Simonsen & Magesa, 2004). There was also the issue with the short shelf life of 3 months at ambient temperature that is limiting to any field study (Weil et al., 2013). The Global Programme for the Elimination of Lymphatic Filariasis (GPELF) multicentre evaluation (Gass et al., 2012) recommended its use as the primary tool for the field because of convenience and ease of use. It is currently the main research tool for mapping and PCT end-point determination (Weil et al., 2013).

More recently, the Alere Filariasis Test Strip (Alere Scarborough, Scarborough, ME, USA) antigen test was developed as an improved field-based antigen test. It was evaluated by Weil et al., (2013) to show improved sensitivity and specificity in comparison with the BinaxNOW Filariasis card test.

1.1.5.3 Antibody Detection

Prior to the use of molecular techniques in the 1990s, diagnostic assays utilised crude antigen extracts from parasites for use in serological techniques. Crude antigen extracts or somatic antigens served well for antibody detection assays in parasitology such as malaria (Demedts et al., 1987; Achidi et al., 2005), toxocariasis (De Savigny, 1980; Jin et al., 2013), trypanosomiasis (Caballero et al., 2007), filariasis (Rajasekariah et al., 1991; Mohanty et al., 2001) and successfully, for leishmaniasis (Rajasekariah et al., 2001; Lakhal et al., 2012). The major drawback for filarial antibody diagnosis was the inherent cross-reactivity of antigen extracts with other helminthic infections. This was difficult to avoid because most LF endemic areas overlap with other related infections such as ascariasis and strongyloidiasis although the use of an IgG4 antibody isotype as the marker improves cross-reaction with non-filaroid helminths (Melrose, 2002).

The advent of recombinant techniques to probe sequences of cDNA libraries from *B. malayi*, (Dissanayake et al., 1992; Chandrashekar et al., 1994), produced some of the most promising recombinant antigens (rAg) for LF diagnosis. The currently existing rAg developed for LF diagnostics is briefly discussed below in Section 1.1.5.3.1.

1.1.5.3.1 Recombinant Antigen SXP

A λ-cNDA library of *B. malayi* adult male was differentially screened, cloned and sequenced by Dissanayake et al., (1992) to produce the rAg SXP-1. It showed reactivity to *W. bancrofti* mainly restricted to IgG4 subclass although SXP-1 is not reactive with *B. malayi* but reacted with samples co-infected with *O.volvulus*, *L. Loa* and *M. perstans* from West Africa. This clone was named Bm-SXP, GeneBank accession number M98813 (Rao et. al., 2000). rAg Bm-SXP-1 and BmR1 was used in a double rAg-based Pan LF ELISA developed by Rahman et al., (2007) for the purpose of detecting *W. bancrofti* and *B. malayi* IgG4. The ELISA was based on the original Bm-SXP1 test by Rahmah et al., (2001a). Unfortunately, the cross-reactivity of both antigens with *L. loa* and *O.*

volvulus means the assay cannot be used in African countries where these infections overlap.

An orthologue of the rAg Bm-SXP-1 was screened in a cDNA library of *W. bancrofti* L_3 and named Wb-SXP-1. The same-species screening method improved on the specificity with *W. bancrofti* up to 100% as described by Rao et al., (2000). It is restricted and useful for IgG4 *W. bancrofti* antibody detection methods. Bm-SXP-1 was utilised in the antibody IgG4 ELISA for urine, developed at Aichi Medical University School of Medicine, Japan by Itoh et al. (2001).

1.1.5.3.2 Recombinant Antigen Bm14

In the early 1990s, the recombinant *B. malayi* protein Bm14 developed for the purpose of filarial antibody detection was described to react against antibodies to both brugian and bancroftian LF. Bm14 (GeneBank accession number M95546) also known as BmM14 is a clone selected and characterised from a *B. malayi* cDNA library by Chandrashekar et al. (1994). Bm14 was found to be 20% identical and 34% similar to the SXP-1 clone from the *B. malayi* cDNA library. The specificity and sensitivity of Bm14 make it a promising antigen for serology testing but cross-reactive with *O. volvulus* and *L. loa*, limiting its use in some African areas of LF where co-infections are present.

1.1.5.3.3 Recombinant Antigen BmR1

A recombinant protein BmR1, GeneBank accession no. AF225296 was produced at the University of Malyasia by Rahmah et al., (2001b) for diagnostic purposes. BmR1 was extensively used for the BrugiaRapid RDT and ELISA since 2001 but found to have a varying reactivity with a range of *B. malayi* clinical diseases. It was found to be cross-reactive with *L. loa* and *O. volvulus* (Rahmah et al., 2001b, 2003; Supali et al., 2004). Noordin et al., (2004) characterised the recombinant antigen using *W. bancrofti, O.volvulus* and *L.loa* cDNA. The screened clones were compared with BmR1 cDNA sequence and found that Wb-BmR1 and BmR1 are identical and share 99.7% homology with Ov-BmR1 and Ll-

BmR. The reactivity with serum samples was restricted to IgG1 and IgG4 and not with IgG2 and IgG3. IgG4 reactivity with serum samples was found to be satisfactory for the purpose of IgG4 detection of brugian filariasis.

1.1.5.3.4 Wb123 Recombinant Antigen

Recently, the recombinant antigen Wb123 was developed using bioinformatics (Kubofcik et al., 2012), to probe for specific antigens of *W. bancrofti* and *B. malayi.* Expressed by L_3 stage larvae of *W. bancrofti*, Bm123 is 82% homologous with Bm, 35% with Ov and 61% to Ll. It is not homologous to proteins from closely related *L. loa* and *O. volvulus* sequences. In an IgG4 LIPS assay, the antigen did not cross-react with non-filaroid *S. stercoralis* and *A. lumbricoides* hookworms and filarial species *L. loa, M. perstans* and *O. volvulus*, with a sensitivity of 98% and specificity of 100% (Kubofcik et al., 2012). These promising results have not been demonstrated in a practical diagnostic assay except for LIPS, a technique that is impractical for the field work that is required by the LF elimination programmes.

1.1.5.4 Molecular Diagnosis

Filariasis parasite DNA detection methods by polymerase chain reaction (PCR) have been developed for *W. bancrofti* (Zhong et al., 1996; Rao et al., 2006; Plichart & Lemoine, 2013), *B. malayi* (Lizotte et al., 1994; Fischer et al., 2000, 2002; Kluber et al., 2001), and for the mosquito vector (Bockarie et al., 2000; Williams et al., 2002; Plichart et al., 2006; Boakye et al., 2007). Although DNA detection was found to be more specific and sensitive than the traditional mf detection (McCarthy, 2000), the high cost of the technology for use in elimination programmes is limiting.

To address the high cost of using DNA detection method, the pool-screen is commonly employed (Gass et al., 2012; Plichart & Lemoine, 2013). It involves the pooling of 10 to 12 individual samples of 10uL to 30uL of blood before testing. Although the role of DNA testing for LF elimination programmes is yet to be re-evaluated (Gass et al., 2012), DNA testing is limited to xenomonitoring

studies for LF assessment, speciation of parasite and detection of cryptic LF infection (McCarthy, 2000).

1.1.6 The Classification and Pathogenesis

The classification of disease manifestation has been based on the linear progression of the disease. But for LF, a spectrum of clinical presentation varies from the pre-patent to the patency stages (Das & Shenoy, 2008) Some recent re-classification have been made based on immunological characteristics (Ottesen, 1989).

Melrose (2002) describes the five widely accepted classifications of LF clinical and sub-clinical disease as, (1) the exposed with no evidence of disease called “endemic normal”; (2) asymptomatic microfilaremia; (3) acute condition with or without mf; (4) chronic disease, lymphoedema or elephantiasis with or without mf and; (5) tropical pulmonary eosinophilia (TPE).

Individuals living in endemic areas who are apparently immune do not show any symptoms (McSorley & Maizels, 2012), are referred to as “endemic normal”. Despite being exposed to LF, this group have no sign of disease, no mf and no antigen detected (MacDonald et al., 2002). Individuals who have evidence of mf or filarial antigen can have the classical symptoms of “asymptomatic microfilaremia”, showing no symptoms of the disease. The reclassification came about due to the availability of improved diagnostic tools that made it possible to detect filarial antigens from those without the apparent disease (Kumaraswami, 2000). It is accepted that detection of antigen indicates mf, a reservoir for ongoing transmission but antigen may also be from a viable adult worm. In any case, a person with the adult worm is considered a carrier (Babu & Nutman, 2012) and, therefore, the risk of infection is present. The asymptomatic condition is the most intriguing because despite living with mf, (sometimes in very high densities per mL of blood) for many years there are no symptoms of the disease. However, it is also believed that pathological changes occur with anyone who has mf, including “asymptomatics” and can be in the form of sub-clinical haematuria or proteinuria and other lymphatic damage in different degrees (Addis & Dreyer, 2000; Nutman & Kumaraswami, 2001; Babu & Nutman). Renal disease (Freedman et al., 1994) or scrotal lymphangiectasia have been observed in infected groups without mf but Ottesen (1980) notes that some individuals may have undetectable mf that cannot be explained.

The acute clinical disease is commonly characterised by recurring filarial fevers that accompany painful inflammation of lymph nodes and vessels in breasts and scrotal area called adenolymphangitis (ADL) (Ottessen, 1980). Recurrent attacks are more frequent than in brugian filariasis (Srividya et al., 1991). *W. bancrofti* infections affect the male genitalia that can lead to funiculitis, epididymitis or orchitis. Inflammation, which may be due to dying adult worms (Babu & Nutman, 2012) can also be accompanied by fungal or bacterial infections. *Wolbachia* bacteria often gain entry from the breaks in the skin, are implicated as the cause of inflammatory-mediated disease and in triggering these filarial fevers (Dreyer et al., 2002).

Chronic Pathology manifests after years of infection (Portono, 1987) and only in a small proportion of the infected population. Increasing severity is seen as age increases (Dennis et al., 1992) and rarely seen in children younger than 15 years old (Kumaraswami, 2000). Hydrocele, lymphoedema, elephantiasis and chyluria are chronic forms of the disease, though, lymphoedema and elephantiasis are less commonly observed in bancroftian filariasis. In brugian filariasis, there are no reports of affected genitals although the arm below the elbow and leg below the knees are mainly involved (Babu & Nutman 2012; Kumaraswami, 2000; Portano, 1987).

TPE is a variant, seen in both brugain and bancroftian filariasis. An immunological reaction mainly to mf that develops in some individuals. TPE causes paroxysmal cough, wheezing at night, fever, adenopathy, malaise, weight loss and increased blood eosinophilia greater than 3000/uL with elevated IgE and anti-filarial antibodies (Babu & Nutaman, 2012). It is limited to Africa, South America and Asia (Boggild et al., 2004), but most prevalent in India and South-east Asia (Pinkston et al., 1987).

The spectrum of clinical disease is thought to be the result of immunological mechanisms that mediate pathologic changes in the progression of disease (Piessens, 1981;Piessens et al., 1987; Ottesen, 1980; Nutman & Kumaraswami, 2001). The fundamental understanding of immunology is the best key to delineating the spectrum of LF disease as discussed by Ottesen (1989). Immunology aspects of disease manifestation will be discussed in a later section (Section 2.4).

1.1.7 The Socioeconomic Effects of Disability

The social impacts and economic burden of the disfiguring disease of LF are observed regardless of cultural differences. The physical suffering and disability contribute to economic hardship, especially for parents whose families rely on them for domestic and financial support. In a study in Ghana and Dominican Republic, women often avoid seeking medical help because they fear being ostracised. They hide their physical disability to preserve social acceptance, be allowed to work in regular roles and access education (Person et al., 2009).

Although no data exists of the social burden, the estimated public health impact and disability-adjusted life years (DALYs) places filariasis second in the world (Ottesen, 1997; Babu & Nutman, 2014), with 40% represented in Africa alone (Gyapong et al., 2000). The economic impact affects individuals due to the cost of medical bills, inability to work and, therefore, the loss of income due to sickness (Melrose, 2004). The loss of productivity impacts the community as a whole. It is seen in 1% of working males in a study in Kenya (Wijers, 1977 cited in Evans, Gelband & Vlassoff, 1993) or 19% of annual working days in a study in Orissa, India (Babu et al., 2002).

In India, Ramiah & Kumar (2000) describe children disadvantaged at school due to absenteeism, lack of understanding and support from teachers. Constant teasing from peers and the community forces these children to drop out of school. Women and men who suffer from lymphoedema and elephantiasis are shunned, shamed and alienated resulting in a reclusive life. For young men, the prospect of marriage is affected, and those married suffer a sexual disability that can impact on family life (Evans et al., 1993; Gyapong et al., 2000).

There are not many studies on the social impact of LF disease evident by the small amount of literature available. Reports from India (Ramaiah & Kumar, 2000), Philippines (Lu et al., 1988; Ramirez et al., 2004), Togo, West Africa (Ziperstein et al., 2014), Ghana (Gyapong et al., 1996), and other countries describe the same social and economic impacts in varying degrees. In the Pacific, recent studies have recognised the of addressing the socioeconomic issues in the community to allow acceptance and help move forward to progressing with elimination efforts (Wynd et al., 2007a, 2007b).

1.1.8 Treatment and Methods to Reduce Disability

1.1.8.1 Drugs

The GPELF strategy to interrupt the transmission of infection is through the administration of single-dose drugs per year over a period of 4 years or more. Transmission is said to be inefficient due to some favourable factors in the biological features of the parasite that make elimination possible (Ottesen, 2006). A review by Chandra (2008) on limiting factors of filarial transmission also includes vector and parasite biology, as well as other natural controls. *W.bancrofti* causing the highest of LF infections is not zoonotic but to infect one host, about 15,500 bites are required. It is these inefficiencies that drugs effective in killing the parasite when repeatedly taken in a PCT programme will reduce the number of mf in the blood to a low level where transmission cannot be sustained. The current regimen is 150-200ug/kg of ivermectin and 400mg albendazole in areas where onchocerciasis is co-endemic and everywhere else is 6mg/kg of diethylcarbamazine (DEC) and 400mg albendazole (WHO, 2013a).

DEC has been a safe drug and without side effects, used for over 50 years (Horton et al., 2000). Although for individuals with onchocerciasis, side effects are adverse due to the death of mf in the lymphatic system (Ottesen, 2006). Ivermectin is recommended for individuals with *L. loa* (Zoure et al., 2011) and to target adult-stage nematodes (Horton et al., 2000), but neurologic side effects have been a concern (Bockarie & Deb, 2010). It has been shown that single doses for these anti-filarial drugs in different strengths and intervals are effective (Addis et al., 2005).

The current regimen is designed for community-wide PCT. For those with clinical symptoms, a separate individual chemotherapy regimen such as the use of doxycycline for 4-6 weeks for associated bacterial infections and reduction of symptoms has been shown to be more effective than current regimens (Hoerauf, 2008). Doxycycline may well be an alternative for ivermectin but it has its limitation because it cannot be used with children under the age of nine (Hoerauf, 2008). The development of other drugs such as moxidectin for onchocerciasis and flubendazole for *O.vulvulus* may also be effective against filariasis and suitable for filariasis co-endemic areas (Bockarie and Deb, 2010).

1.1.8.2 Disability Management

LF infected individuals with acute, chronic and advanced clinical disease suffer physically and socially from the disability throughout their lives. Second of the two aims of GPELF is to reduce the suffering of those who are affected. Because morbid clinical manifestations are irreversible and cannot be cured (Dreyer et al., 2002), morbidity management and prevention are important strategies to alleviate suffering (Addis et.al., 2010; Dreyer 2002). It is estimated that out of the 120 million infected worldwide, there are 14 million who suffer from lymphoedema. Basic lymphoedema management has been a method practised by Dreyer and her colleagues in Brazil since the 1980s. Comprehensive observations have contributed to the development of new methods of implementing compression bandaging (Addiss et al., 2010), hygiene, skin-care and physiotherapy as standard practice for clinical and home-based management (Dreyer et al., 2002).

Acute filarial lymphangitis attacks are caused by the death of adult worms as they cause blockage, leading to lymphatic system damage and acute dermatolymphangioadenitis (ADLA) attacks as identified by Dreyer and colleagues (Brantus, 2009). Repeated ADLA attacks cause further damage to the peripheral lymphatic channels leading to elephantiasis. Morbidity management is, therefore, essential in reducing acute attacks as a preventative measure and in some cases, can have reversal effects (Addis et al., 2010).

Morbidity management programmes are low, with only 27 participants out of 53 countries in 2011 (WHO, 2010). Surveys in Sri Lanka (Yahathugoda et al., 2005) and India (Schellekens et al., 2005) reveals morbidity management is not known to physicians and not prescribed to those who are affected. In the Dominican Republic, reports of local and cultural practices and self-medication using topical antibiotics on affected areas are common (Person et al., 2006). Where morbidity management programmes are implemented, reductions are observed. In Burkina Faso, a reduction of 39.1% of acute attacks (Jullien et al., 2011) and 69% in Haiti (Addis et al., 2010) has been reported. Addis (2013) adds that low programme participation is due to competing elimination objectives for diseases other than LF and a lack for sustained compassion to drive the cause. The importance of an integrated approach (discussed in

Section 2.3.1) is, therefore, imperative to implementing an effective management programme in a global scale to reach those who need it (Ramaiah & Ottesen, 2014).

1.1.8.3 Vaccines

Current elimination strategies for Africa are not sufficient in controlling the disease long-term (McGraw & O'Neill, 2013). This is due to overlapping infections that prevent available drugs to be used and most unlikely to be effective (Babayan, Allen & Taylor, 2012). The availability of vaccines will be integral for a sustained interruption of transmission (Keiser & Nutman, 2002). Adding to the need for vaccines are the growing concerns on drug resistance (Samykutty et al., 2010) that needs to be taken into consideration if PCT is the main strategy for transmission interruption.

Irradiated larval vaccines have been investigated but these have their own limitations where protection is limited to the L_3 stage as seen by Babayan et al. (2006) and Goff et al. (1997). The poor understanding of the parasite-host interaction and the natural course of disease contribute to the challenges of vaccine development. Vaccines based on multivalent recombinant protein had been demonstrated to protect against *B. malayi* in a mouse model (Samykutty et al., 2010) up to 95% protection in L_3 challenge (Dakshinamoorthy et al., 2013). This may be sufficiently effective as a prophylactic vaccine against LF. Vaccines against *B. malayi* and *W. bancrofti*, possibly a cross protection against *Loa loa* and onchocerciasis have also shown 100% protection in rodent system, but are yet to be demonstrated on non-human primate studies (Kalyanasundaram, 2011). To this day, no vaccine has been demonstrated in humans (Samykutty et al., 2010).

1.2 THE GLOBAL PROGRAMME

1.2.1 History of the Programme

In the US Centers for Disease Control and Prevention (CDC) International Task Force for Disease Eradication Report (1993), LF was one of the six diseases identified as

eradicable, or potentially eradicable. This is due to the availability of effective anti-filarial drugs. In the 1997 50th World Health Assembly (WHA50.29), LF was tabled as a global public health problem for the elimination. The private-public partnership of WHO and GPELF was formed later in 2000, with the aims to support the elimination goal by 2020. The role of GPELF is to organise fundraising, advocacy and communication while WHO coordinates policies and strategies to achieve elimination goals (Dennis et al., 1992; Ichimori et al., 2014). The aims of GPELF are to (1) stop the spread of infection by interrupting transmission and; (2) reduce the suffering of those who are affected.

WHO has received phenomenal support through commitments of long-term drug donations from GlaxoSmithKline (GSK) for albendazole, Merck & Co., Inc. for ivermectin and Eisai for DEC (Ichimori et al., 2014; Ottesen et al., 2008). These drugs have made a remarkable difference for the elimination efforts. To interrupt transmission, the strategy was to use a single dose of a combination of two drugs over a period of five or more years to entire at-risk populations (WHO, 2013a). The repeated mass drug administration (MDA) or PCT is said to be effective in breaking the LF transmission by killing the parasite to very low levels, a threshold where transmission cannot be sustained (Ottesen, 2006; Bockarie et al., 2009). This threshold is < 2% antigenaemia in *W. bancrofti* where *Anopheles* and *Culex* are the main vectors. In *Aedes* areas, this is <1% antigenaemia prevalence. In Brugian areas, the threshold is <2% antibody prevalence (WHO, 2013a). The implementation of PCT has been successful in the past by the reduction of microfilariae to nearly zero levels (Bockarie et al., 2009; Rebollo & Bockarie, 2013).

One of the two goals of the GPELF is to help reduce the suffering of an estimated 120 million infected worldwide and the 14 million who suffer from lymphoedema. Because morbid clinical manifestations are irreversible and cannot be cured (Dreyer et al., 2002), morbidity management and control is an important strategy to alleviate the suffering, and prevent disability of those who are affected (Addis et.al., 2010; Dreyer 2002).

The integration of resources for all neglected tropical diseases (NTDs) that overlap in 56 of 58 poor and disease-endemic countries (Hotez et al., 2009), were identified as crucial in the global elimination programmes (Hotez et al., 2008, 2009; Burkot et al., 2006; Ichimori et al., 2014; Alexander, 2015). In 2012, the London Declaration on

NTDs (www.unitingtocombatntds.org) and the WHO Roadmap for Implementation to overcome NTDs endemic in 149 countries (WHO, 2012) has officially launched integration strategies to meet NTD elimination goals. This new global strategy was also supported by the Global Alliance to Eliminate Lymphatic Filariasis (GAELF) in the 7th meeting in November 2012 (Brady & GAELF, 2014). These initiatives aim to accelerate the elimination efforts of eleven chosen NTDs by: (a) providing accessible PCT programmes to those who require it; (b) implementing and integrated vector control; (c) intensifying morbidity management on case-detection basis; (d) preventing human-animal suffering by zoonosis and provision of clean water and; (e) promoting sanitation and hygiene (WHO, 2012).

1.2.2 Current Successes

After 14 years of improved diagnostic tools, screening methods, vector control, MDA, morbidity management and other elimination activities, the progress of elimination programmes have been remarkable (Ramiah & Ottesen, 2014). An assessment based on *risk-of-infection* was published by Hooper et al., (2014) estimating a 46% reduction of the at-risk population to 789 million. This was nearly half in 2012 and 59% fall based on a *reduction-in-the-burden* assessment. By the end of 2013, 60 of the remaining 72 endemic countries will have implemented PCT programmes (WHO, 2013a). The concerted effort to accelerate elimination programmes of neglected tropical diseases announced by WHO (2012) will conceivably progress to meet the objectives of eliminating LF in 2020, PCT coverage expected at 100% for about 695 million people. At the time of writing this thesis (2015), challenges remain for the global programme. The WHO report and strategic plan (WHO, 2010) summarises the current successes halfway and highlights the priorities for the second half of the programme. It includes scaling up and reaching out with respect to the challenges.

1.2.3 Current Challenges

The WHO Progress Report and Strategic Plan (WHO, 2010) towards elimination by 2020 identified three current challenges:

(1) programme implementation;
(2) geographical coverage of PCT and;
(3) criteria for stopping PCT and surveillance.

To meet the goals by 2020, implementation is a priority for the eighteen remaining countries still to start PCT. The lack of suitable drugs for countries co-endemic for *L. loa* is one of the main problems for non-implementation of programmes in fifteen countries in Africa (Bockarie & Deb, 2010; Brady, 2014). Also, the current destabilisation of communities brought about by conflict has been an impediment to the start of programme implementation. There are currently 13 countries in Africa affected by conflict (WHO, 2010). For areas that have started implementation, the lack of geographic coverage and an acceptable, unified method for end-point assessment and surveillance are the major issues impeding progress.

1.2.4 The Need for Antibody Assays

The GAELF strategy to interrupt transmission of LF through PCT is implemented by (1) mapping of LF transmission areas to determine geographical distribution; (2) implementing PCT where needed over 5 or more years; (3) determining an end-point for stopping intervention; (4) surveillance after stopping PCT and; (5) verifying if transmission has been interrupted (Robello & Bockarie, 2013). Diagnostic tools are crucial throughout these programme stages to provide information such as prevalence rates, intervention efficacy and interruption of transmission for programmatic among others, important in programmatic decision-making. The study by Bergquist, Johansen & Utzinger (2009) highlights the significance of a comprehensive diagnostic approach in helminthic elimination programmes as efforts progresses towards its goals. Diagnostic tools must be complementary to the current epidemiological situation of each stage of elimination. The use of suitable tests of highest sensitivity and specificity are most crucial at the endgame of elimination. In most of the helminthic infections, some rounds of PCT consequently decrease antigen and mf prevalence. Antigen tests that were once sensitive for the mapping stages are no longer suitable as a tool for the low-prevalence community. Antibody detection becomes a more suitable marker to detect immunological changes in a community.

Serological testing for epidemiological studies were used as early as the 1970s and successfully demonstrated in malaria eradication programmes as a tool for assessing changes in exposure (Drakeley & Cook, 2009). For LF PCT programmes, antibody assays are crucial for scale-down and endgame strategies of the global LF elimination programme. Antigens appear up to 18 months after the establishment of adult worms

(Steel et al., 2012b), a long time to wait before antigen can be detected by assays. By this time, transmission can potentially be established in the community and the elimination efforts will be lost. Since antibody appears earlier after exposure to infective L_3, it better serves as a marker for early detection of infection (Steel et al., 2012b). Children born in LF endemic areas develop antibodies as young children, making antibody detection a powerful index for infection. These sentinel children are the basis for assessing changes in transmission of infection (Lammie et al., 2004; Grady et al., 2007; Joseph & Melrose, 2010).

The availability of improved antibody diagnostic tests based on recombinant antigens provides the elimination programme with a reliable tool to help in programmatic decisions. Correct assessment of transmission status is vital as a majority of countries approach the end of PCT programmes. Why is antibody testing important for the end-game? The rationale for using antibody as the index for transmission in elimination programmes is summarised below:

(1) after repeated drug treatment, microfilaraemia and antigenaemia are reduced significantly to low levels, below the detection limits of antigen tests (Section 2.2.4.2), making antigen detection unsuitable as a tool for transmission assessment;

(2) antigen persists in blood up to three years after drug treatment (Schuetz et al., 2000), therefore, PCT results in community transmission assessments give inaccurate results for programme decision-making and;

(3) young children born during or after the start of PCT can be used as a sentinel group for transmission surveillance. They should be free of infection and will develop antibodies if exposed to infective L_3, a powerful indication of ongoing transmission.

1.2.5 Determining the End-point and Hot Spots

WHO issued a new guideline in monitoring and assessment of PCT programmes (WHO, 2011). These guidelines are activities and strategies for the transmission assessment survey (TAS) that aim to determine when assessment units (AUs) can

stop PCT or whether PCT needs to be continued. Despite the arguments against the use of antigen detection in AUs where antigens levels are very low, TAS recommends the use of BinaxNOW card test for bancroftian filariasis antigen testing. For brugian filariasis, the antibody RDT BrugiaRapid was recommended. It seems, after more than halfway through the elimination programmes, there is a lack of a unified and comprehensive method of assessing and verifying whether the transmission has ceased. This is a programmatic challenge (WHO, 2010), a gap that is recognised to prevail in PCT programmes and can be addressed by the use of a comprehensive method of transmission assessment incorporating antibody tests.

The Progress Report of WHO (2010) recognises the importance of antibody assays for the critical stage of stopping PCT programmes as outlined in a list of programmatic challenges. During the first ten years of the programme, the need for diagnostic assays was emphasized by Melrose et al., (2004), Lammie et al., (2004), Weil (2005), Weil & Ramzy, (2007) and Grady et al., (2007). These authors hailed antibody testing as the most appropriate and useful in detecting end-point of transmission and surveillance studies.

The significance of antibody testing was realised with the use of the Bm14 Antibody CELISA (Cellabs Pty Ltd, Brookvale, Australia) for comprehensive studies of LF spatial clustering and surveillance in the Pacific Island Countries and Territories (PICT). A series of transmission assessment studies were conducted by Joseph (2010) as part of the requirements for the degree of Doctor of Philosophy at JCU.

In Samoa, a transmission assessment was conducted after seven rounds of PCT from 1999 to 2006. It was established that spatial clustering or “hot-spots” exist by observing young children less than ten years old who were born within the PCT programmes. By using antibody as the index for transmission, four AUs were observed with varying degrees of antibody prevalence, an evidence of spatial clustering of transmission (Joseph et al., 2010). Transmission assessment studies in Samoa, Tonga, Vanuatu (Joseph et al., 2011a.) and five Samoan AUs (Joseph et al., 2011b) were also conducted to determine the value of using the antibody test as a complimentary method for assessment. Mapping of infection (mf, antigen and IgG4 antibody) in the AUs studied gave useful information of current residual infection, and the corresponding antibody levels. The strategic assessment used in these studies is important for steering future programme decisions in the PICT.

Another fine example of transmission assessment using antibody test against Bm14 has been demonstrated in a recent publication of a comprehensive study in Sri Lanka after six years of PCT (Rao, et al., 2014). The study used the WHO TAS guidelines using antigen for school-based assessments coupled with antibody testing. Rao et al., highlights the downside of using a universal TAS for areas with significantly reduced mf and antigen rates because it was not sensitive and was not sufficient to show an interruption of transmission. There is no one-fit-all assessment strategy because each AU is unique. The method used in this study demonstrates an excellent comprehensive assessment that is appropriate for each AU. Based on the studies, it recommends the use of antibody testing for children and parasite DNA detection for comprehensive surveillance programmes.

Since some of the programmatic challenges are due to the failure of some AUs to meet the end-points, the importance of continuous assessments in low transmission areas need to be highlighted to ensure that efforts are not lost.

1.3 THE IMMUNOLOGY OF LF

1.3.1 Background of LF Immunology

The wide spectrum of LF disease is a result of the immunological reaction of the host to parasite interactions. Immunology is the main discipline that drives our continued gain in knowledge of the complexity of LF disease (Ottesen,1989) which makes the role of immunodiagnostics important. Historically, children were excluded in studies of the spectrum of LF disease because they are rarely seen to be affected. Witt & Ottesen (2001) discuss the recognition of children as an important consideration in the immunological study of LF because LF is an infection of childhood. The availability of improved diagnostic assays has revealed the early acquisition of infection by children living in an endemic area and the occurrence of some subclinical manifestation of disease of those who do not observe any clinical manifestation of disease.
The host immunity plays an important role in immunoregulating the mechanisms that work against the filarial L_3. This ensures that the parasites can persist in the host without destroying it (Das & Shenoy, 2008). It is also believed that the co-evolution of host and the parasite in an endemic setting has allowed a protective immunity

phenomenon that can be observed in asymptomatic conditions. The same co-evolution theory is supported by an influential Presidential Address written by Beaver (1970). Beaver's comprehensive review of the "filariasis without microfilariae" condition and has made points of other different variations of the disease.

A review by Rajan (2005) also agrees that the study of infection in endemic residents prevails but understanding the natural course of disease in individuals with naïve immunity will give a clearer explanation. The ideal situation is to follow the natural course of disease in one individual from the early years of life. He points out that humans either mount an immune response to terminate infection without any pathology as seen in a comprehensive studies of World War 2 American military forces in the Pacific by Wartman published in 1947. After years of follow-up studies, there has been no significant number of individuals developing mf. This is in contrast to the observations of Portano (1978) in a study of transmigrants from a non-endemic area of Irian Jaya in Indonesia. This group was re-settled to Flores, endemic for brugian filariasis. A significant number was found to have developed mf over time as early as two years. Follow-up studies also found a significant number developing elephantiasis. Beaver (1970) proposes a fit and non-fit in the host-parasite relationship but it does not clearly explain why the Irian Jaya transmigrants show the sequelae of LF. It seems that prolonged exposure to transmission is one major factor. However, according to McNulty et al., (2013), the variety of observations may also be due to the differences in LF species.

Although these studies make some logical point based on the current knowledge of the dynamics of immunology, there are still underlying mechanisms that are not clearly explained despite a large amount of studies available. The study of non-endemic LF infections may not give useful clues for the endemic situation because as explained earlier, there is a strong belief that disease manifestation is dependent on the immunological interactions of host and parasite. This approach may still be distant from explaining the mechanisms of the sequelae of the disease in endemic areas because of the varied immunological disposition of both non-endemic and endemic groups. There may be an accumulation of other factors that contribute to various immunological changes. The later sections will briefly cover immunological topics of interest with regards to immunodiagnostics of LF.

1.3.2 Understanding the Immunology of LF Infection

When foreign objects including microparasites such as viruses, bacteria or protozoans, and macroparasites such as helminths enter the human body, it triggers the immune system to mount immunological defences to protect itself and expel the foreign object or organism. Immune mechanisms of man are seen to have co-evolved with parasites that infect them (Beaver, 1970; Ottesen, 1989; Maizels et al., 1993; Allen & Maizels, 2011; McSorley & Maizels, 2012). Unlike other microparasite infections that multiply in the host, helminths reproduce sexually and produce progeny in high numbers, a crucial transmission stage for its survival (Maizels et al., 1993).

The body's first line of defence following penetration of the skin barrier by an invader is the innate immunity, a rapid, non-specific immunological reaction with an arsenal of white blood cells and phagocytes to attack invaders, and natural killer cells to remove infected cells. Most infections can be cleared efficiently by antigen presenting cells (APC) of the innate system called dendritic cells, without the involvement of other immune mechanisms (Babu & Nutman, 2013). Though in the case of persistent infections such as large helminthic parasites (Maizels et al., 1993), the adaptive immune system is activated because prolonged innate immune reaction can be damaging to healthy cells.

The adaptive immune system is the next line of defence with immune mechanisms that are mounted slower and more specifically to expel the invader. The adaptive immune system responds with the proliferation of T-cells and B-cells. In T helper subsets, TH1 is characterised by producing interferon gamma (IFNγ), interleukin (IL)-2 and tumor necrosis factor beta (TNFβ) that function as inflammatory mediators and selectively activate macrophages as defence against intracellular pathogens. TH2, on the other hand, produces cytokines interleukin (IL) IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 that elicit a vigorous antibody response specific to the antigen. It stimulates B-cell and eosinophil development, mechanisms mainly against intestinal nematodes (Maizels et al., 1993; Romagnani, et al., 1997; Romagnani, 2000). A good understanding of LF immunology will help in delineating the complexity of the disease. However, mechanisms are still not clear and cannot be substantiated because these immunological responses do not correlate with disease manifestation (Piessens, 1981; Adjobimey & Hoerauf, 2010; Bundy et al., 2001). A multitude of varying

information from investigations in both animal and human observations exist (Maizels et al., 2000).

1.3.3 Cell-Mediated Response of TH1 and TH2

The TH1/TH2 paradigm is a concept established with animal models where polarisation occurs with one system activated and the other muted (London et al., 1998). TH2 response is believed to be a helminthic infection defence mechanism (London et al., 1998). In LF, a strong TH2 response and a downregulation of TH1 is known to occur in humans who are microfilaremic and no obvious acute or chronic clinical pathology (Maizels, Allen & Yazdanbakhsh, 2000), but may have sub-clinical pathological changes (MacDonald et al., 2002). TH2 immune responds in high levels of CD4+, T cells that produce immunoregulatory IL-10 (Mahanty & Nutman, 1995; Mahanty, 1996), IL-5 (Maizels et al.,1993, 2000; McSorley & Maizels, 2012) , and IL-4, producing an elevated IgG4, IgE and IgG1 antibody response (McSorely and Maizels, 2012).

Maizels et al., (2000) observe a wide display of IL4/IgG4 and IgA as a result of a TH2 hyper-response and a lowered IgE response in “endemic normal”. This observation conflicts with the widely observed IL4/IgG4, IgE response seen in asymptomatic individuals with high mf loads (Maizels et al., 2000; Sahu et al., 2008). The ability of an individual to mount a strong, responsive TH2 may have bearing on the development of clinical pathology (MacDonald et al., 2002; Adjobimey & Hoerauf, 2010).

The unresponsive TH1 is marked by the decrease in IL-2, IFNγ (Babu et al., 2006) that activates macrophages (London et al., 1998). A diminished B-cell and Ag-specific proliferation (Babu et al., 2005) and the expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) inhibitory marker (Steel & Nutman, 2003) that activates neutrophils and NK cells. These function to attack the parasite by releasing nitrogen intermediates and nitric oxide into the surface of parasites (Babu & Nutman, 2012). TH1 is mainly inflammatory response and can contribute to tissue damage and may be due to the downregulation by the adaptive immune system (London et al., 1998). An array of other TH1 suppressed regulatory function have been described (Muraille & Leo, 1998), though it is not clear if TH1 is responsible for the development of clinical pathology in LF (MacDonald et al., 2002). It is however thought to be a response

against the specific antigen (Mahanty & Nutman, 1995). The unresponsive T-cell is not unique to helminths and quite common, seen with other diseases such as visceral leishmaniasis and leprosy (T-cell, PHA), tuberculosis (TGFβ), schistosomiasis and paracoccidioidomycosis (IFNγ), all of which return to normal function after chemotherapy (Maizels et al., 2000).

1.3.4 Humoral Response of IgG Subclass

Early immunological investigations on LF endemic individuals have observed a high IgG antibody response in the presence of IgE. The proportion of subclasses varied, depending on the clinical manifestation of the disease (Rajan & Gundlapalli, 1997). The study by Ottesen et al., (1985) was one of the first publications to investigate IgG subclasses and the corresponding clinical forms of LF. In elephantiasis patients where mf is not usually present, IgG1, IgG2 and IgG3 are in higher levels than IgG4 (Kurniawan et al., 1993; Rajan & Gundlapalli, 1997). A study by Nielsen et al., (2002) has seen high levels of IgG3 in individuals with advanced lymphoedema as compared to the decline of IgG4 in the early stages of the disease. It may indicate a switching of antibody subclasses where the decline in IgG4 is a signal of a pathological change or the clearing of parasites (Bal et al., 2003). Although a different observation by Simonsen et al. (2002) on IgG3 does not support this. The group's East African study found IgG3 to be significantly higher in a low transmission area compared to a high transmission area. The low transmission area observed a ratio of IgG4 /IgE that corresponds to the theory of protective immunity. It indicates that there are other factors at play resulting in resistance to infection. Transmission intensity has been thought to play a part in the variation of disease progression and a drive for the acquisition of herd immunity.

Subclass IgG1 is known to be a response specific to microbial infection Adjobimey & Hoerauf (2010), but found to be negatively associated with mf and may represent a response against L_3 (Simonsen et al., 2002). There has been some debate that IgG1 may have a role as an early marker for diagnostics. It has been associated as a prominent immune response in LF infections and may well represent an early signal to infection, more so than the IgG4 that is strongly associated with mf.

Ottesen (1989) notes that immune reactions against LF may better be explained by studying non-endemic normal populations whose exposure to LF do not trigger a

downregulation of immune system. Non-endemic studies may give some insight to the pathology of LF Rajan (2005). It appears that in these conditions, there is a significant IgG1 response that needs to be defined. The study conducted by Melrose et al., (2000) of non-endemic expatriate mine workers in an endemic area in Papua New Guinea found IgG1 prevalence differed between those who worked outside and were exposed to the environment when compared to those who worked inside offices. Correlated IgG1 high levels and increased time of exposure in the environment is suggestive of a response to parasite exposure. A similar study of Australian defence personnel deployed to Timor-Leste by Frances et al., (2008) investigated the IgG1 and IgG4 antibody response to LF. Exposure to LF was evident with 49/907 subjects with IgG1 seroconversion compared to 1/944 IgG4 seroconversion, a strong indication of an IgG1 prevalence. The non-conversion of IgG4 for this non-endemic population supports IgG4 as a marker for active infection. This holds true because observations show that not many will go on to develop the disease, possibly due to clearance of invading parasite.

In a study by Kurniwan-Atmadja et al. (1998), the reactivity of somatic antigen extracts from different parasite life cycle stages was investigated. It was found that L_3 surface antigen extracts are strongly reactive with IgG1 rather than IgG4, suggesting that IgG1 has a role in early detection of exposure. It has also been observed that the antibody response increases with age (Njenga et al., 2007), a common theme in age-profile studies. If IgG4 is a specific response against mf and adult worm antigens, perhaps, there is a distinct switch of antibody subclasses from L_3-specific IgG1 and as the disease progresses, IgG4 is presented. This means that the identification of a specific IgG1 may be suitable in detecting ongoing transmission for PCT assessments. The study has been followed up from the work of Day, Gregory & Maizels (1991) where IgG antibody responses were observed in age-specific immunity of young children exposed to L_3. By using L_3 antigens to determine IgG reactivity, age-dependent IgG1 is seen in the youngest group. Although both studies presented very interesting findings with respect to IgG1 and associated early filarial exposure, the antigens used were from crude parasite antigens. These antigens as described previously were said to be cross-reactive.

One study based on the recombinant Bm14 that supports the IgG1 theory is the age-specific studies in Kenya by Njenga et al., (2007). In this study, similar results were found with regards to L_3 -specific IgG1 response in early filarial infection. Children in

the age group 4-11 years were found with higher IgG1 response than IgG4, suggestive of L_3 exposure. The rise of IgG4 subclass seen in the older age group is indicative of a subclass switch from IgG3 to IgG4 that occurs as infection progresses, a sign possibly related to pathological changes. This study also supports the potential use of Bm14 in surveillance studies for elimination programmes in different endemic areas. The rAg Bm14 being suitable for use with the different IgG subclasses.

1.3.5 Protective Immunity and IgG4 Subclass

The humoral IgG antibody response, including IgE is produced vigorously against specific antigenic attack by the adaptive immune system. In the presence of high mf loads in individuals living in endemic areas, the prominent IgG4 and an IgE response have been observed (Ottesen et al., 1985; Lal & Ottesen, 1988; Garraud et al., 1995; King et al., 1993; Haarbrink et al., 1999; Terhell et al., 1996, 2000, 2002; Neilsen et al., 2002; Hussain et al., 1987). Induced by mf, IgG4 levels can reach as high as 95% of the total IgG in infected patients (Ottesen et al., 1985). The dominance of IgG4 has made it the widely accepted marker used for antibody diagnostics and an indicator for active infection.

High levels of IgG and IgE have been observed in infected patients who are antigen positive (Ottesen & Nutman, 1992; Nutman & Kumaraswami, 2001), or with adult worms (Dreyer et al., 1996). This reaction was also correlated with TPE, an allergic reaction associated with IgE due to the dying mf in the lymphatics. IgE is produced by B-cells and regulatory IL-4 and IL-13 but in the presence of IL-10 and TGFβ, the reaction leans towards switching to a dominant IgG4 (McSorely and Maizels, 2012). Antibody IgG4 is believed to have a major role in protective immunity and its blocking activity is well known for inhibiting IgE allergic responses to antigen (Ottesen et al., 1981; Hussain et al., 1992; Garraud et al., 1995). IgG4 blocks by attaching to mast cells and eosinophils at the antibody fixing sites.

The protective immunity triggers a class switching of antibodies to the non-inflammatory IgG4 (Babu & Nutman, 2014) and possibly a parasitic mechanism to evade the host immune system (Adjobimey & Hoerauf, 2010). The production of antibodies in the adaptive immunity is to protect the body from antigenic attack. However, questions are also raised if this state contributes to disease pathogenesis (Piessens et al., 1987; Neilsen et al., 2002; Terhell et al., 2000).

Children born to mothers with filarial infections acquire immunity to parasite antigens that allows them to mount the TH2 response, indicated by elevated levels of IgG4 and IgE (Terhell et al., 2002; Weil et al., 1983). Children as young as 18 months old born in high endemic areas with high transmission intensity mount specific IgG4 and IgE responses in comparison to those in lower transmission intensity areas (Terhell et al., 2000;).

Innate immune B-cells produce IgG4 and IgE as a response to the T-cell producing IL4 and IL13 but IL21 has a role in the production of IgG4 as well as IgG1 (Adjobimey and Hoerauf, 2010). The prolonged persistence of antigen being released by mf results in continuous antibody production by B-cells but the extent to which antibodies can protect the host is not clearly understood (Cross, 1996; McSorley and Maizels, 2012). The dominant IgG4 may have other important roles in the development of protective immunity (Sahu et al., 2008; Adjobimey & Hoerauf, 2010), however this needs to be substantiated (Ravindran et al., 2003). A study by Van der Neut Kolfschoten et al., (2007) has demonstrated the anti-inflammatory reactivity of IgG4 in rhesus monkey model. Several human observations have also showed that previously infected with mf, the clearance of mf by chemotherapy resulted in the absence of IgG4 in some individuals who later on developed clinical pathology (Kurniawan et al., 1993). These arguments were said to be reasonable indications of the protective role of IgG4, with its presence, progression to lymphoedema or elephantiasis is prevented.

1.3.6 Filariasis-specific IgG4 Marker

The inherent problem with cross-reactivity using crude antigen preparations has been a limitation for LF research in the past. There has been no alternative until the advent of state-of-the-art molecular techniques. The introduction of recombinant antigens developed against brugian or bancroftian filariasis made it possible to develop assays that are highly specific. IgG4 antibody is said to be non-specific because of its association with other helminthic infections. However, the use of a combination of a recombinant antigen reactive against both burgian and bancroftian filariasis and coupled with a monoclonal antibody detection against IgG4 makes for a robust, immunochemical assay.

This area in LF diagnostic research has been underutilised with only two other assays, the BrugiaRapid for *B. malayi* and the cocktail rAg Pan LF ELISA for both *B. malayi* and *W. bancrofti*. These assays have filled the need for antibody assays for LF but may not be adequate for the global PCT programmes. In addition, these are restricted to IgG4 assays. The rAg Bm14 is highly specific to both *B. malayi* and *W. bancrofti* have not been used to its full potential. The standardisation and optimisation commercialisation of the Bm14 antibody ELISA will make the assay widely available for the LF elimination community.

1.4 SUMMARY

After more than ten years of the GPELF, many countries are approaching the end of PCT programmes that is the strategic approach to eliminating LF by 2020. This review has identified the following key areas in LF research that needs to be addressed as elimination efforts move forward:

(1) A gap exists where the need for antibody tests had not been adequately addressed. Why is an antibody test needed?

(a) Antigen tests are no longer adequate for use at the assessment and surveillance stages of elimination because of the lack of sensitivity in low-level antigen and mf areas. Antibody becomes the most suitable method of testing in transmission assessment and surveillance studies.

(b) The value of antigen tests diminishes because filarial antigen persists in blood up to three years after drug treatment. Programmatic decision making that rely on transmission assessment studies can potentially impede the progress of elimination programmes.

(c) Using the antibody responses of young children born during or after the start of PCT is a powerful approach to transmission surveillance. Sentinel group should be free of infection and if

exposed to the presence of L_3, will develop antibodies, a powerful indication of ongoing transmission.

(2) The current tests used for the GPELF TAS may not be adequate as one of the challenges reported by WHO (2010) is the requirement of suitable assays for stopping PCT programmes. The role of antibody testing is essential for transmission assessments. Therefore, the development of a commercially available recombinant–based Bm14 antibody assays fulfil this gap. Why are anti-IgG4 Bm-14 antibody-based assays needed?

(a) The use of recombinant antigen Bm14 resolves the problems with past antibody assays based on crude antigen preparations that were cross-reactive with other helminthic infections.

(b) The recombinant antigen Bm14 is reactive for both *W.bancrofti* and *B.malayi* infections. It is also adaptable to different IgG subclass antibody markers, making it suitable for use in immunological studies.

(c) A rapid test dipstick version of the assay will address the need for a convenient field-based diagnostic tool.

(3) Studies of age-profile and the role of IgG1 and IgG4 antibody isotypes were based on crude antigen extracts. The newly standardised recombinant-based Bm14 Antibody CELISA was utilised to present IgG1 and IgG4 age-profile of *W. bancrofti* infection based on a highly specific assay.

(4) What are the quality and regulatory requirements that scientists need to consider when developing a novel diagnostic test, and how can it be translated to a commercial product for the wider research and clinical laboratory? Because Australia is the first country to harmonise IVD regulatory reforms for the future, diagnostic assay researchers and developers can benefit from the information based on ‘lessons learnt’. This thesis will address the need for information or guidance on how to translate a research diagnostic product to a commercially available test using the Bm14-based antibody assays as a template.

CHAPTER 2

STANDARDISATION AND OPTIMISATION OF A NEW ANTIBODY ELISA BASED ON RECOMBINANT ANTIGEN Bm14

2.1 INTRODUCTION

Following the fiftieth World Health Assembly (WHA50.29) (WHO, 1997), the GPELF was formed in year 2000 with the aim to eliminate lymphatic filariasis as a public health problem by 2020. The two goals were to stop the spread of LF infection by interrupting transmission and, to alleviate the suffering of those who are infected with the debilitating disease. The strategy to interrupt transmission was the MDA or PCT in whole populations mapped as endemic for LF. The rationale for using this strategy alone is based on the premise that mf in a community can be reduced below a threshold where transmission can no longer be sustained (Ottesen, 2006; Bockarie et al., 2009). This threshold is < 2% antigenaemia in *W. bancrofti* where *Anopheles* and *Culex* are the main vectors. In *Aedes* areas, this is <1% antigenaemia prevalence. In Brugian areas, the threshold is <2% antibody prevalence (WHO, 2013a).

Transmission is believed to be unsustainable at very low levels of antigen or mf, even without the administration of anti-filarial drugs (WHO, 2013a). PCT is to be administered over a 5 -year period with coverage of more than 65% of an implementation unit (IU). An end-point assessment is to be conducted to determine whether PCT can be stopped otherwise, additional rounds are administered. A surveillance period after the cessation of PCT is to be assessed for the completion of an elimination programme.

Diagnostic tools for use in the elimination programmes have a crucial role in achieving the goal of the GPELF. The call for the development of reliable diagnostic tools (Lammie et al., 2004; Melrose et al., 2004; Weil, 2005; Weil & Ramzy, 2007; Grady et al., 2007) has been topical during the first half of the GPELF 20-year elimination period. The currently employed diagnostic tests for the elimination programmes at that time are the mf counts, Og4C3 Tropbio filarial antigen ELISA, and the ICT BinaxNOW

antigen card test. More than ten years onward, there has been no widely accepted diagnostic tools for assessing the progress of elimination programmes and its surveillance after PCT. The recent WHO progress report and strategic plan for 2010-2020 (WHO, 2010) indicate the urgent need for guidelines for stopping PCT and surveillance. Mf detection and antigen rapid diagnostic tests (RDT) are currently being used but these may not be suitable for transmission assessments for stopping PCT or surveillance after PCT has ceased. After repeated PCT, antigen levels fall below detection limits therefore antigen tests are no longer sensitive (Melrose et al., 2004). Also, Filarial antigen can remain up to three years after drug intervention, as found by Schuetz et al., (2000) in a post-treatment surveillance study using the ICT BinaxNOW card test in Haiti. This implies that alternative diagnostic tests to antigen detection are required because the value of current antigen tests diminishes especially where transmission is high. The use of antigen tests in PCT monitoring and surveillence also needs to to be well-qualified to ensure correct reporting of transmission status of IUs.

The use of antibody testing for elimination programmes is not a new strategy. The role of antibody testing to assess transmission was first investigated by Mathews et al., (1969) for malaria eradication in the West Indies. Successful malaria elimination programmes after this time have relied on the use of antibody diagnostic tools to validate elimination efforts. For the filariasis research community, the value of antibody testing has also been widely discussed as a significant tool in the end-point determination and surveillance stages of the elimination programmes. For surveillance studies, antibody can be detected earlier in children born after PCT implementation, the sentinel group. If antibody is detected in the sentinel group, it becomes a powerful method to indicate that transmission is ongoing. It is agreed in general that an antibody test needs to be developed to complement the currently used diagnostic tests for elimination programmes.

Previous antibody tests were based on crude whole worm native antigens that posed a lot of cross-reaction problems (Melrose 2004, p.45). To be more useful as a tool for elimination programmes, a new antibody test has to meet certain requirements. An antibody test must be:

(a) based upon well characterised recombinant antigens;
(b) reactive with all three LF species;
(c) have excellent sensitivity and specificity;

(d) produced as a standardised commercially-produced kit;
(e) produced under high-quality control standards with on-going quality assurance;
(f) produced in batches to satisfy demand, but at an affordable price;
(g) have excellent transport and storage stability under tropical conditions and;
(h) produced preferably in an ELISA and rapid test format.

For every new diagnostic product development, market requirements are an important consideration in defining the outcome. The items outlined above were used to define the development of a new antibody test using the available recombinant antigen Bm14.

ELISA was chosen as the platform for a diagnostic test because it is one of the most reliable assays that can address all the requirements of the LF global elimination. It is a suitable low-tech format that does not require major laboratory infrastructure, yet sophisticated enough to achieve excellent sensitivity (Voller, Bartlett & Bidwell, 1978). It can detect low or early antibodies in sentinel children during the monitoring and surveillance stages of PCT programmes if constructed carefully to detect the lower limit. It is inexpensive enough for the tracing and mapping of residual infection. Also, an ELISA can be developed to achieve excellent stability to address reliability and robustness required for use in tropical and sub-tropical developing countries with limited laboratory resources.

The first ELISA utilising the recombinant antigen (rAg) Bm14-GST was a research assay developed by Gary Weil's group at The School of Medicine, Washington University, St Louis, MO, USA. The original research assay was a rough and ready research grade ELISA evaluated in Egypt by Weil et al., (1995). The assay requires each laboratory to prepare ELISA plates and buffers before testing can be carried out. A method of preparation that is not consistent from batch-to-batch making multi-centre comparisons almost impossible. The research grade method used long serum incubation times and prepared in a research laboratory setting without performance validation. The assay can only be manufactured small-scale, it is not standardised and does not undergo quality assurance. The kit production is impractical in a research laboratory and cannot meet the large-scale requirements for elimination programmes. Because of these reasons, there is a need to develop a standardised antibody test that is manufactured in a commercial setting to meet the large-scale requirement of a global programme. The antibody assay must also be produced under a Quality

Management System (QMS) to meet International Standards of IVD manufacturing (ISO13485).

To construct a new antibody ELISA acceptable for use, the specific GPELF requirements for a new antibody assay will be addressed. The standardisation and optimisation will focus on the lower limit of detection to be a useful tool for the monitoring and surveillance stages of PCT programmes. This will enable the detection of low-level antibodies in sentinel groups. Since background noise can cause reactivity levels to read higher optical density (OD), and sometimes higher than the cut-off value determined for the assay, unacceptable false positive readings can occur. Background noise shall be an important consideration across the construction stages to ensure that non-specific reactivity originating from buffer composition, components from serum and, other methodology processes that can illicit background noise is eliminated. The elimination of inherent background noise ensures correctness of result and high assay performance. The development will follow a series of experiments to select the best raw material for use and optimisation experiments to determine the most suitable parameters for assay reagents. In addition to aiming for specific product performance, the standardisation and optimisation of the ELISA will include in-built designs that following international standards of In-vitro Diagnostic (IVD) manufacture, ISO13485 (ISO, 2012). Implementing requirements of ISO13485 during the development of the Bm14 ELISA prepares the kit for the transition to a commercial-ready ELISA for use in both filarial research community as well as for the clinical laboratory.

This chapter aims to standardise a new antibody ELISA based on rAg Bm14 targeted at meeting the assay requirements of a well-characterised recombinant antigen for brugian and bancroftian filariasis. Experiments for construction, optimisation and standardisation employ the principles of ELISA based on the work of Engvall & Perlmann, the pioneers of the technology (Engvall & Perlmann,1971,1972). Experimentation of parameters for buffers, blockers, plates and kinetics of the assay is exhaustive and impossible to describe in detail in this chapter therefore, high-level experiments of the most important aspects of ELISA development will be presented.

Procedures used as references for the development of ELISA are by Voller, Bartlett & Bidwell (1978), Voller et al., (1974, 1976), Burgess (1988), Kemeny & Challacombe (1989) and doctoral dissertation by de Sevigny (1980).

2.2 AIMS

The aims for the work described in this chapter are to address the need for a new diagnostic tool for LF as stated by GAELF:

(1) To standardise and optimise an antibody ELISA based upon a recombinant antigen Bm14, suitable for use with LF elimination programmes;

(2) To design a commercial-ready ELISA under a GMP manufacturing guidelines compliant with international standards and;

(3) To optimise the ELISA for increased sensitivity and specificity.

2.3 MATERIALS AND METHODS

2.3.1 The ELISA Protocol

The ELISA protocol summary described below is an indirect method, a widely used method for measuring antibody in almost all human and animal infections as described by Voller & Bidwell (1986). The standard developed method is modified according to the ELISA component being investigated in each step. This summary can be used as a guide for assay development.

2.3.1.1 Preparation and Coating of ELISA plates

ELISA plates were prepared by coating a specific concentration of antigen in 0.05M carbonate-bicarbonate buffer at pH 9.6. Each well of a flat-bottom high binding ELISA plate, (Greiner Bio-One, Cat. No. 705-071, Frickenhausen, Germany) was coated with100uL each well and incubate overnight (18-21 hours) at 2-8^{o}C. The next day, wells were flicked empty to remove the solution and tap-dried on layers of laboratory tissue to remove residue. Added was 250uL/well of post-coat for each well and incubated for 45 minutes at 37^{o}C. After incubation, wells were emptied by flicking, tap-dried on laboratory tissue to remove residue. Plates were air-dried before using or if storing plates, these were sealed foil bag with desiccant sachets and stored at 2-8^{o}C.

2.3.1.2 ELISA Protocol for Experiments

All components were brought to room temperature before performing the assay. Serum samples were prepared by diluting at 1:100 in a 1x sample buffer using phosphate buffered saline + Tween (1x PBS/T) and used at 100uL per well. Samples were incubated at 37^{o}C in a humidity chamber for 60 minutes to allow binding of the antibody. Plates were washed four times with 1x PBS/T using an automatic plate washer (Multiwash III Microplate Washer, Tricontinent, UK) to remove any unbound antibody. The conjugate was prepared to the correct working dilution using a Conjugate Diluent and 100uL used for each well before incubation at 37^{o}C in a humidity chamber for 30 minutes. After incubation, plates were washed four times with 1x PBS/T to remove unbound conjugate. TMB substrate was prepared to working concentration using a substrate buffer and plated at 100uL/well.

Plates were left for 15 minutes in the dark at room temperature to allow colour development of complex and the reaction stopped using 0.1M orthophosphoric stopping solution. Plates were scanned at 450/620nm dual wavelength.

2.3.2 Interpretation of Results

The assay development experiments were described and followed with results under each method (Section 2.3). Interpretations appear under their respective sections as these results are the basis for making decisions for the preceding steps of assay format and construction.

Student's t-test was used to analyse the results statistically and comparisons were made between two sets of independent data. A 95% confidence level was used and results reported as t (degrees of freedom) = test value, p = 2-tailed significance.

One-way Analysis of Variance (ANOVA) was used to test comparisons of OD means between three or more sets of independent data. Comparison analysis used IBM SPSS Statistics software version 22.

The signal-to-noise ratio was used to express the general performance of ELISA on test samples where the emphasis on background or noise was required. The signal-

to-noise ratio (S/N) was calculated as S/N = positive control / negative control. A high S/N value >10 is said to be acceptable sensitivity for an ELISA (Rajasekariah et al., 2003).

2.3.3 Selection of ELISA Critical Materials

2.3.3.1 Evaluation of Recombinant Antigen Bm14

To determine the Bm14 antigen of choice for the development of the assay, rAg Bm14-HIS (HIS-tag purified) and BM14-GST (GST purified) were validated. An antigen reactivity comparison used a set of non-endemic negative reference samples (Figure 2.1a) and a set of low reactive endemic samples (Figure 2.1b) in an experimental ELISA. The ELISA plate was constructed by coating rAg at 2ug/mL concentration and a 0.5% Casein post-coat using the method in Section 2.3.1.

The result in Figure 2.1a shows the reaction of non-endemic samples on Bm14-HIS and Bm14-GST plates. There was a significant difference is reactivity between the antigens (t(19) = -4.147, p=0.0005). This result suggests that non-endemic samples do not react and have no background when tested using the Bm14-HIS plates as compared to Bm14-GST.

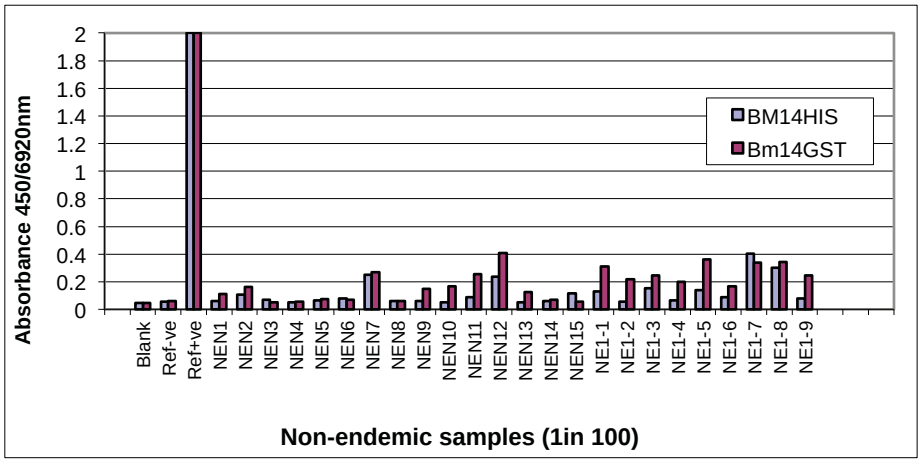

Figure 2.1a: Recombinant Antigen Evaluation. Using non-endemic serum samples, Bm14-HIS coated plate shows a significantly lower reactivity as compared with Bm14-GST coated plate.

The results for endemic samples were plotted separately (Fig 2.1b) to show reactivity for both antigens. The visual difference observed in the graph is confirmed as significant (t(14) = 2.751, p=0.0005), Bm14-HIS being more reactive as compared to Bm14-GST.

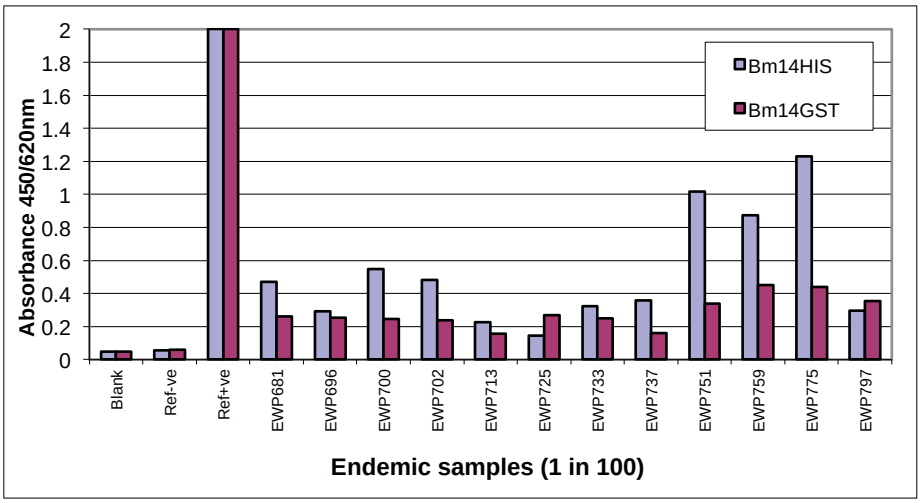

Figure 2.1b - Antigen Evaluation. A comparison of antigen reactivity using endemic samples shows a significantly higher reaction when using rAg Bm14-HIS plates as compared to Bm14-GST coated plates.

To check for background noise that may be attributed by the coating antigen, Bm14-HIS and Bm14-GST antigens and the respective vector blanks, CAT-HIS and GST at (2ug/mL) were prepared on a plate and post-coated with 0.5% Casein. The plate preparation and ELISA were performed as per methods in Section 2.3.1. To observe whether vector blanks interfere with low-level antibodies, 20 non-endemic samples and ten low-reactive endemic samples were included in the test.

Figure 2.2a shows the sample set results for Bm14-HIS and its vector blank CAT-HIS. A significant difference in the mean OD values (t(19) = 3.144, p = 0.005) was seen when non-endemic samples were tested with the rAg slightly higher than its vector blank mean. Low-reactive endemic samples display a

good signal-to-noise ratio with CAT-HIS vector blank below OD 0.1, confirmed by statistical analysis, a significant difference (t(9) = -3.7, p = 0.005). The results indicate that there is no interfering background attributed by CAT-HIS vector blank therefore Bm14-HIS is a suitable coating antigen.

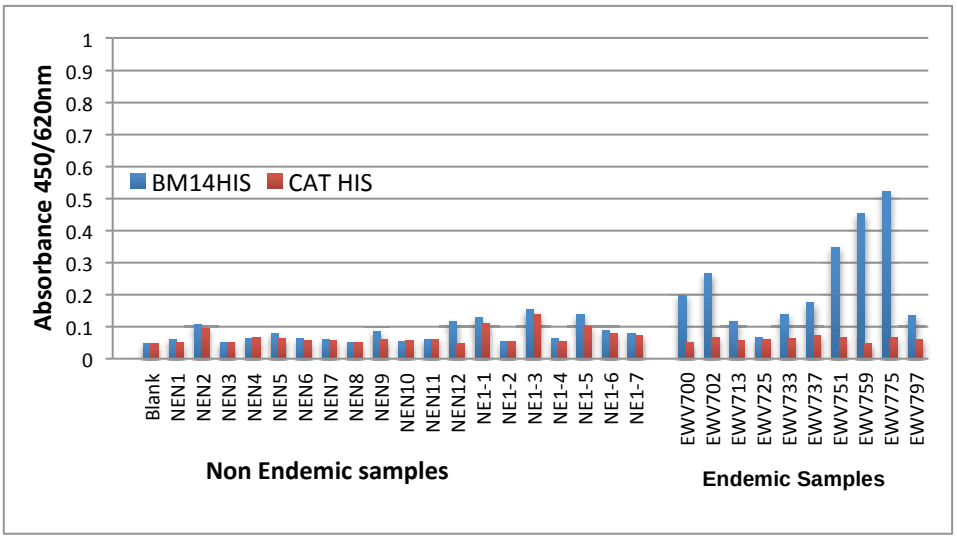

Figure 2.2a: Comparison of Bm14-HIS and vector blank CAT-HIS. CAT-HIS vector blank does not interfere with both non-endemic and low-reactive non-endemic samples making the Bm14-HIS the coating antigen of choice.

Figure 2.2b shows the results for Bm14-GST and GST vector blank. Using non-endemic samples, some background (> OD 0.2) was observed for both the rAg and its vector blank. Statistical analysis (t (19) = 1.556, p= 0.136) indicates equal background reactivity for both the Bm14-GST and its vector blank GST. In contrast, visual results of endemic samples tested shows some reactivity. No interfering background with the GST vector blank which are reading less than OD 0.2, a significant difference (t(9) = 3.833, p = 0.004). Due to the interference seen when non-endemic samples were used with plates coated with Bm14-GST, the rAg will no longer be used for further experimentation.

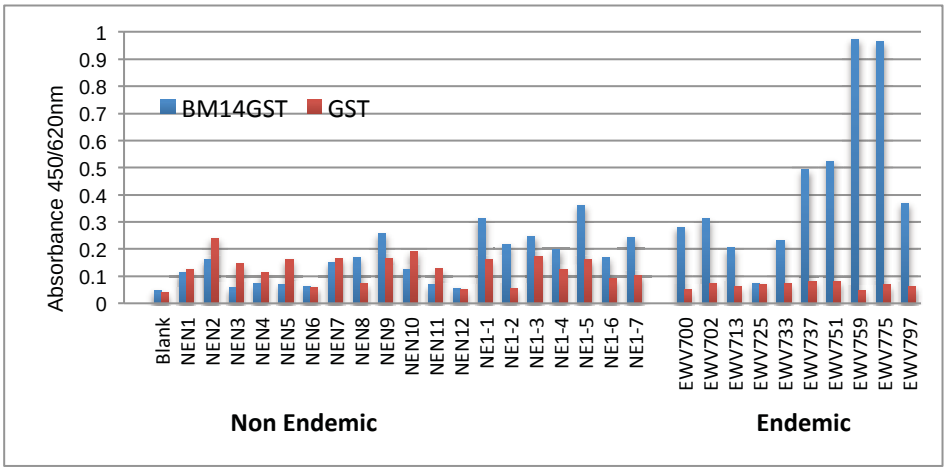

Figure 2.2b Bm14-GST and vector blank GST. Bm14-GST rAg shows some background reactivity when non-endemic samples are used. Results for endemic samples show good reactivity,

2.3.3.2 Evaluation and Preparation of Reference Serum Samples

Negative serum samples were collected from individuals who live in non-endemic Australia and USA, referred to as "non-endemic samples". Samples were confirmed negative for travel or residence in a LF endemic area except those samples with a prefix NEN. There was no travel background information available for these samples. Endemic serum samples were collected from individuals who live in LF endemic areas of Papua New Guinea, Samoa, Egypt and Sri Lanka. Samples EWP, EWV and EG and EM were selected as reference positive samples, pre-determined and characterised based on mf status. All samples donated to this project were collected within appropriate ethical guidelines and were given prefixes for sample identification purposes. Appendix 1 summarises the samples and the prefixes used in the studies.

2.3.3.3 Reference Positive Controls

Positive serum (EWP) from PNG with reactivity reading > OD1.5 were pooled and used as a reference positive (Ref +ve). Medium reacting sera (~OD 0.8-1.5) and, low reacting sera (~OD 0.4 -0.8) from the sample set EWV were also

included in the reference panel. These samples were used randomly and as convenient in the experimental ELISAs. Serum sample Kit+ve is a well-characterised serum found to be extremely reactive. Dilution up to ~10,000 therefore Kit +ve sample was pre-diluted as stock control and supplied as a 1:100 dilution in the final ELISA kit to give an OD 3.0 (+/-0.3). The reference serum Kit +ve was used to make appropriate dilutions of high, medium and low reacting control standards used to verify assay drifts from variations of assay performance during optimisation and final versions of the kit.

2.3.3.4 Reference Negative Controls

The quality control (QC) negative reference control (Ref -ve) is a serum sample from an individual living in a non-endemic country with a reading < OD 0.2 used primarily as a reference QC control during the experimental ELISAs. A commercially available pooled kit negative control (Kit –ve) (MPBiologicals, Cat. No. 2930149, CA, USA) reading of < OD 0.20 in an ELISA was included.

The reference QC serum panel was tested using the method in Section 2.3.1. Figure 2.3 shows a 1 in 100 dilution, the highly reactive kit reference (kit+ve) and QC reference (Ref+ve) samples read >OD 3.0 and <OD3.5. A median reactive reference positive was >OD 2.0 and <OD 2.5 and the low reacting positive control reads >OD0.5 and <OD1.0. Negative reference samples were reading well below OD 0.2, the expected cut-off value for the assay. The signal-to-noise ratio was >10 for Kit reference (SN = 43) and QC reference (SN = 51). The optimal serum dilution for use in the assay was determined at 1 in 100.

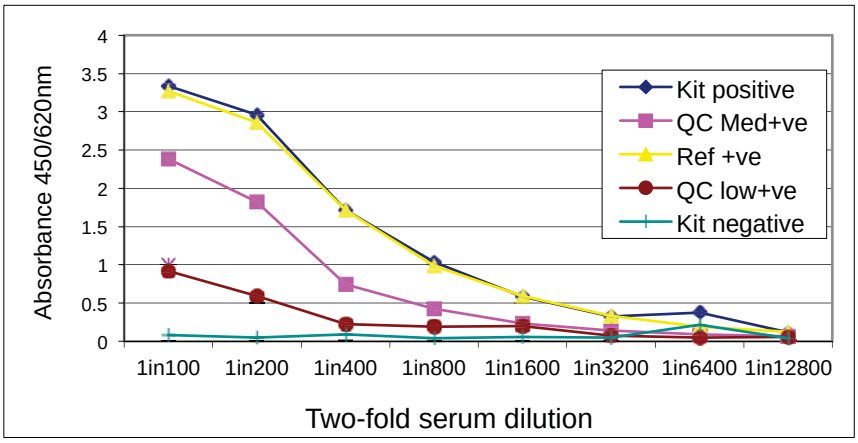

Figure 2.3: Reactivity of QC reference controls. A double dilution of serum controls used to check kit performance.

2.3.4 Selection of Solid-phase ELISA Microwell Plates

Three plates were compared (Fig. 2.4) to select for the best solid-phase, plastic-binding capacity ELISA plate. Evaluated were: (a) flat bottom, solid, flexible polyvinyl (PV) plate (Falcon, Becton Dickinson, Cat. No. 35-3912, NJ, USA); (b) flat bottom, 12 x 8-strip well, high binding polystyrene (PS) plate. (Greiner Bio-One, Cat. no. 705071, Germany) and; (c) 12 x 8-strip well PS plate from Nunc (Thermo Fisher Scientific, Cat. No. 446470, Denmark).

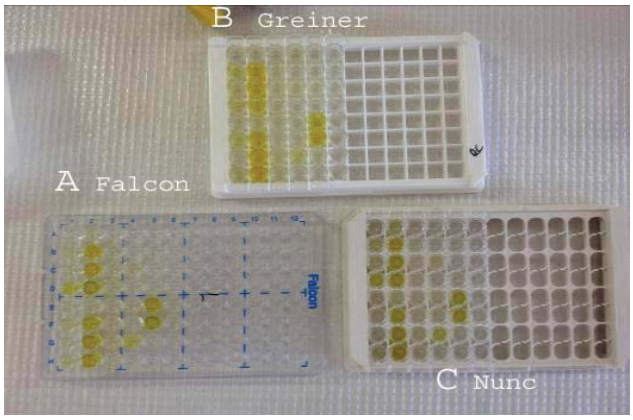

Figure 2.4: Kinds of ELISA plates for comparison. (a) Falcon PV solid plate, (b) high binding Greiner 12 x 8-well breakable strip plate and, (c) Nunc 12 x 8 non-breakable strip plate.

Each plate was coated using 2ug/mL rAg Bm14-HIS with a post-coat 0.5% Casein. Plate preparation and ELISA followed the method outlined in Section 2.3.1.

Statistical analysis shows no significant difference in OD means between all three plates using non-endemic and endemic normal ($p = 0.412$) and endemic samples ($p = 0.916$). However, signal-to-noise ratio shows that Greiner PS has the lowest background with the highest signal-to-noise ratio (SN=57) followed by Falcon PV (SN=25) and Nunc PS (SN=19). Greiner plate was selected for the Bm14 ELISA based on the performance and the excellent signal-to-noise ratio (Fig. 2.5). Other considerations for the choice are the convenience of using strip plates, and the availability and commercial-ready supply of these plates in Australia.

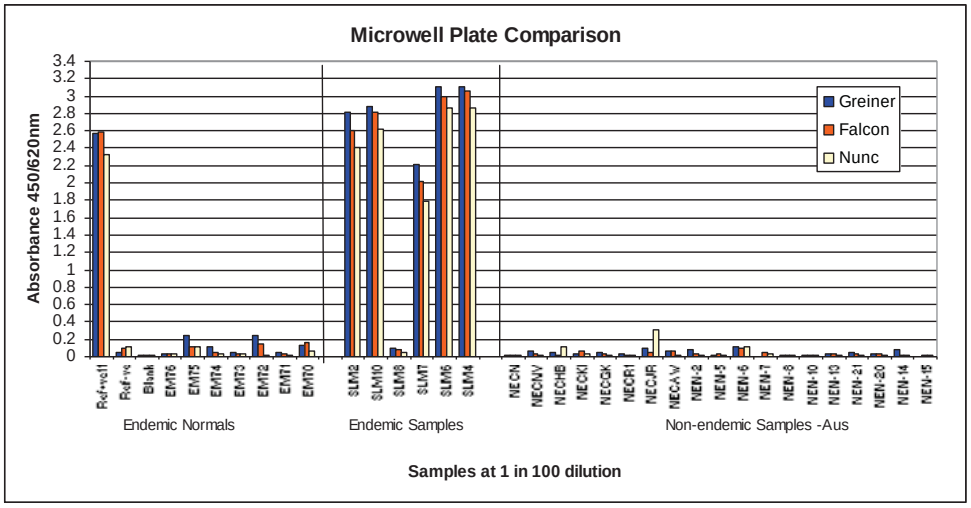

Figure 2.5: Reactivity of three ELISA plates. The Greiner plate demonstrates a high signal and low background compared to Falcon and Nunc plates**.**

2.3.5 Conjugation of anti-IgG4 Antibody

2.3.5.1 Purification

IgG4 monoclonal antibody (MAb) mouse ascites fluid from clones HP6023 (ATCC, Cat.no. CRL-1753, VA, USA) and HP6025 (ATCC, CRL-1775, VA, USA), were purified using the ammonium sulphate precipitation method described by Hudson and Hay (1980). After purification, the antibody was dialysed for 72 hours in a dialysis buffer at 2-8^{o}C with three buffer changes over a 24-hour period. After dialysis, the MAb was prepared for conjugation by determining the concentration in a spectrophotometer at A_{280} nanometer (nm). Concentration in milligrams per millilitre (mg/mL) was calculated as concentration = absorbance x Ig factor (0.74 for IgG) x Dilution Factor (DF). The MAb yield mg/mL was calculated as total purified MAb (mg)/ascitic fluid (mL). Table 2.1 below summarises the purification of both MAb clones HP6023 and HP6025.

Table 2.1 Purification of anti-IgG4: Results summary

	HP6023	HP6025
Ascitic fluid (mL)	10 mL	10mL
Purified MAb (mL)	10mL	5mL
MAb concentration (A280 x 0.74 x DF)	0.208 x 0.74 x 10 = **1.5 mg/mL**	0.156 x 0.74 x 10 = **1.2 mg/mL**
Yield in mg/mL (Total purified MAb/ starting ascites fluid volume)	15.4 mg / 10mL = **1.2 mg/mL**	6.0 mg / 10mL = **0.6 mg/mL**

2.3.5.2 Conjugation

Purified MAbs were conjugated to horseradish peroxidase (HRP) (Sigma, P6782, St. Louis, MO USA) using the conjugation procedure of Nakane & Kawaoi (1974) and purified by saturated ammonium sulphate precipitation method (Hundson & Hay, 1980). A minimum of 7.8 milligrams of purified antibody from each clone was conjugated. The hemin content was determined by reading the absorbance ratio Reinheitszahl (RZ) at A_{403} and A_{275} and the RZ value calculated as RZ = A_{403} / $A_{275.}$ Each mother conjugate was evaluated by a two-fold titration in an ELISA using the reference positive control (Ref+ve). The optimal dilution for use that will give an acceptable signal-to-noise ratio for the reference negative and positive controls was determined (method Section 2.3.1). Table 2.2 below shows the conjugation results and the end-use dilution for IgG4 conjugate HP6023 and HP6025.

Table 2.2 Summary of HRP conjugation of anti-IgG4 antibody

	HP6023	HP6025
Total mg purified MAb	7.8mg	7.8mg
RZ value (A403 / A475)	1.89 / 1.96 **= 0.96**	0.372 / 0.734 **= 0.51**
ELISA titration approximate end-use dilution.	1 in 2000	1 in 400
Signal-to-Noise Ratio (SN = positive OD / negative OD)	EWV55(2.019/0.098) = 20 EWV53(1.491/0.098) = 15 EWV58(1.172/0.079) = 12	EWV55(1.158/0.196) = 5 EWV53(0.436/0.196) = 2 EWV58(0.309/0.196)=1.5

2.3.5.3 Evaluation of Conjugates

Conjugates HP6023 and HP6025 were compared to look at performance and reactivity, to determine the conjugate of choice. Figure 2.6a graph shows non-endemic sample results with HP6025 with higher background with some of these samples (>OD 0.2) as compared with HP6023 which have ODs less than 0.2, a difference also confirmed as significant by statistical analysis (t (39) = -9.760, p = .0001).

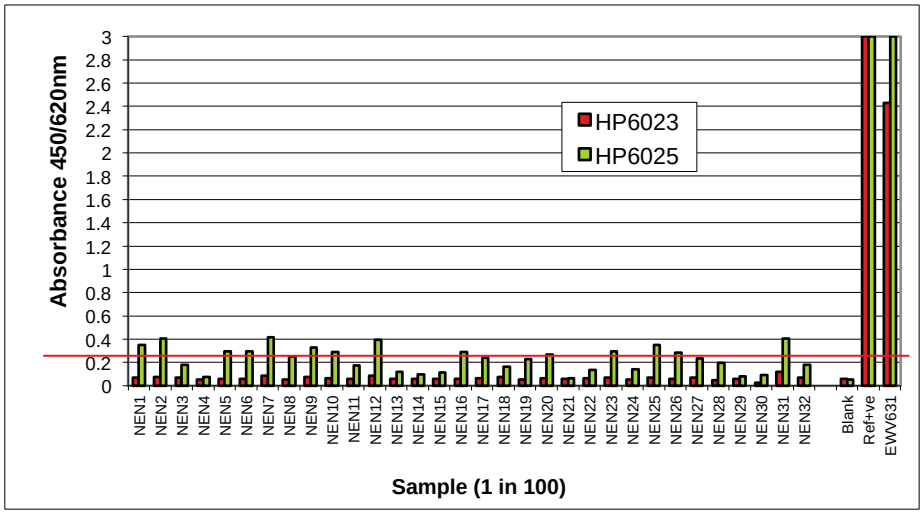

Figure 2.6a: **Conjugate comparison using non-endemic samples.** Higher reactivity with HP6025 conjugate is seen on non-endemic samples. All samples tested using HP6023 conjugate remains below the cut-off value OD 0.2 (red line).

Results for endemic samples in Figure 2.6b below, shows a more reactive observation for the HP6023 conjugate. Statistical analysis confirms a significant difference in the OD means (t (12) = 3.408, p = .005) as compared to HP6025. With HP6023 demonstrating better reactivity and no background, it is, therefore, the preferred anti-IgG4 antibody for use in conjugates.

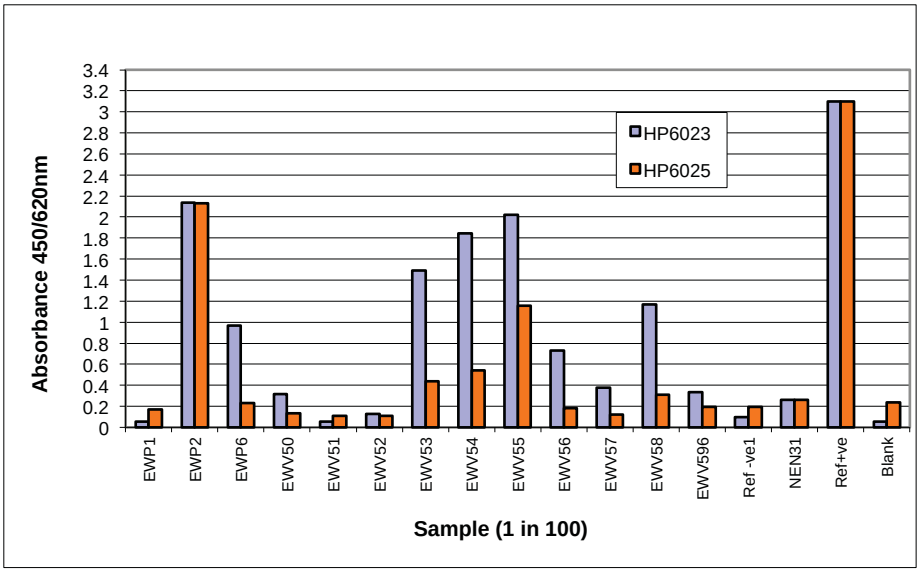

Figure 2.6b: Conjugate comparison using endemic samples. The graph shows visual and statistically significant difference in reactivity with HP6023 showing a good high signal with low background as compared to HP6025.

2.3.6 Optimisation Experiments

2.3.6.1 Optimal Ag Coating Concentration

The optimal rAg concentration for use was determined by a chequerboard assay. The antigen was diluted two-fold in coating buffer starting from the top row to bottom row of a 96-well plate with the highest concentration of 2mg/mL down to the lowest concentration at 0.0156 ug/mL. The serum sample (Ref +ve) was diluted from 1 in 100, two-fold across the plate. Also included in the assay on the last column are sample blanks (PBS/T) for all antigen concentrations. The assay follows the protocol outlined in Section 2.3.1.

A standard curve was constructed from the chequerboard results and shown in Figure 2.7a. The graph shows the reaction of reference positive sample (Ref+ve) at different dilutions against the antigen concentration. The highest signal (OD 3.5) of sample dilution, 1 in 100 can be achieved with a low antigen blank (OD 0.035) and a Ref-ve (OD 0.151), a signal-to-noise ratio of 23.

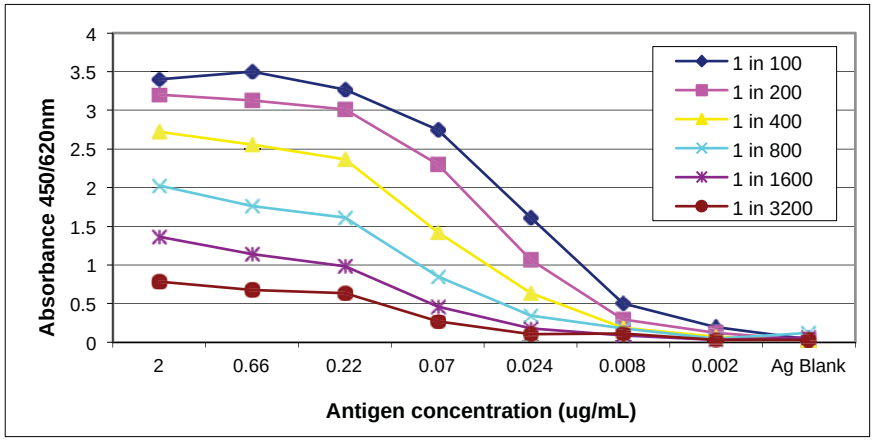

Figure 2.7a: Chequerboard assay for antigen concentration. An optimal concentration at 1 in 100 is seen at a concentration of 0.66ug/mL.

Figure 2.7b graph shows the observed reaction of the reference sample (Ref+ve) and the reference negative (Ref-ve) at 1 in 100 dilution against the antigen concentration. The graph was presented to focus on the reactivity of the sample controls. At a concentration of 2ug/mL, the graph shows a saturation of immobilised antigen on the plate. Observed was a hook effect, and a rising peak where the optimal antigen concentration level is at approximately 0.66 ug/mL.The negative pool consistently stays below OD 0.2, therefore, the optimal concentration determined for Bm14 rAg for use in the ELISA is 0.66ug/mL while the serum dilution was 1 in 100. For reasons of economy, the concentration used for manufacturing was adjusted to 0.6ug/mL for coating. At this level, there was no loss of reactivity as the Ref+ve serum sample remains above OD3.0.

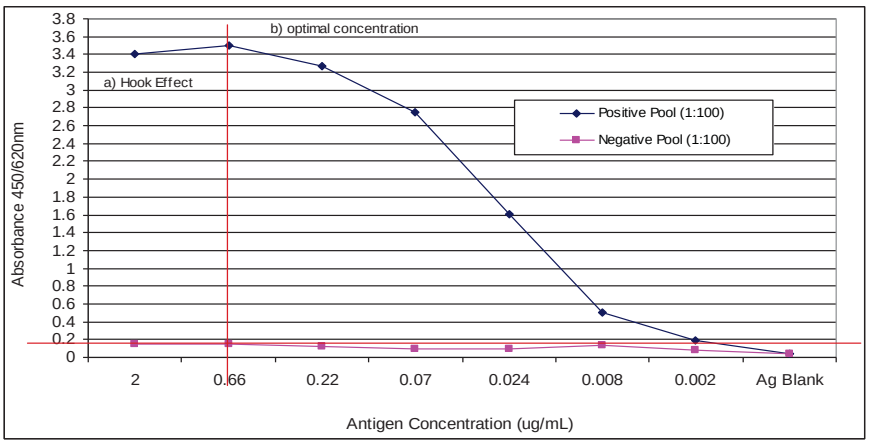

Figure 2.7b: Reactivity of reference controls. An optimised antigen concentration showing the reaction of the Ref+ve sample. A saturation point seen as (a) hook effect and the optimal concentration seen at (b) peak.

2.3.6.2 Optimising the ELISA Plate

2.3.6.2.1 Post-coat Blocking

An experiment comparing the reactivity of plates prepared with a post-coat and without post-coat was performed using the ELISA method in Section 2.3.1. The objective as to determine blocking effects of a post-coat on filter paper blood spot eluates. The investigation was included due to reported end-user problems with the use of filter paper blood spot eluates on prototype kits (G. Weil, personal communication, 20 April 2009; H. Joseph, personal communication, 06 April 2009). Statistical analysis on the results would show no significant difference if a post-coat were to be used when preparing the plates ($t(7) = 1.836$, $p = .109$). Visual analysis on the graph below (Fig. 2.8) shows that EUP pool, an endemic pool of eluates from filter paper samples (without mf), has demonstrated a high reaction (OD 2.247) and reaction lowered when using a post-coated plate (OD 1.281).

Background noise and plate stability were taken into consideration when choosing to use a post-coating step. It is evident that filter paper blood spots require dilution at 1:400 to remove the possibility of non-specific binding that was causing some background reaction.

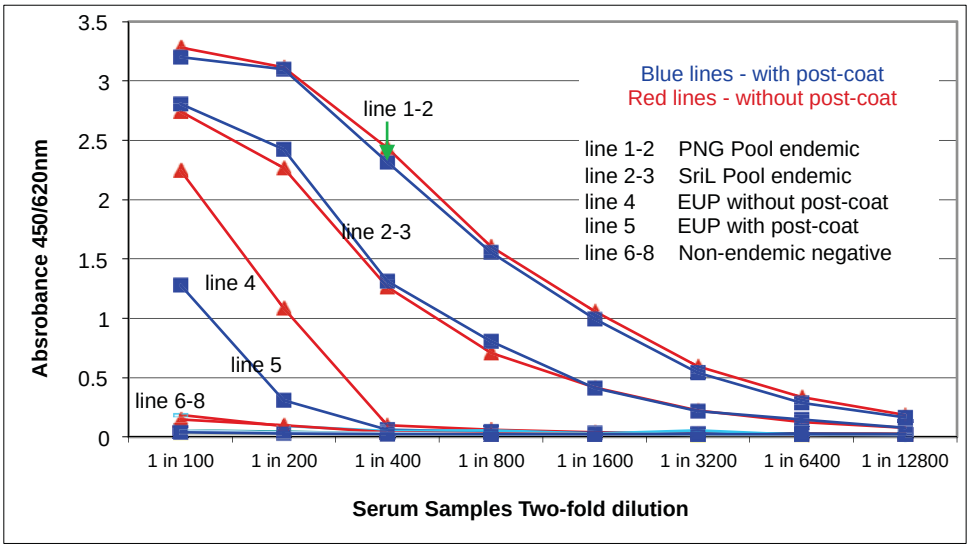

Figure 2.8: Post-coat vs. No post-coat. A comparison of results using reference samples.

2.3.6.2.2 Coating Buffer System

The recombinant antigen Bm14 was prepared in 5 different microwell plate coating systems. The combinations aim to determine the optimal combination of antigen coating buffer and the post-coating buffer (Table 2.3). Coating of plates were prepared as in method (Section 2.3.1) and labelled A-E respective of the coating system used.

Table 2.3 Summary of the coating buffers used for plates.

	Antigen Coating Buffer	Post-coating Buffer
A	Carbonate-bicarbonate (0.25M)	No post-coat
B	Carbonate-bicarbonate (0.25M)	0.5% Sucrose
C	Carbonate-bicarbonate (0.25M)	0.5% Casein
D	Carbonate-bicarbonate (0.25M) + 0.001% BSA + 0.5% sucrose = pH9.6	PBS-T + 5% NCS
E	Carbonate-bicarbonate (0.25M) + 0.001% BSA + 0.5% sucrose = pH8.6	PBS-T + 5% NCS

A protein-based post-coat functions as a stabiliser for rAg-coated wells and enables longer refrigerated storage. A technique also used to eliminate any background noise by blocking other non-specific molecules from binding to the plastic surface. To select a suitable post-coat for use, an accelerated stability study was performed by subjecting strips of wells from plates A-E to three different temperatures:

(a) at 37^0C to simulate the most extreme of temperature conditions the kits may be subjected during transit;

(b) at room temperature to simulate variable temperature changes and;

(c) at 2-8^0C to simulate the plate at the recommended storage temperature.

A control ELISA marked Day 0 was performed by titrating the reference positive serum sample on the 8-well strip using the ELISA method in Section 2.3.1.

After 49 days of testing, the accelerated stability trial was terminated. Figure 2.9a below shows the stability results of each combination of plate coating system at 2-8^{o}C. Starting at day 26, the Ref+ve sample tested on plate coating system D sharply decreases in reactivity and falls below the acceptable specification of OD 2.5 at day 49. Plate coating buffer systems A and B remain stable above OD 3.0. Plate coating system C and E are both dropping in reactivity at day 49. The result for control temperature 2-8^{o}C suggests stability for plate coating system A and B for the 49 days observation period.

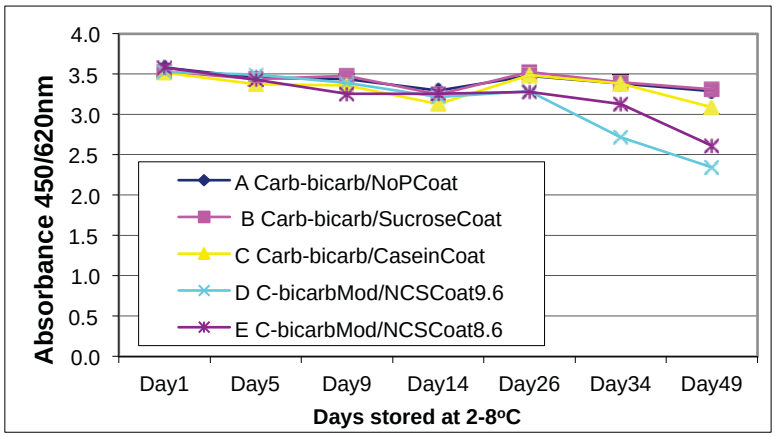

Figure 2.9a: Accelerated stability at 2-8^{o}C. Stability trial at 2-8^{o}C storage temperature.

Figure 2.9b shows the stability of test plates after storing for 49 days at room temperature. Coating systems A and B appear to be the most stable system, seen to remain reactive with an OD reading above 3.0 up to 49 testing days. The plate coating systems C and E drops off sharply by day 26 below the Ref+ve sample specification of OD 2.5. Coating system D is unstable with reactivity reduction below the specification of OD 2.5 at day 9 and continues to fall throughout the testing period.

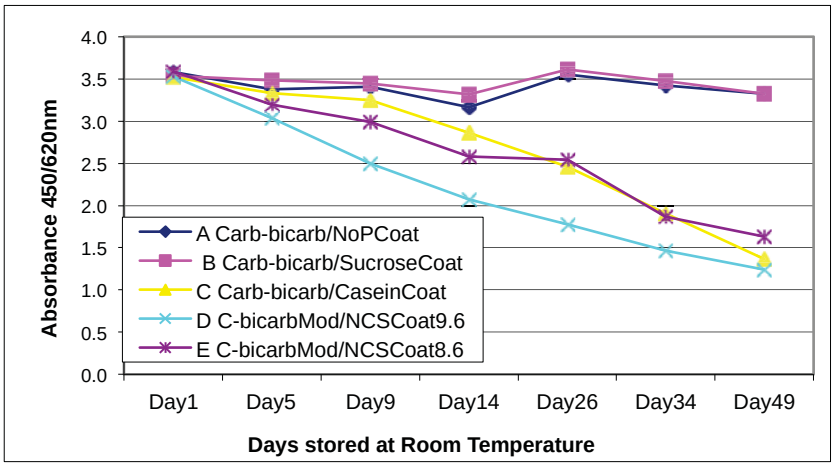

Figure 2.9b: Accelerated stability at Room Temperature. Results of accelerated stability trial at room temperature. The graph shows a clearer picture of the stability of Plate Coating Systems A and B.

After 49 days at 37^{o}C (Figure 2.9c), plate-coating systems A and B remain stable above OD 3.0. Plate coating systems C, D and E fail the stability trials as observed by the drop in OD reading below the Ref+ve sample specification of OD 2.5 by day 9. The accelerated stability result identifies the use of antigen coating buffer carbonate-bicarbonate as the suitable buffer with a sucrose post-coating solution. Although plates without the post-coating step pass accelerated stability tests, post-coating experiments under Section 2.3.2.2 suggests the need for a post-coating step to reduce non-specific background.

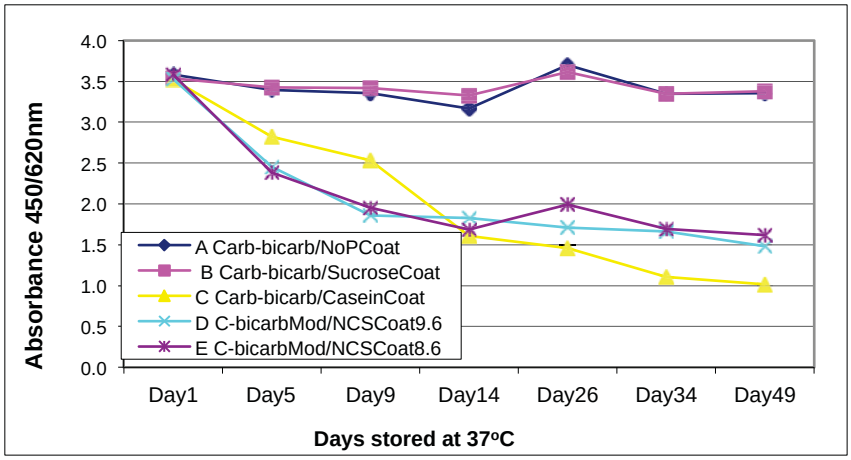

Figure 2.9c: Accelerated stability at 37^{o}C. Results of accelerated stability trial at 37^{o}C showing excellent stability of buffer systems A and B.

2.3.7 Kinetics Experiments

2.3.7.1 Practical Assay Temperature

Positioning of samples in the outer wells of an ELISA plate or "edge effect" is said to affect the resulting OD values. To avoid such variation, it is suggested that samples be incubated at room temperature until it reaches the maximum reaction, at a plateau (G. Burgess, personal communication, 2 July 2009). Incubation temperature for the assay was pre-determined at 37^{o}C rather than room temperature, which can be variable from laboratory to laboratory around the globe. Kinetics of sample binding should include determination of a temperature at which binding is optimal.The decision to use a set temperature of 37^{o}C is a compromise that has to be made to ensure repeatability and reproducibility.

2.3.7.2 Assay Incubation Time

To investigate the optimal serum sample incubation time and temperature, reference serum samples were titrated two-fold across the plate in six sets. A chequerboard ELISA was performed as per method in Section 2.3.1. Each sample set was incubated to bind for: 15, 30, 60, 90, 120 and 150 minutes. Figure 2.10a below shows the kinetics of sample binding time at 37^{o}C. The sample Positive Pool (high) diluted 1:100 can achieve maximum binding within 15 minutes, with no changes regardless of longer incubation time, due to antibody saturation of the rAg coated plastic surface. Positive Pool (Low) increases in signal over time, reaching OD 1.367 for 60 minutes and OD 1.494 for 90 minutes incubation time. The negative pool indicates no reactivity at the cut off value (COV) OD 0.2 when incubated at a maximum of 60 minutes, after which, the OD value increases above the cut-off, an OD of 0.316 for the Negative Pool after 120 minutes of incubation. The results indicate that 60 minutes incubation is sufficient to give low reacting samples enough binding time that will give a detectable OD value while keeping the background to a minimum, below the cut-off value of OD 0.2. The calculated S/N value for Positive Pool High (SN=49) and Positive Pool Low (SN=20) at 1:100 serum sample dilution.

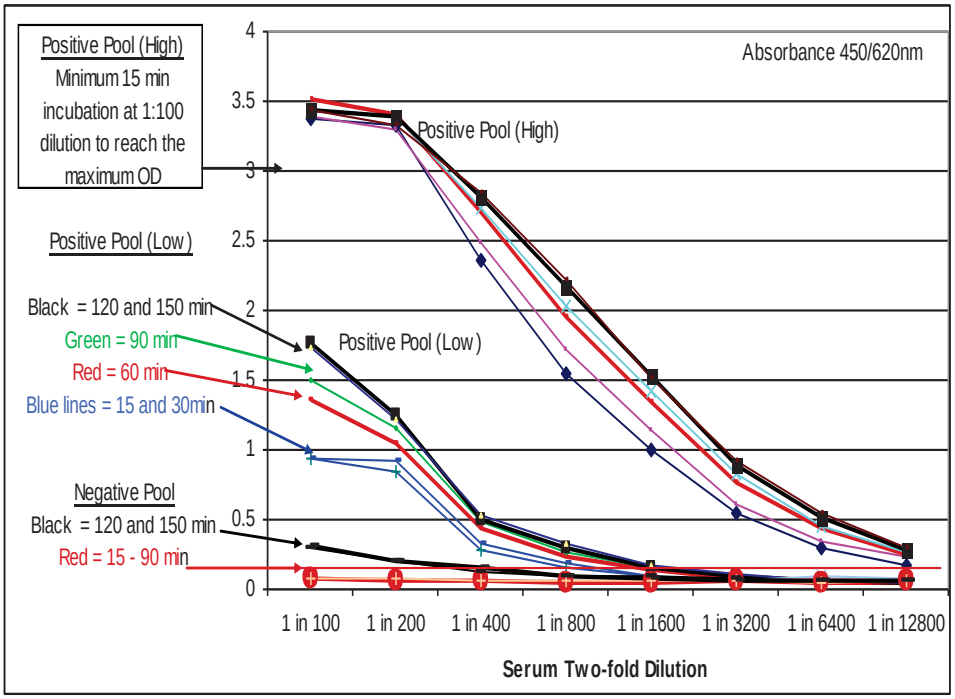

Figure 2.10a: Sample dilution vs incubation time - Chequerboard assay. The Positive Pool (High) reaches a maximum OD at a minimum incubation time of 15 min. A good signal and low background can be achieved by incubating at a minimum of 60 minutes at 37^{o}C.

The results for the references observed at 1 in 100 dilution were plotted as OD value against time in Figure 2.10b demonstrates the kinetics of binding at 37^{o}C incubation temperature and the reaction in a different perspective. The high positive signal is already at saturation level after 15 minutes and does not change if it is left to incubate longer. The Positive Pool (Low) increased in the signal after 30 minutes and reaches a plateau after 2 hours. The negative pool remains below OD 0.2 up to 1.5 hours after which, an increase in signal as high as OD 0.316. This indicates a non-specific reaction that needs to be avoided therefore an incubation time of 60 minutes was used.

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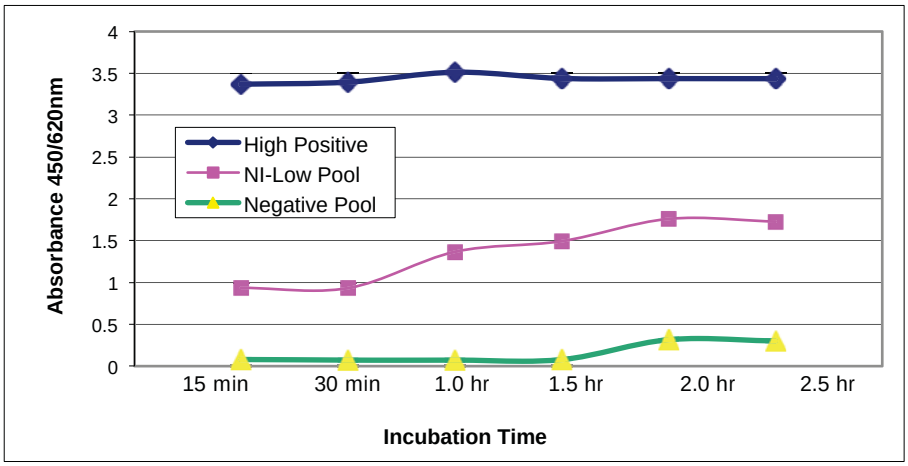


Figure 2.10b: Kinetics of analyte binding. A closer look at the reaction of 1:100 sample dilution showing the Negative Pool sample OD increase above the cut-off value of OD0.2 after 90 minutes incubation. At 60 and 90 minutes, the Positive Pool (Low) gives an OD of 1.367 and 1.494 respectively.

2.3.7.3 Conjugate reaction time

The kinetics of reaction time for each conjugate was investigated to determine the optimal incubation time for antibody-conjugate binding. Essentially, the old research grade assay took up to 2 hours for a conjugate reaction step, however, faster binding times of a pre-determined 37^{o}C incubation temperature can be achieved in a well-optimised ELISA. To investigate the reaction times of both HP6023 and HP6025 conjugate, reference samples were tested in the current working ELISA method described in Section 2.3.1. Two 8-well strips were used to run a negative and positive reference sample. A set of negative and positive control was removed from the chamber during the incubation step. The reaction was developed with a substrate, stopped, and read at A_{620nm} on a plate reader after 3, 6, 10, 15, 20, 25 and 30 minutes. The OD values were plotted to look at the reaction time as shown in Figure 2.11 graph below.

The graph shows the binding complex of IgG4 antibody to the conjugated antibody. A steady increase in signal was observed from 3 minutes up to 20 minutes where the signal reaches a maximum then exhibits a plateau. The optimal reaction time for conjugate incubation is between 20 to 30 minutes for both HP6025 and HP6023 conjugate.

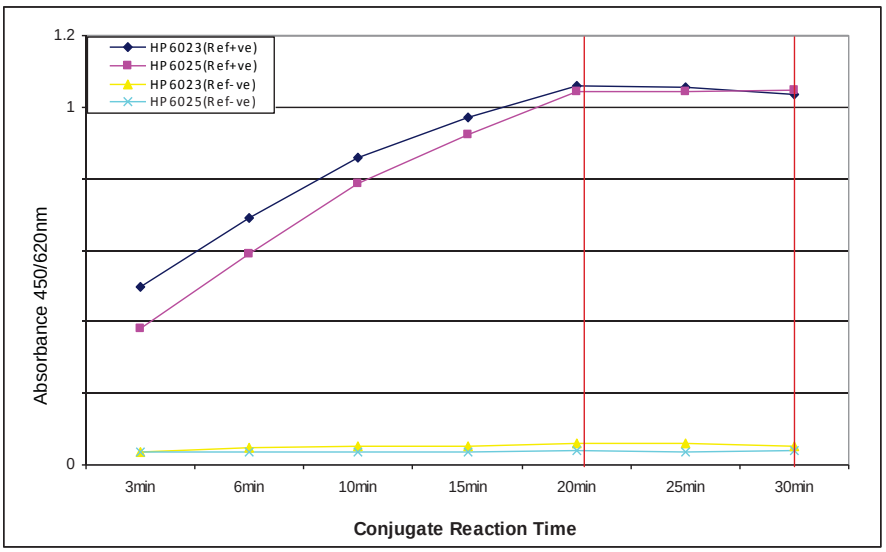


Figure 2.11: Determination of optimal conjugate binding time. Conjugate reaches a maximum reaction after 20 minutes of incubation at 37^{o}C. Reaction for HP6023 (blue line) conjugate is seen to be higher than that of HP6025 (pink line).

2.3.7.4 Substrate Development Time

Three chromogenic substrates were evaluated for use in the ELISA: (a) 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma, St Louis, MO, UAS, cat.no. T2885); (b) two-step TMB (KPL Kirkegaard & Perry Laboratories, Inc., MD, USA, Cat. No.50-62-01) and; (c) one-step component 2,2'-amino-di(3 ethyl-benzthiazoline-6-sulphonic acid (ABTS) (Kirkegaard & Perry Laboratories, Inc, MD, USA, Cat. No. 50-66-18). Substrates are chromogenic solutions used to react with the bound antibody complex to give a colour reaction. The intensity of the colour reaction is equivalent to the bound antibody present in the sample.

The Sigma TMB used is a non-carcinogenic chromogenic substrate in powder form used at a low concentration of 0.48g/100mL prepared as a 20x concentrate. The TMB concentrate was diluted to a working 1x buffer using a citric acid based substrate buffer before use. The KPL TMB is a two-step component that requires mixing equal volume of component A and B as per manufacturer's instructions. The ABTS was a one-step component used as neat and no dilutions required.

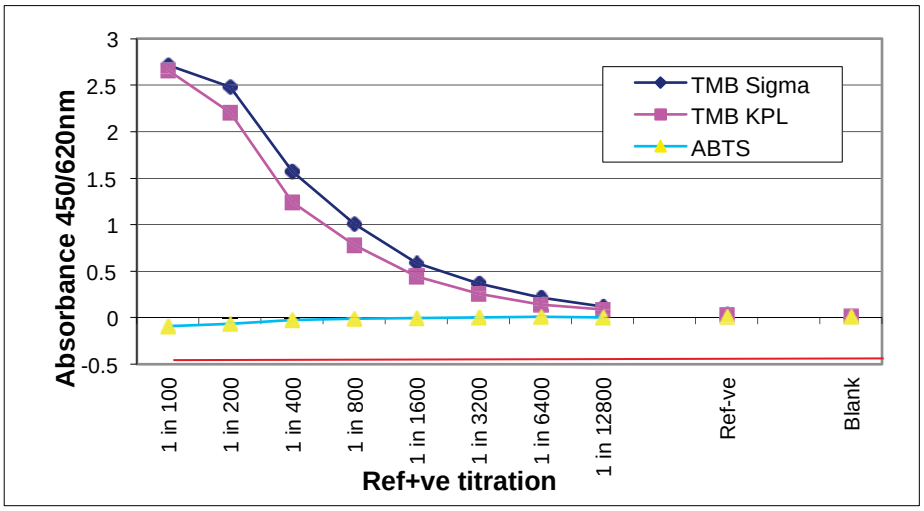


Figure 2.12a: Substrate development comparison. ABTS substrate shows a very slow development requiring up to 60 minutes compared to TMB substrates

The substrates were evaluated by the working ELISA method in Section 2.3.1. A sample set that includes a reference negative and positive were used to carry out the reactions. Before the end of the conjugate washing step, each substrate solution was prepared to ensure that the substrates were loaded on the well one after the other, avoiding time lags. TMB substrates were read at $A_{450/620}$nm dual wavelength and ABTS read at A_{410}nm. Figure 2.12a shows a graph of the substrate development at 15 minutes. The ABTS reacted very slowly compared to TMB substrate.

Figure 2.12b below shows the visual colour development of substrates being compared on the ELISA plate. An added value of using the sensitive TMB is to

enable laboratories without plate readers to interpret results quickly by visual reading of blue colour complex.

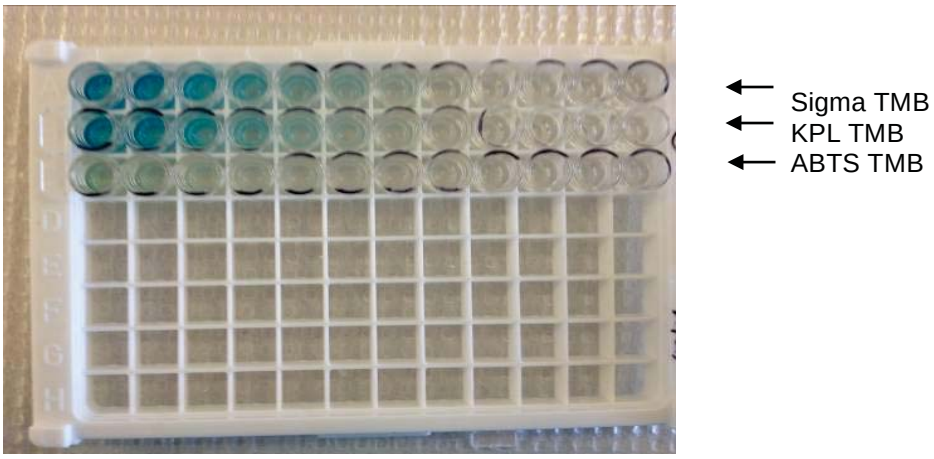


Figure 2.12b: Visual reading of ELISA after addition of TMB.
TMB substrates develop the colour complex after 15 minutes as compared to ABTS which took more than 60 minutes.

2.3.8 Background Noise Correction

2.3.8.1 Sample Absorption

In order to eliminate some non-specific binding by competition observed with some non-endemic negative samples, a sample diluent containing an irrelevant protein such as calf serum (CS) was introduced. Production costs need to be taken into consideration hence, the use of a less expensive newborn calf serum (NCS). The sample diluent was formulated to absorb any non-specific components contained in plasma or serum samples that may illicit background, thus allowing efficient antigen-antibody binding. Samples were diluted 1:100 using the sample diluent. An ELISA was performed following the method in Section 2.3.1.

A number of non-endemic negative samples and a set of endemic samples were tested to look at any absorbing effects of NCS-based Sample Diluent in comparison to the use of PBS/T as the sample diluent (Fig 2.13). Non-endemic samples shows a significant difference when tested statistically ($t(22) = -2.609$,

p = .016). The Serum Diluent reactivity is slightly lower than the set of samples tested using PBS/T as a sample diluent. In contrast, the endemic samples show no difference when compared (t (17) = -1.861, p = 0.08).

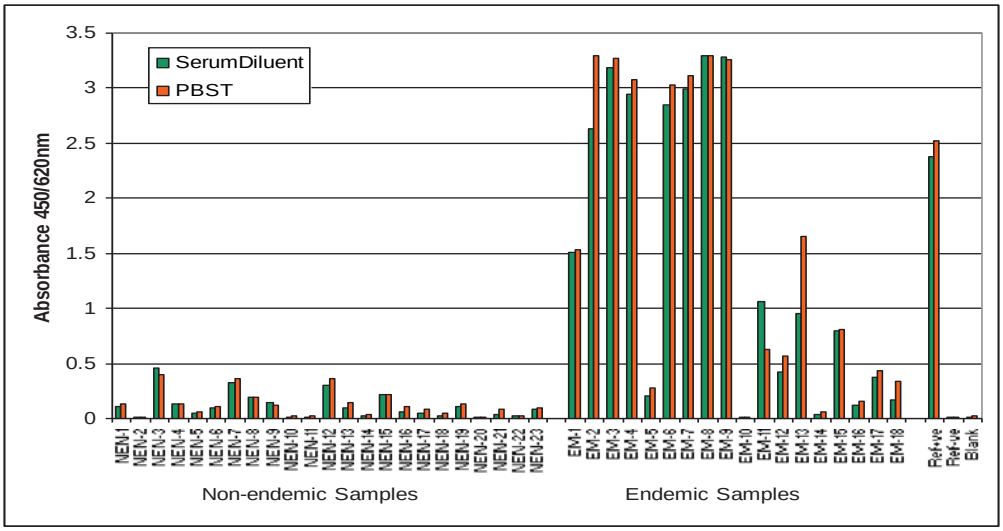


Figure 2.13: Comparison of serum diluent blocking effect. Non-endemic samples shows a significant difference with a FCS-based Sample Diluent slightly lower when compared to a PBS/T sample diluent. Endemic samples do not show any significant difference when tested statistically.

2.3.8.2 Conjugate Diluent

Tris-based diluent with newborn calf serum was formulated as a conjugate diluent to give the conjugated antibodies some stability in solution. However, the use of such a diluent may potentially have a negative effect by binding non-specifically by competition, resulting in an inhibited reaction. It may also improve the results by absorbing out non-specific components that can contribute to background noise. An ELISA experiment using the method in Section 2.3.1 was completed to determine the effect of using the conjugate diluent. Figure 2.14a graph shows a panel of endemic samples tested at random to look at the effect of the conjugate diluent. Out of the 5 positive samples reading more than >OD 0.2, NCS-based conjugate diluent visually

demonstrates higher reactivity compared to using PBS/T. Though, statistical analysis tests show no significant difference in the results ($t(6) = -1.840$, $p = 0.115$).

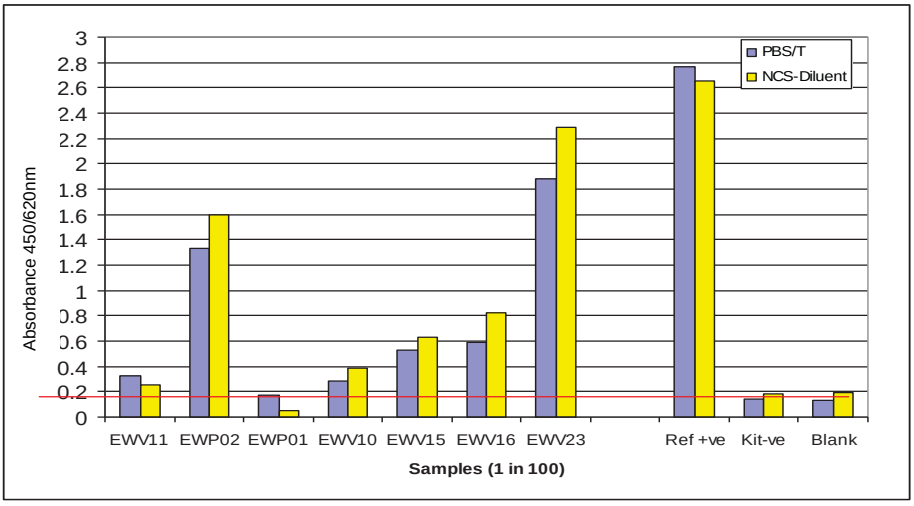


Figure 2.14a: Comparison of conjugate diluent using endemic samples.
The results for NCS diluent shows a visually higher reaction compared to PBS/T however, there is no significant difference between the two conjugate diluents when tested statistically.

The graph in Figure 2.14b is shown to give a closer visual analysis of the comparison between reference samples diluted with the NCS diluent and with PBS/T alone despite the statistical test result. Visual analysis on the reactivity based on OD values show that NCS diluent consistently lowers the Ref -ve (NCS) reactivity below OD 0.2 as compared to a higher Ref -ve result when diluting with PBST. This result is significant when dealing with an assay cut-off value (COV), despite the statistical analysis test result seen in the experiment above (Fig. 2.14a.). Based on this visual analysis, the use of a sample diluent will improve the specificity of the assay.

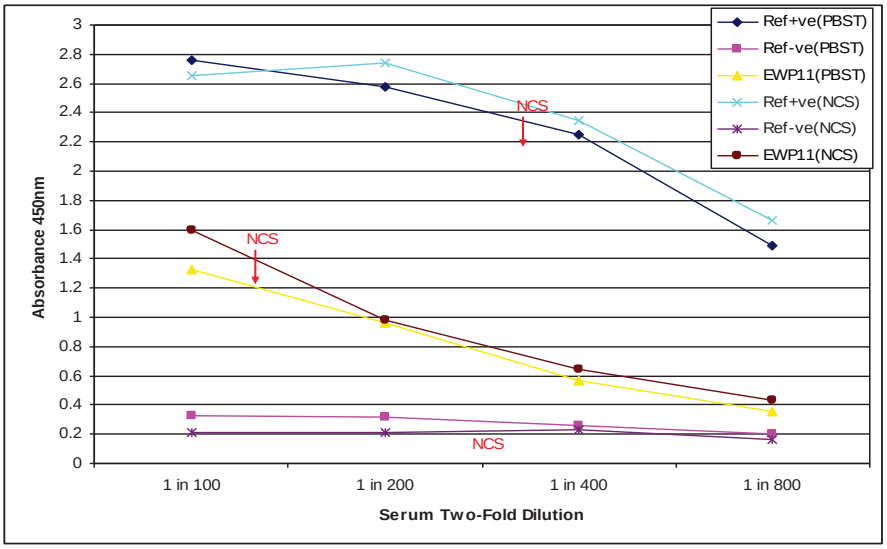


Figure 2.14b: Conjugate diluent blocking effect. NCS-based conjugate diluent is observed to lower the OD readings of the negative reference below OD 0.2. Graph shown for visual purposes.

2.3.9 ELISA Standard Buffers

2.3.9.1 Wash Buffer

PBS/T wash buffer is a standard buffer used in most commercial ELISA. Prepared as a 20x concentrate using salts in reverse osmosis (RO) water, the concentrate was pre-diluted to working concentration (1x) before use. The wash buffer PBS/T was used for washing the plate after sample incubation and conjugate incubation to remove unbound material before the next ELISA step.

2.3.9.2 Stopping Solution

An appropriate stopping solution to terminate the colour development of the antigen-antibody colour complex during the substrate step was selected by experimental comparison of the available acid solutions. Orthophosphoric Acid stopping solution (1.0% H_3PO_4), hydrochloric acid stopping solution (6.5% HCl) and sulphuric acid stopping solution (10.7% H_2SO_4) were tested on the working ELISA method of Section 2.3.1. Substrate development was stopped by the addition of stopping solution after 15, 20, 25, 30 and 60 minutes (Fig. 2.15b). The graph in Figure 2.15a below shows the titration of the negative and positive controls following a typical curve.

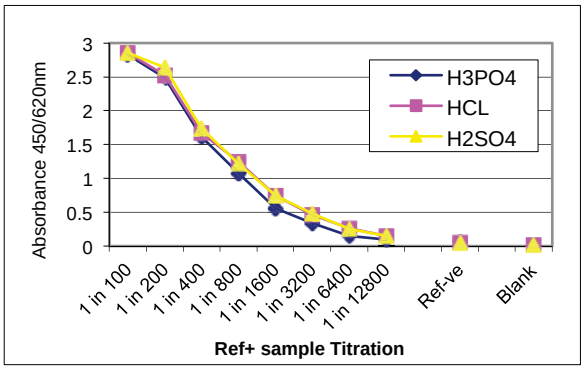


Figure 2.15a: Stopping solution Evaluation. ELISA results using three different stopping solutions at A450/620nm.

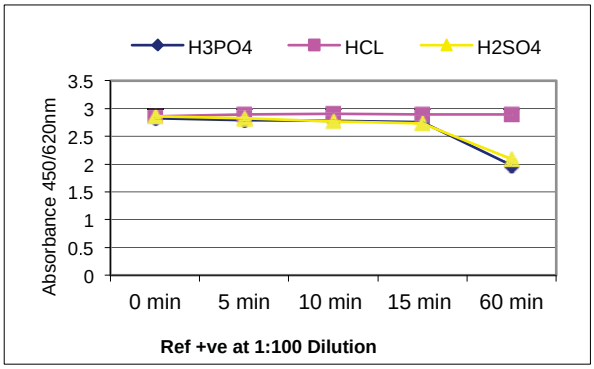


Figure 2.15b: Reaction of stopping solution over time. HCl stopping solution is observed to be stable after 15 minutes of stopping

2.3.10 Plate Reading

The ELISA optical density (OD) results throughout the experiments were obtained by reading the plate straight after the addition of stopping solution. The readings were carried out using a spectrophotometer (Molecular Devices, VMax, CA, USA) plate reader with a single wavelength A_{450nm} and dual wavelength $A_{450/620}$nm. A calibration check using colorimeter strips (Dri-Dye Check Strips 450, Awareness Technology, FL, USA) was performed every six months to maintain and validate the instrument ensuring correct absorbance readings for all assays.

Qualitative results were also obtained by visualisation of a blue colour complex 15 minutes after the addition of substrate buffer. Development of the colour complex was stopped by the addition of the stop solution that turns the blue colour complex yellow. The colour intensity observed is relative to the amount of antibody present in the sample, the positive and negative control reactions being the visual guides to the qualitative readings.

2.4 RESULTS

2.4.1 The Standardised Bm14 ELISA

2.4.1.1 The Final Kit

The final standardised Bm14 Filariasis Antibody CELISA, product designation KF3 was provided as a ready to use kit containing 5 x 96 recombinant antigen-coated ELISA plates (FAMW) stored in a heat-sealed, snap-lock foil bag with silica gel sachets, 1 x 0.6 mL conjugate at 100x concentrate (FAPO), 1 x 60mL conjugate diluent (FACD), 1 x 0.05 mL positive control (FAPC) and 1 x 05 mL negative control (FANC), 1 x 60 mL sample diluent at 10x concentrate (FASD) ,1 x 3 mL 3,3',5,5'-tetramethyl benzidine (TMB) substrate at 20x concentrate (FASC), 1 x 60 mL substrate buffer (FASB), 1 x 250 mL PBS/T wash buffer at 20x concentrate (FAPT), 2 x 30 mL stopping solution (FASS) and 1 kit insert (LF3) for instructions for use (IFU). Note that the codes in brackets refer to the respective components coding system, also used as identification code in the final standardised kit. Appendix 2 shows the final kit IFU.

2.4.1.2 Plate Preparation

The microwell plates (Greiner BioOne, Cat. no. 705071, Germany) were prepared using 0.6ug/mL recombinant antigen Bm14 concentration in carbonate-bicarbonate buffer. Plates were incubated overnight at 2-8^{o}C in a humid chamber for 18-24 hours. After the incubation period, the antigen solution was flicked empty and plates were tap-dried on laboratory tissue to remove excess antigen solution. Post-coating buffer was added and incubated in a humid chamber for a further 45 minutes at room temperature. After the post-coating step, the plates were flicked empty and tap-dried on laboratory tissue to remove excess post-coat solution before air-drying upside-down in the 37^{o}C incubator. Once dry, the plates were placed in ELISA foil bags with two indicating silica gel sachets before heat sealing. Plates were stored at 2-8^{o}C until all component QC tests have been passed and kits were ready to be assembled and completed.

2.4.1.3 The Final Bm14 ELISA Protocol

Components were brought to room temperature before performing the assay. The sample diluent was prepared by diluting 1 in 10 with distilled water for use in diluting test samples. The 20x concentrate PBS/T was diluted 1 in 20 (1x wash buffer) with distilled water for use as washing buffer. The negative control, positive control and test samples were prepared by diluting to 1 in 100 (1:100) with 1x sample diluent. 100uL of each diluted test sample including controls were added to r-Ag coated ELISA wells and the plate placed in a humid chamber and incubated at 37oC for 60 minutes. Five minutes before the end of the incubation period, the 100x concentrate HRP-conjugate was diluted 1 in 100 (1x conjugate) using the conjugate buffer. The ELISA wells were washed four times using an automatic plate washer followed by adding 100uL of the 1x conjugate. The wells were placed back in the humidity chamber and incubated at 37^{o}C for 45 minutes. Five minutes before the end of the incubation period, the 20x TMB substrate concentrate was diluted to 1 in 20 (1x substrate) using the substrate buffer. The wells were washed four times as before, followed by adding 100uL of the 1x substrate to each ELISA well. Colour complex was developed for 15 minutes in the dark, after which reaction was stopped using 100uL of the stopping solution. After mixing by gently tapping the plate, the wells were read photometrically using an ELISA plate reader at A_{450}nm/A_{620}nm dual wavelength or single wavelength A_{450}nm.

2.5 DISCUSSION

During the standardisation and optimisation process, there were standard industry practices and principles of assay development that were avoided in favour of practicality. An example was the decision to use Greiner polystyrene plates that were more expensive than the polyvinyl plates. Since commercial kit considerations include economies of scale, using polystyrene high binding plates with lower optimal rAg concentrations offsets the high cost of polystyrene plates that required higher antigen concentrations. Another method avoided was the experimentation of optimal temperature to use. Because a room temperature incubation is variable in laboratories around the globe, the use of a 37^{o}C incubation temperature is better controlled. The

assay development and design considered the reduction of many other factors that affect variability.

Some ELISA systems where proteins were successfully immobilised on solid phase, remain stable for many years when post-coated using an irrelevant protein such as bovine serum albumin (BSA), casein or calf serum. In the plate coating experiments, the antigen optimisation, coating buffer and post-coating or post-coating buffers were investigated in conjunction with each other and the combination of buffers called the "coating buffer system". This optimisation work was initiated by the problems encountered with the kit stability of the prototype version that were sent to a number of external validation laboratories. One laboratory's storage room broke down during the weekend and kits were subjected to temperatures around 25^{o}C to 35^{o}C for two days. After investigation, the plates were found to be unstable when subjected to temperatures higher than the recommended storage of 2-8^{o}C. This is not acceptable because these kits are to be shipped globally to tropical areas in minimum cold chain transportation therefore assay robustness is a design requirement. The overall kit needs to withstand high temperatures for at least 5-7 days in transit and stable for the given 12 months shelf life. It was therefore required to optimise the three parameters, the recombinant coating concentration, and a coating buffer system, antigen coating buffer and post-coating buffer combinations. The resulting plate using a standard carbonate buffer as the coating buffer and a sucrose-based post-coating buffer was stable.

The prototype kit used a modified carbonate coating buffer that contained a small percentage of calf serum. It was thought that the serum contained in the buffer may be inhibiting the availability of antigen binding sites on the surface by competitive binding and, therefore, was not optimal. Experiments confirmed the blocking effect of serum and the use of carbonate-bicarbonate buffer has increased the sensitivity significantly. During the changes, the kit design has been adjusted carefully to ensure that the panel of reference serum OD values from the development and prototype stages did not change on the new kit version. This increase in sensitivity was observed by the multi-centre evaluations as background that increased the OD values that resulted in high false positive readings when using antibody negative samples on filter paper blood spots. It must be noted that the Bm14 ELISA standardisation was optimised for serum samples. The use of filter paper blood spots as the source of antibody had not been included as a potential method of sample collection at the beginning of the

ELISA design and development stages. There has been no communicated requirement for the use of filter paper blood spots until after the the prototype evaluation feedback has been received by the manufacturing laboratory. There is now a requirement to develop a method so the kit can be used with filter paper blood spots. The results of high background problems during the multi-trails are further discussed in Chapter 7.

Optimisation experiments are an important activity in ELISA development and understanding the kinetics of chemical and biological materials and buffers used in the assay is crucial. The sample binding was investigated (Section 2.3.3.2) using a chequerboard assay to look at two parameters in one assay, the sample dilution and how it reacts when incubated for a length of time at 37^{o}C. The results clearly demonstrate that an optimal reaction can be achieved in 1 hour. Modern technologies have made a great deal of improvements on materials such as plastic solid-phase microwells that do not require pre-treatments allowing improved efficiency in ELISA kinetics.

The final ELISA components included a sample diluent and a conjugate diluent despite statistical analysis of experimental results indicating that the use of these additional buffers does not make any significant difference in reducing background. Visual analysis of graphs and OD values show that in some non-endemic samples, the OD was reduced from OD 0.4 to OD 0.2, a difference (+/-) of 0.2. In ELISA terms, a difference of (+/-) 0.2 can be critical in determining whether a sample is negative or positive. In sentinel children samples, we need to be able to detect those that are low reacting.

It must be noted that modern cutting-edge technologies have now saturated the diagnostics market with products that can improve the ELISA. Plate stabilising buffers or special conjugate diluents, all these products can be used in the construction of an ELISA however, there are dangers in using these commercial buffers. Commercial buffers can be passed on from one company to another under mergers or company take-overs that the quality and composition may change over time. For assay manufacturers, problems caused by the raw material quality and supply can have detrimental effects on a well-designed assay. Troubleshooting component issues is time-consuming and can potentially halt the manufacturing and supply of kits and jeopardise the progress of ongoing PCT and other monitoring programmes. Because

of this reason, all buffers formulated in the Bm14 ELISA are standard buffers that have been carefully evaluated by experimentation. A real-time stability has been completed to validate the stability of the plate, the conjugate, and the kit as a whole (shown in Chapter 3).

This development, standardisation and optimisation chapter continues to the next chapter to cover validation experiments and other requirements for a commercial-ready product.

CHAPTER 3

ASSAY PERFORMANCE VALIDATION AND QUALITY ASSURANCE OF Bm14 FILARIASIS ANTIBODY ELISA

3.1 INTRODUCTION

In Australia, all diagnostic tests manufactured or imported for use within the country require registration with the in-vitro medical device (IVD) regulatory body. The Australian Therapeutics Goods Administration (TGA) is the regulating body for diagnostic tests classified as IVD. In most countries, the governmental Department of Health regulates IVD supply and manufacturing as part of a role in protecting the country's health and wellbeing. All diagnostic tests for registration in the TGA database require an assessment to ensure that tests are safe. The manufacturing of tests needs to conform to international standards of IVD manufacture (ISO13485). Part of conformity to ISO13485 is a verification and validation process. These are documented experiments that are used as evidence that detection of the specific analyte is of the highest accuracy and precision (Jacobson, 1996). This stage is important for defining a research grade and laboratory-developed tests from a standardised commercialised diagnostic test. All commercialised diagnostic tests for both clinical or research use that are made available in the market, have to undergo the process of verification and validation. This evidence-based medicine is an essential requirement of diagnostic tests before they can be used as a legal tool for human in-vitro testing.

At the beginning of the product development project, it was crucial to have detailed specifications defined for the required assay characteristics. Measures of performance characteristics are specificity and sensitivity, cross-reactivity, stability, repeatability and reproducibility.

Sensitivity and specificity, positive predictive value (PPV) and negative predictive value (NPV) are the terms used for probabilities when describing diagnostic quality measures. An indication of acceptable test perfromance is to specify more than 80% sensitivity and specificity. The prevalence of the disease in a population being studied

is an important factor in the usefulness of these diagnostic measures. Akobeng (2007) explains that the application of PPV and NPV can only be meaningful for a certain area because these measures are based on the prevalence in that particular area. The samples used for validation experiments in this chapter were from a mixed set of different endemic and non-endemic areas. PPV and NPV measures were not included for internal records, but these will be determined by external epidemiological studies and evaluations using the Bm14 ELISA. A list of epidemiological studies and evaluations that determine the diagnostic measures of the Bm14 ELISA are discussed in Chapter 7.

Cut-off value (COV) is an ELISA specification used to define the diagnostic threshold or the level of antibody activity. COV determines the negative or positive status of a given sample (Wright, et al., 1993). Methods of determining COV can be used such as frequency distribution method or the mean plus three standard deviation (mean + 3SD) method (Rajasekariah et al., 2003). Using a standard deviation set at two or three times the mean is common (Kurstak, 1985), or it can be tailored to suit a particular specificity requirement. The COV is based on the mean OD value of some non-endemic samples assumed to have no antibody response to a particular infection. Because the level of sensitivity of an assay is heavily dependent on a COV, each laboratory can determine a suitable value for a particular study based on internal negative samples. Commercially available assays often pre-determine COV based on samples from a non-endemic area as part of a complete product test specification. Whichever method is used to determine a COV, it needs to be carefully selected as it directly affects the sensitivity that is required of an assay.

Validation of kit stability used both, real-time and accelerated temperature simulation methods. Stability is crucial for the life of the test as well as logistics. It addresses the variable temperatures in the transit of kits to tropical and sub-tropical areas. Developing countries do not have efficient transportation and adequate cold-chain distribution. It is, therefore, required that a kit be robust and stable to withstand at least one week of air and road transportation time, with minimal cold packaging using ice packs.

Diagnostic tests need to be durable so performance can be precise. The test needs to be repeatable and reproducible. Repeatability, also known as the intra-assay precision measures the agreement of ELISA results when different operators complete the

same test, using the same method and under the same controlled conditions. Reproducibility, also known as inter-assay precision uses the same repeatability method but extended to demonstrate the repeatability of a test by different operators in different laboratory conditions. Reproducibility tests are more open to errors due to the introduction of a variety of factors but must be within an acceptable range. A standardised, robust assay design can usually eliminate problems associated with reproducibility and repeatability.

Verification is implemented after the development stage to determine whether the kit meets its particular purpose. It confirms that all the product requirements have been addressed, and performance measures have been met using the prototype kit as a functional proof of principle. The equivalent process is a series of experiments to evaluate the diagnostic performance in real situations. In filariasis research, the method of evaluation commonly used is the *multi-centre trials*.

Multi-centre trials involve three or more independent laboratories performing the same experiments. This method of evaluation is practical in terms of assay performance demonstration and measurement of assay characteristics. Comparisons of diagnostic measurements such as sensitivity and specificity can be made directly. However, it is crucial that multi-centre trials are carefully planned and controlled from the finest detail such as kit procurement and logistics to ELISA experience and practice of technicians. Diagnostic kit performance is being tested, therefore, all parameters must be performed exactly to the protocol. Failure to do this can result to poor results that may not be a reflection of assay performance, but a result of introduced variables that interfere with assay performance. Some examples may be the use of different positive controls that make it impossible for direct comparisons. The use of different sizes of blood spot samples on filter paper, ELISA techniques and training and laboratory equipment used. Some of these errors have been observed with the Bm14 Antibody CELISA multi-centre evaluations and are discussed in Chapter 7. Common variables that affect diagnostic assays and performance and the importance a validation process is discussed by Jacobson (1998).

ELISA kits were manufactured in a standardised process by following a quality management system (QMS) using ISO13485. All development and manufacturing procedures are written in standard operating procedures (SOPs) that are strictly

followed for each batch production to maintain and monitor kit performance long-term. The high level of control and verification process ensures traceability under a QMS.

This chapter covers the components of assay verification and validation of product performance, a process requirement before commercialisation can take place. It demonstrates the output of safety and quality expected to be built-in every stage of the design and development and verifies that the Bm14 Antibody CELISA will perform its intended purpose.

3.2 AIMS

The works in this chapter cover the final assay verification and validation, in preparation for a commercial-ready Bm14 Antibody CELISA. The verification and validation processes aim to evaluate the kit performance and demonstrate that the original requirements for a new antibody test has been met. The areas of kit performance verified and validated are:

(a) cut-off value for determining negative and positive;
(b) diagnostic measures, sensitivity and specificity;
(c) cross-reactivity;
(d) repeatability and reproducibility
(e) stability and;
(f) Quality control procedures

3.3 MATERIALS AND METHOD

3.3.1 ELISA Methods for Validation Tests

All validation tests for IgG4 antibody used the final kit format Bm14 Lymphatic Filariasis Antibody CELISA. Methods followed the kit insert instructions version LF3.7 provided in Appendix 2.

Circulating filarial antigen (CFA) testing for validation studies used the Tropbio Og4C3 Antigen ELISA (Tropbio, Townsville, QLD, Australia) kit insert provided in Appendix 4 as the gold standard.

The cut-off value was determined by calculating the total negative mean OD plus 3 Standard Deviation (SD) (Rajasekeriah et al., 2003, Weil et al., 2011).

3.3.2 Determination of an assay cut-off

An initial cut-off was determined by testing 47 non-endemic samples from Australia using the experimental method as described previously (Chapter 2, Section 2.3.1). The cut-off was used as a guide during the development and optimisation of the assay improved performance. Therefore, a new cut-off was determined for the final assay conditions using a larger number of samples. There were 80 samples from Australian residents and 67 from residents of Manila, Philippines, a non-endemic region for LF, a total of 147 non-endemic samples. These samples were not well characterised, and no clinical history was available. However, testing of these samples has shown no reactivity in the ELISA, therefore, deemed suitable as a sample set for the purpose of determining a cut-off value.

3.3.3 Sensitivity and Specificity of the Bm14 ELISA

A non-endemic and endemic sets of samples tested for Og4C3 ELISA CFA as the gold standard were used to determine the diagnostic accuracy (sensitivity and specificity) of the Bm14 ELISA.

Included in the specificity test were 128 serum samples from individuals living in non-endemic Australia. 120 of which were breast cancer patients without background information on exposure to LF and 8 were confirmed samples having no prior travel to LF endemic areas. Also included was 123 non-reactive serum samples from a non-endemic region, Manila in the Philippines. There were 13 samples from the USA with no prior travel to or residence in an LF-endemic area. A total of 264 non-endemic samples. A total of 152 samples from New Ireland, PNG testing positive for CFA were used to determine the sensitivity.

Estimation of sensitivity and specificity was determined using the 2 x 2 table method for diagnostic tests (Muller & Buttner 2009, Akobeng 2007).

Table 3.1: Diagnostic Test 2x2 Table Method. P is the point prevalence of the disease. T is a positive test result and t is a negative test result. D represents disease that is present and d represents absence of disease.

		True Health Status	
		D	d
Decision of Diagnostic Test	T	D ∩ T	d ∩ T
	t	D ∩ f	D ∩ t

Sensitivity $P(T|D) = \frac{P(T \cap D)}{P(D)}$ Specificity $P(t|d) = \frac{P(t \cap d)}{P(d)}$

3.3.4 Cross-reactivity Studies

Cross-reactivity studies were performed by testing available well-characterised serum samples donated by the reference laboratory at Parasitology Unit, ICPMR, Westmead Hospital, Sydney, Australia. A set of samples confirmed positive for strongyloidiasis, schistosomiasis, malaria, dengue, toxoplasmosis and Chagas disease were included in a sample set along with CFA and positive mf samples as a reference.

3.3.5 Assay Repeatability and Reproducibility.

Repeatability or intra-assay variation and Reproducibility or inter-assay variation was validated by testing the ELISA in three laboratories at Laboratory A (Cellabs Pty Ltd, Brookvale, NSW, Australia), Laboratory B (School of Public Health, Tropical Medicine and Rehabilitation Sciences, JCU, Townsville, Queensland, Australia) and Laboratory C (CDC, Atlanta, GA, USA).

Repeatability tests were performed at Laboratory A by following the summarized method in Table 3.2 below. “Operator only” investigates the performance variability with a controlled assay by one operator and one batch of kits. Operator and batch variability is an extended test with three operators testing three different batches of kits. The Reproducibility used the same method but extended to investigate three different operators, batch and laboratory conditions.

Variations within these assays were calculated by using average OD values to determine the standard deviation (SD) and calculating the percentage coefficient of variation (CV) = (SD / Mean)*(100). A CV (%) of less than 5% is considered to be excellent repeatability and reproducibility. CV 5% - 10% is acceptable and CV more than 10% indicates that standardisation of assay conditions and kit production needs further review to improve performance.

Table 3.2 Repeatability and Reproducibility Method: Table summarising the different ELISA conditions to determine the repeatability and reproducibility of the

	Repeatability (Intra-assay variation)		**Reproducibility (Inter-assay variation)**	
	Operator Only	**Operator and Batch**	**Operator Only**	**Operator and Batch**
Operators	*One*	*Three*	*Three*	*Three*
Sample Controls	*Positive samples: (High and Medium) Negative sample Blank*		*Positive samples: (High and Medium) Negative sample Blank*	
Batch of kits	*One*	*Three*	*One*	*Three*
Laboratory	*One laboratory*		*Three*	*Three*
ELISA repetitions	*Eight*	*Three/batch*	*Three/batch*	

3.3.6 Kit Stability

The final standardised version of the ELISA was tested for stability using the international standard ISO 23640 version 2013 for Stability Testing of In-vitro Diagnostic Reagent as a guide. A total of three kit batches were used for the real-time stability tests at the prescribed storage temperature of 2-8^{o}C. A control was established by performing an ELISA using the kit positive control and a blank, the results were recorded as day 0 and tested at a minimum of one test per month for 12 months.

To accept the stability results, the kit Positive Control (FAPC) at the dual wavelength (A450/620) was expected to read >OD 2.5. The kit Negative Control (FANC) was expected to read <OD 0.2 to pass specification. The kit batch shelf-life specification was 12 months from the time the kit QC was completed and QC passed.

3.3.7 Verification of Batch Performance: Quality Control

3.3.7.1 Regulatory Bodies and Standards

Quality Control methods were documented as SOP for commercial production. QMS procedures comply with international standards for the manufacture of IVD Medical Device (ISO13485) and other normative documents required for certification under TGA IVD Framework 2014 and CE Marking (for European certification). These were documented starting from receipt of raw materials from the supplier to intermediate quality control (QC) methods, individual components and a final kit QC. The methods below describe each stage of QC test performed during the manufacture of the commercial-ready Bm14 ELISA kit. TGA certification for commercialisation is covered in detail in Chapter 5.

3.3.7.2 Incoming Raw Material Quality Control Method

Raw materials for critical components were tested prior to being released for use in the production of ELISA plates and conjugate. Plate-coating rAg Bm14, detector anti-IgG4 antibodies in raw ascitic fluid form for conjugation, and kit control serum were subject to evaluation as they are considered critical raw

materials. QC procedures outlined below were implemented to verify each critical component.

3.3.7.2.1 Quality Control - rAg Bm14

Each batch of rAg Bm14 received from the supplier as a coating material for ELISA plates was checked for antigen reactivity before using at the appropriate coating concentration. A QC test was completed by coating the antigen at concentrations 2, 1, 0.5, 0.25, 0.125 ug/mL on microwell plates (Greiner Bio-One, Cat No. 705-071, Frickenhausen, Germany). The following plate coating method described previously (Chapter 2, Section 2.3.1.1) was used. Once microwell plates were dried and ready for testing, the reference sample panel of high, medium, low reactors and a negative sample were used to test the reactivity of each of the batches. A sample blank was included to check for any background variation caused by each batch of rAg.

3.3.7.2.2 Quality Control - IgG4 antibody

IgG4 monoclonal antibody HP6023 for use in the conjugation process was supplied as raw ascitic fluid material. The material was evaluated for reactivity by constructing an IgG4 ELISA. Human IgG4 myeloma (Calbiochem, Cat# 400126, Merck-Millipore, Darmstadt, Germany) was coated on a plate at 1ug/mL concentration in 0.2M Carbonate-bicarbonate buffer and incubated for two hours at 37^{o}C. The solution was removed by flicking out and tap-drying the plate on laboratory tissue to remove the excess fluid before drying upside-down in the 37^{o}C incubator. After drying in the incubator for two hours, the IgG4 ELISA plates were ready to use for the QC test of anti-IgG4 monoclonal antibody reactivity.

Each batch of ascitic fluid was tested by serially diluting two-fold in PBS from 1:500 to 1:64,000. Each dilution was loaded onto a well, and the plate incubated for one hour at 37^{o}C. After incubation, the plate was flicked empty and tap-dried on laboratory tissue to remove excess fluid. The plate was washed four times in a plate washer with PBS/T. A secondary antibody, rabbit anti-mouse HRP-conjugate (Sigma-Aldrich,

Cat# A9044, St Louis, MO, USA) was used at 1 in 8000 and incubated for 30 minutes at 37^{o}C. The colour complex was developed using a TMB substrate (FASC) from a reference Bm14 ELISA kit, diluted 1 in 20 with kit substrate buffer (FASB). The reaction was stopped using the kit stopping solution (FASS). The plate was scanned using a plate reader (Molecular Devices, E-max, CA, USA) at dual wavelength A450/620nm. Reactive batches detectable at OD>1.0 at 1 in 1000 dilution were passed and released for use in HRP conjugation process.

3.3.7.2.3 Quality Control – HRP Conjugate

Each batch of anti-IgG4 HRP conjugate was tested for reactivity by using the Bm14 ELISA for both the negative and positive controls. The "mother" or "neat" conjugate was prepared by serially diluting two-fold down the plate starting from 1:500. Blank wells were included in the assay to check for any non-specific reaction that may be attributed by the conjugate.

A good working conjugate is expected to work optimally without any background at approximately 1:8000 dilution for ELISA. However, acceptability for use in the kit production is dependent on whether the reference serum reactivity fall within kit control specifications (Appendix 2).

3.3.7.2.4 Quality Control – kit controls

Positive control sera for the kit was tested for safety by external services (Pacific Laboratories PaLms, Royal North Shore Hospital, St Leonards, NSW). HIV and hepatitis B antigen (HBAg) were tested according to current TGA legislation TGO34 (Appendix 3) for the use of human-derived materials such as serum or plasma as controls in human diagnostic devices. All sera or plasma used as controls for a batch of commercial kits must be negative for HIV and Hepatitis B surface Ag (HBsAg) using specific TGA approved test kits.

Kit negative and positive serum or plasma verified HIV and HBsAg free were released for use as kit controls. These were evaluated by testing using the Bm14 ELISA to check for reactivity and confirm that it meets the OD specifications. Positive control > OD 2.0 and for a negative control <OD 0.2 at both single wavelength A450nm and dual wavelength A450/620nm.

All quality control results and methods were adequately documented under a GMP system to ensure traceability and compliance to kit specification.

3.3.7.3 ELISA Kit Quality Control Procedures

3.3.7.3.1 Quality Control – Critical Component

The kit conjugate and the coated plates are the two critical components of the kit requiring a thorough QC before using as components for a batch of kits. Component QC testing at stage one was performed by using the reference sample panel in an ELISA to test a new batch of plates or conjugate including a reference batch for comparison. A reference batch was the previously passed batch of kits within its expiry date. The aim was to determine whether the critical component was comparable to a previously passed batch. This method ensures assay repeatability and batch uniformity.

3.3.7.3.2 Quality Control – Batch

Once the critical component has satisfactorily passed stage 1 QC testing, the batch was used as a component for a kit. Stage 2 QC tests all components put together to make a new kit. To perform a kit batch QC test at stage 2, an ELISA was performed using a previously released batch as the reference kit. The reference panel of serum was used to check for acceptable performance comparison within the specifications before a batch is released for commercial purposes.

3.3.7.3.3 Specification for Acceptance

A QC reference panel of serum samples (gold standard) were used for both stages one and two QC testing. The panel included a negative (<OD 0.2), a high positive (>OD 2.5), low median positive (>OD 1.0, <OD 2.0) and a low positive (>OD 0.4 and <OD 0.8). A sample blank (<OD 0.1) was included in each ELISA for background checks.

3.3.7.4 Controlling Batch Performance drift

Batch performance drift can occur even in controlled ELISA manufacturing processes if kit performance is not effectively monitored. In addition to the use of a prescribed OD specification for the reference serum panel or "gold standard", plotting of OD results for every batch QC was useful in monitoring a potential performance drift. These are seen as inconsistencies of kit manufacture that can affect the performance of the kit.

A graph was constructed by plotting the OD results of reference serum panel each time the batch QC was performed (Figure 3.11) This graph shows the historic results of the same reference serum panel tested on every batch manufactured. A potential assay performance drift was detected if results show a gradual increase or decrease outside the OD specifications for each reference serum, or consistently outside the OD specifications.

3.3.8 Verification of ELISA edge effect

The edge effect and assay conditions of the ELISA were validated by randomly placing a positive control, negative control and sample blank wells on a plate. A humidity chamber was created by placing a few layers of wet laboratory tissue at the bottom of a plastic box with a lid. The plate was placed inside the sealed humidity chamber and incubated at 37^{o}C in a water bath (Grant SUB14, Grant Instruments, Hertz, England). The temperature was kept constant by +/- 0.2 degrees using a minimum-maximum temperature monitor, (-50/+70^{o}C Digital Thermometer, Alla France, Chemille, France).

3.3.9 Internal Validation of Kit Performance

Before testing the CFA status of samples to be used for the internal validation, a cut-off value was determined for the Tropbio Og4C3. There were 150 non-endemic serum samples used to determine the COV using the Negative Mean +3SD method.

To perform the internal validation, serum samples from different parts of PNG, Egypt, Sri Lanka, Philippines, USA and Australia were tested using Bm14 ELISA kit batch KF329. Due to the difficulty in procuring samples to determine mf status for internal diagnostic validation, analyses of performance can only be based on the CFA test results using the Tropbio Og4C3 kit. Some of these samples were received with mf status and additional antigen status information by the ICT/BinaxNOW card rapid test. Therefore, additional information will be taken into consideration.

To interpret the test results, assumptions were made that a high CFA positive (>OD 0.8) will be IgG4 antibody positive. Samples negative for CFA and positive for the antibody may mark an early infection where CFA is currently undetectable, but antibody presence is already detectable by the Bm14 ELISA. Other unexpected results and observations were be repeated for confirmation and results reported for discussion.

3.4 RESULTS

3.4.1 Determination of Cut-off

An initial set of non-endemic serum samples from residents of Australia (n=9) and USA (n=38), a total of 47 were tested to determine a cut-off value that can be used as a guide during the development of the assay (Table 3.3). A cut-off value of OD 0.211 was used as a guide. Another set of 142 non-endemic samples including the 47 samples from the initial set was tested on the final Bm14 ELISA (method in Appendix 2). An OD of 0.192 was determined as the cut-off value, rounded up to OD 0.2.

Table 3.3: Cut-off values for the assay. The cut-off for the final assay was rounded up to OD 0.2 as determined by testing 142 samples from a non-endemic population.

	Optimisation cut-off	**Verification cut-off**
Number of samples	47	142
Mean	0.088	0.061
Standard Deviation	0.041	0.043
Cut-off value	**OD 0.211**	**OD 0.192**

3.4.2 Sensitivity and Specificity

ELISA results to determine the assay sensitivity and specificity are shown on Table 3.4 below. The values in red were the percentage calculations for actual numbers written in black. The assay sensitivity was determined at 100% where 151 confirmed positive for CFA were also antibody positive by the Bm14 assay. Out of the 152 samples from non-endemic populations, one was at the equivocal area at OD 0.295, a sample from Australia. This sample was tested twice to confirm OD reading and marked as a positive reactive following the definitions of the assay cut-off-value due to unconfirmed clinical records, history of travel to endemic LF or exposure. It is possible that this was a non-specific reaction. The determined specificity was 98% using the available sample sets.

Table 3.4: Quality measures of a diagnostic test. A total of 416 serum samples were used to determine the diagnostic accuracy with a calculated sensitivity of 100% and specificity of 98%.

Diagnostic Test **Sensitivity = 100%** **Specificity = 98%**		True LF Status		
		Confirmed CFA positive	Non-Endemic Samples	TOTAL
Decision of Bm14 ELISA	Positive	151 36%	1 1%	152 37%
	Negative	0 0.0	264 63%	264 63%
TOTAL * percentage calculations		151 36%	265 64%	Total Samples 416 100%

3.4.3 Cross-Reactivity

The graph (Figure 3.1) shows the test results of samples positive for toxoplasmosis, Chagas disease, schistosomiasis, Dengue, malaria and strongyloidiasis. One sample from Cameroon, positive for malaria, had a slightly elevated reaction compared to the rest of the samples; however, the value remains <OD 0.2, the cut-off value for the assay. All samples other than those positive for LF had OD values less than 0.2 confirming no cross-reactivity when using the Bm14 ELISA.

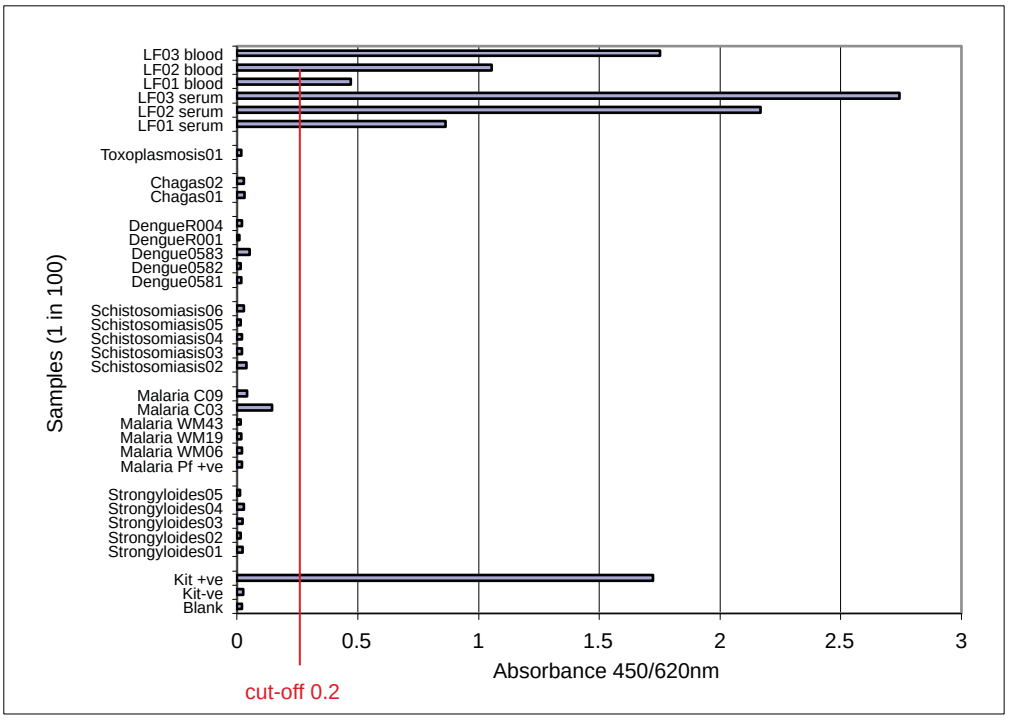


Figure 3.1: Cross-reactivity testing. Available serum samples were tested to verify the cross-reactivity of the Bm14 antigen used in the ELISA. All serum samples positive for seven unrelated diseases did not react in the Bm14 assay.

3.4.4 Assay Repeatability and Reproducibility

Repeatability and reproducibility for the assay were completed by three different laboratory locations, and the results were calculated as averages to give the CV (%). The results show excellent repeatability at Lab A with CV (%) for both methods 1A and 1B below the maximum limit 5% CV. Reproducibility observed for the three laboratories meets the specification with both methods 2A and 2B below the maximum limit 5% CV. The excellent results indicate a high level of uniformity, precision and standardisation of the assay (Table 3.5).

Table 3.5: Repeatability and Reproducibility Table. The average CV (%) results for Method 1 and Method 2 sets of testing meets specifications below the maximum limit of 5% CV indicates a high level of assay manufacturing quality and standardisation.

	Repeatability CV (%)			**Reproducibility CV (%)**		
	OpA	OpB	OpC	**A**	**B**	**C**
Method 1A 1 operator/1 batch/8 tests/ lab A	**2.369**					
Method 1B 3 operators/3 batches/ 1 test/ lab A	2.283	3.038	7.449			
	Avg. 4.257					
Method 2A 3 operators/1 batch/ labs A,B,C				2.409	2.848	3.440
				Avg. 2.899		
Method 2B 3 operators/3 batches/labs A,B,C				4.34	5.04	3.102
				Avg. 3.326		

3.4.5 Stability Trials

Real-time Kit stability studies observations for positive control results at 1:100 dilution is shown on the graph below (Figure 3.3), using kit batch KF326, KF329 and KF330 at the recommended storage temperature of 2-8^{o}C. The trial demonstrates the stability of the kit up to 20+ months of monitoring.

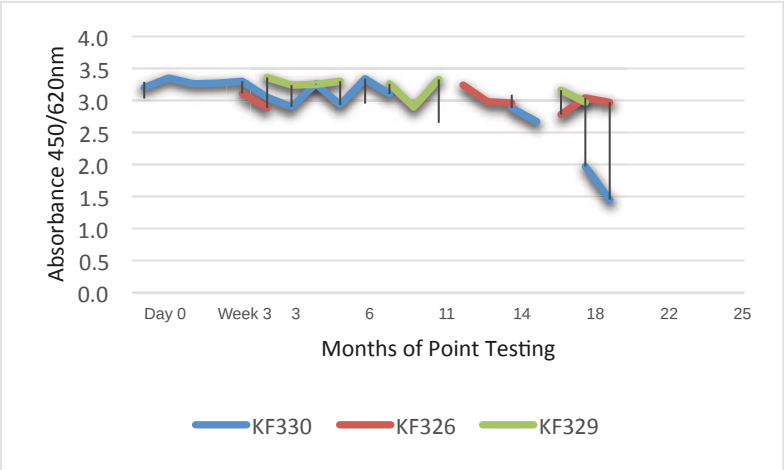


Figure 3.3: Bm14 ELISA Kit Real Time Stability at 2-8C. The kit remains stable at the storage temperature of 2-8^{o}C for months after the prescribed shelf-life of 12 months, up to the maximum 24 months testing date for kit

Stability studies commenced in January 2010 and concluded at the beginning of August 2011 (Table 3.6). Kit control positive (FAPC) meets the specification of >OD 2.5 and negative control (FANC) meets the specification of < OD 0. The shelf-life specification of 12 months for the standardised kit is therefore validated and deemed compliant to requirement.

Table 3.6: Results of stability trials at 2-8^{o}C storage temperature. Three batches of kits used for real-time stability trials passes stability specifications of 12 months.

Batch	IFU	Commence/End	Results	Comments
KF326	LF3.4	24/8/11-9/08/13	PASS	Stable up to 24+ months testing dates – OD2.377 at 24mo
KF329	LF3.5	25/05/12-14/05/14	PASS	Stable up to 23+ months testing dates – OD2.976 at 23mo
KF330	LF3.6	3/09/12-14/05/14	PASS	Stable up to 20+ months testing date – OD1.452at 20mo

3.4.6 Verification of Batch Performance: Quality Control

3.4.6.1 Quality Control Results – rAg Bm14

QC test results for batch 08-1108 Bm14 rAg for plate coating was plotted on a graph (Fig.3.4) to show the reactivity when used with a positive reference control. The positive reference control was a highly reactive serum sample with specifications OD 2.5 – 3.5. At the optimised antigen concentration of 0.66ug/mL (pink curve), the reference control was within specification therefore batch 08-1108 passed QC test and released as material for plate production.

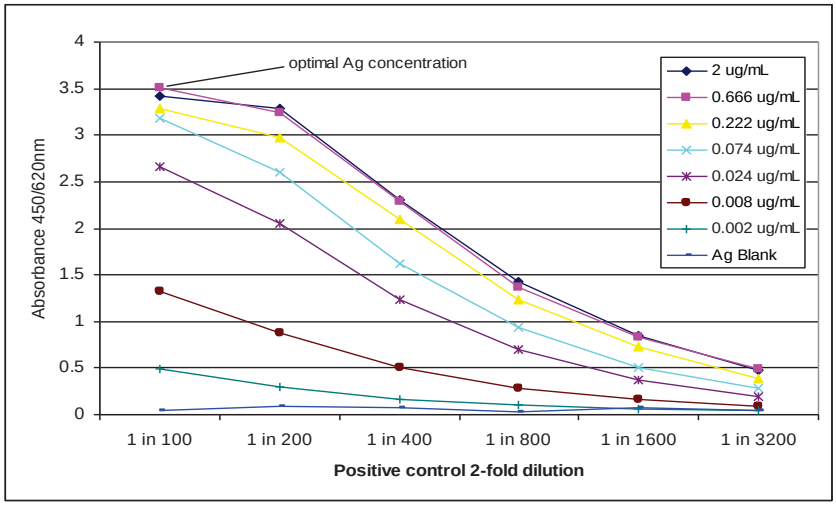


Figure 3.4: Recombinant antigen batch QC test. The antigen batch passes QC testing when used at the specified optimal concentration of antigen at 0.66 ug/mL.

3.4.6.2 Quality Control Results - anti-IgG4 antibody

Three batches of ascitic fluid IgG4 antibody material for conjugation were tested and compared to an internal reference (Ref AF). Figure 3.5 shows the reactivity of each batch of the ascitic fluid tested. Both batches IIR#1524 (tapping sample) and IIR#1524S (batch) were highly reactive and met the minimum specification for acceptance (OD>1.0 at 1 in 1000 dilution). These batches of antibody were passed and released as material for use in HRP conjugation. One batch IIR#144, however, fails QC testing showing a negative reaction (green line).

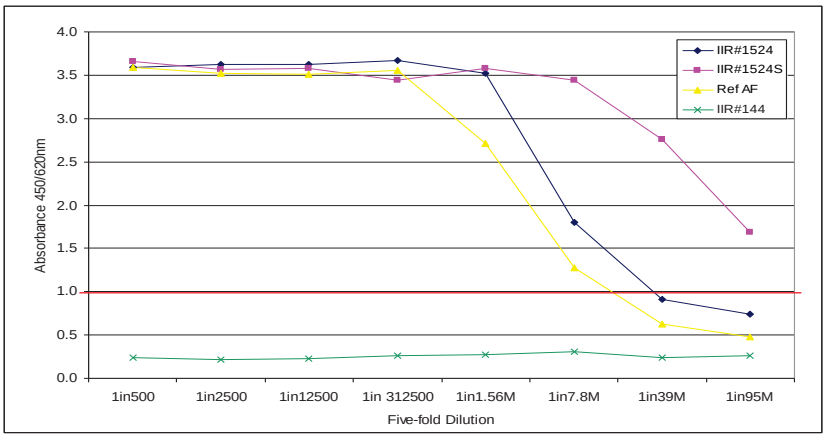


Figure 3.5: Quality Control of secondary antibody. A crude anti-IgG4 ELISA was used to test for the reactivity of ascitic fluid batches before using as material for the production of anti-IgG4 HRP Conjugate for kits.

3.4.6.3 Quality Control Results – Neat Conjugate

Figure 3.6 below shows the different reactivity of five "neat" or "mother" conjugate batches before pre-dilution. Line 1 marks the cut-off value at OD 0.2. Line 2 marks Batch 7 (pink curve) at the point where reference positive control reads OD 3.0, and conjugate dilution is approximately 1:750. At this level, the conjugate fails QC despite the positive reference OD reading because the background is higher than the cut-off. Batch 8 was non-reactive. Line 3 marks Batch 5 (blue curve) and line 4 marks Batch 9 (green curve). At this point, the positive control is at OD 3.0, and the conjugate dilution is approximately 1:3000 and 1:40,000 respectively. The negative control was below OD 0.2 which meets the specification. Therefore, both batches pass QC.

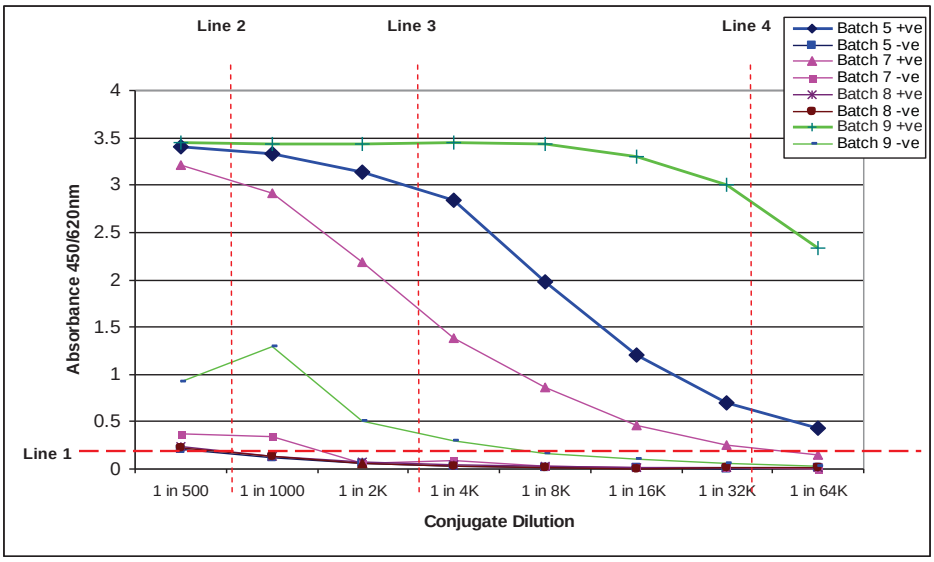


Figure 3.6: Anti-IgG4 conjugate evaluation. Conjugate Batch 5 (blue curve) and Batch 9 (green curve) pass QC specification however Batch 7 (pink curve) and Batch 8 (non-reactive and not visible in the graph) failed QC evaluation.

3.4.6.4 QC Results - Negative and Positive Control

Serum or plasma samples used as controls for kits were evaluated for reactivity. Figure 3.6 shows two out of six strongly reactive plasma batches that meet the specification for a positive control (>OD2.5). PC-08 and PC-09 were

suitable for use as kit positive controls. PCFC, PCSL and PC-07 did not meet the specification but have relatively good reaction making these samples suitable as reference QC material only. Negative serum pool (MP Biomedicals Australia, Seven Hills, NSW) was tested in the same assay and passed the QC test with OD reading below 0.2. This indicates that there was no background caused by the sample and can be used as a negative control for kit production.

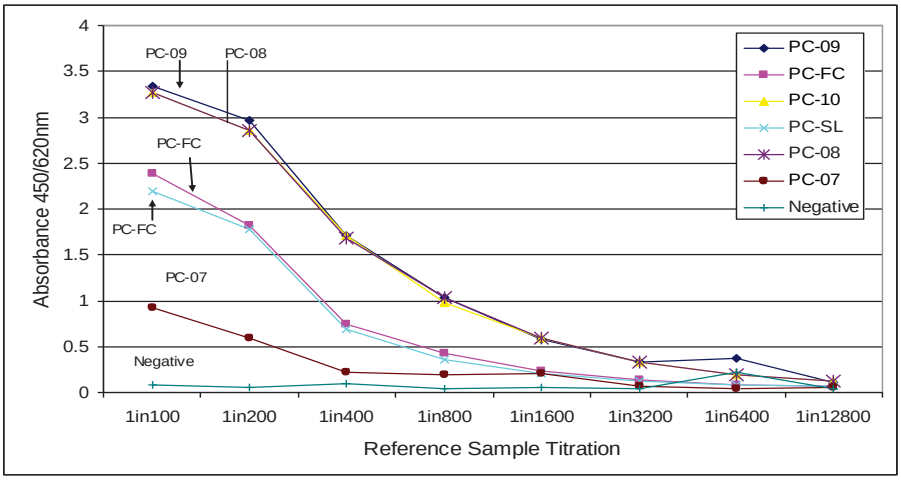


Figure 3.7: A typical ELISA graph of positive and negative control evaluation. Each batch of control serum was evaluated to look at the reactivity. Once deemed QC passed, the material was used for ELISA kit production.

3.4.6.5 Final ELISA Kit Quality Control

3.4.6.5.1 QC Results - Coated ELISA plates

Each new batch of antigen-coated ELISA plates (FAMW) were QC tested before using as a component in a batch of kits. Figure 3.8 below shows the graph of QC results for the latest batch of plates (FAMW19) compared to previous batches of plates. Y-error bars show a 10% +/- acceptability criteria for reproducibility. Yellow shaded areas show the OD specification for the panel of control sera. All plates tested in this ELISA passed QC and released for use in a batch of kits.

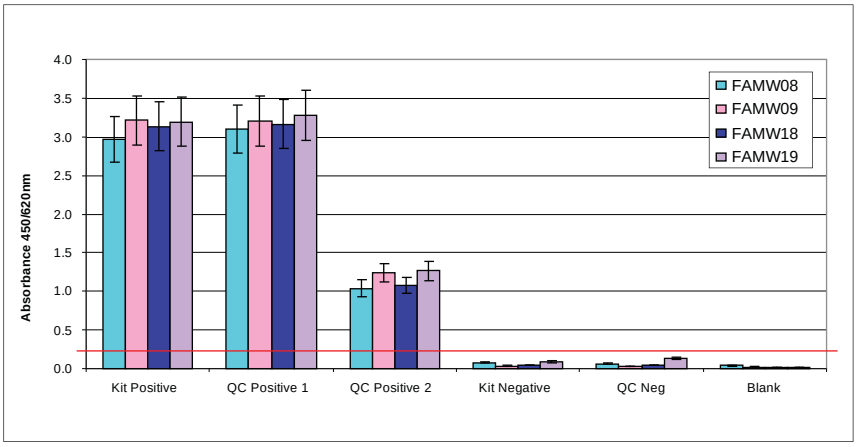


Figure 3.8: A typical QC test for a batch of ELISA plates. The latest batch of plates FAMW19 (lilac bar) was compared to the previous batches of plates at the time of production. The batch was comparable and passes the plate QC testing.

3.4.6.5.2 Quality Control – Kit Conjugate

Batches of pre-diluted kit conjugate were QC tested to determine if the performance meets specifications. Figure 3.9 below shows the typical QC results of 5 batches of kit conjugates (PO11 – PO14). At the positive control dilution of 1:100, all five kit conjugates passed QC. Batch PO14 was slightly higher than the specifications, therefore, was adjusted by diluting to a lower concentration and re-testing again until it met the specifications (between OD 2.5 and 3.5). The negative reactivity was slightly higher but under the cut-off OD 0.2.

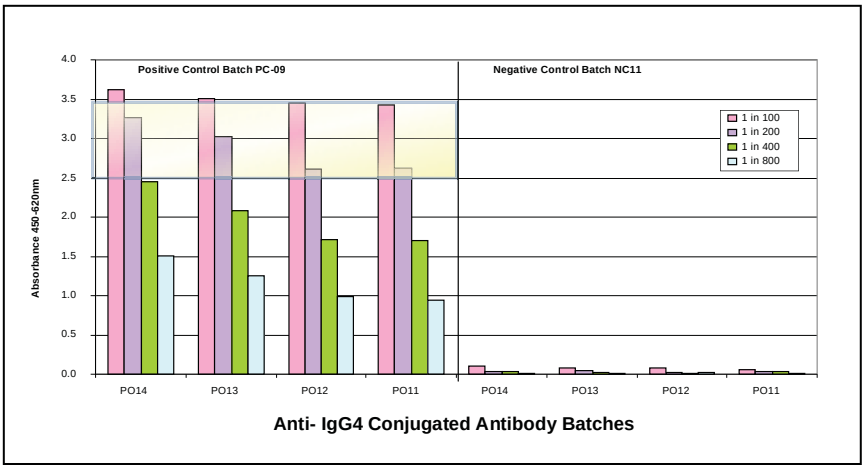


Figure 3.9: Kit conjugate QC test. A number of conjugates were QC tested to determine the reactivity of a freshly made conjugate (PO14) in comparison to previously released conjugates (PO13-PO11). The yellow shade indicates the specification, area of acceptance (OD 2.5-3.5).

3.4.6.5.3 Quality Control – Batch

Figure 3.10 shows the typical QC result of a new batch of kits (KF322) in comparison to five previously passed batches of kits. The yellow shaded areas indicate the acceptance range for each reference serum in the panel. The new batch KF322 meets the specification, therefore, passes QC and ready for release.

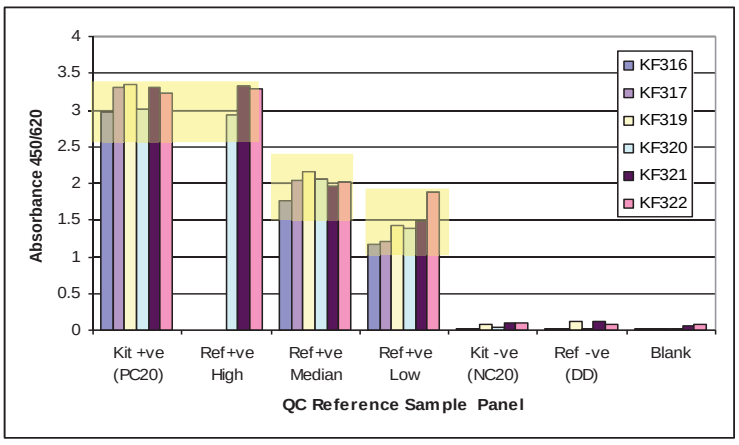


Figure 3.10: Final QC test of a batch of kits. Bm14 kit batch KF322 was tested by comparing the reactivity of kit controls and the panel of reference serum samples. The kit is comparable to previous batches and passes QC.

3.4.6.4 Monitoring of Batch Performance Drift

The graph below (Fig. 3.11) was constructed by taking final batch QC results of the reference serum panel and plotted each time a batch of kits was manufactured. A good consistent kit was observed with six consecutive batches of kits where the reference positives Ref+ve High and Ref+ve Median were consistently within specification. Ref+ve Low shows a gradual increase in OD values however this was not consistent with the rest of the reference panel therefore not an indication of assay drift rather, an issue with the preparation of reference control.

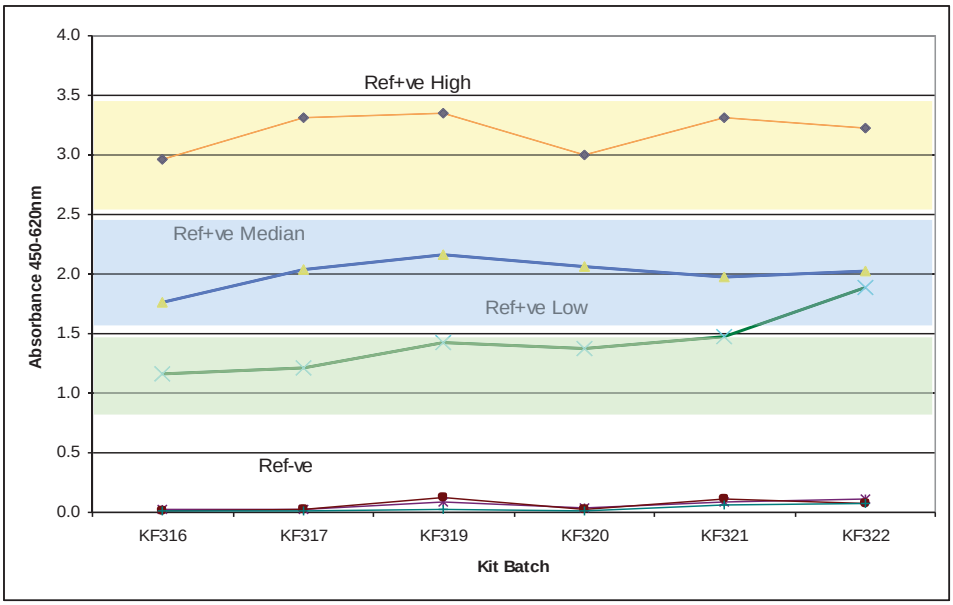


Figure 3.11: How to monitor assay drift. The same reference positive samples were used each time a batch of kits were QC tested. The OD results are plotted to monitor the level of performance for released batches of kit. Batch results must remain within the shaded areas, according to specification.

3.4.7 ELISA Edge Effect

ELISA incubation conditions were validated by the results below (Fig. 3.12). A coefficient of variation (CV) expressed as a percentage was used to determine any variation with the OD reading of 47 wells of positive control. A consistent reactivity of kit positive and kit negative was observed with a CV= 4.4%.

1	2	3	4	5	6	7	8	9	10	11	12
0.067	1.612		1.621	1.661		1.649		0.006	0.064	0.009	0.009
0.06	1.558		1.805		1.511		1.697		1.794	1.7	1.703
0.008					0.068				1.605	1.653	1.637
0.016	1.6		1.641		0.074	1.778					1.603
1.7	1.63				0.008	1.84			1.636	1.703	1.655
	1.858	1.619		1.627	0.007	1.582	1.73			1.762	1.7
1.791		1.622	1.63	1.702	1.683		1.684		1.687	1.832	1.752
1.767	1.708	1.604	1.609	1.656	1.643	1.652	0.075	0.004	0.008	0.008	1.652

Legend: Yellow = kit negative control
Tan = blank

Blank cells = no samples
Cells with values = kit positive control

Figure 3.12: ELISA Edge Effect. Raw data of a plate randomly loaded with positive and negative control to look at any edge effect of using the recommended incubation method of keeping the plate in a humid chamber at 37^{o}C conditions.

3.4.8 Internal Validation of Kit Performance

Kit performance was validated in-house using available laboratory serum bank samples. The results were summarised below (Table 3.7). The serum samples were qualified by tested for CFA using the Tropbio Og4C3 ELISA version II. The NE set 7 samples found 1/265 positive for antibody however, this sample does not have a full history to determine if the reactivity is non-specific.

Table 3.7: Summary of results for internal validation.

Sample Set	Total samples	Ab -ve CFA -ve	Ab +ve CFA +ve	Ab +ve CFA -ve	Ab -ve CFA +ve
PNG Set 1	18	4	5	17	
PNG Set 2	23	13	6	12	
Egypt Set 3	51	0	50	0	1
PNG (Naru) Set 4	9	1	6	2	
PNG (New Ireland) Set 5	160	25	67	68	
PNG Set 6	75	10	33	32	3
NE Set 7	265	264	0	1	0

3.5 DISCUSSION

The validation studies completed in this chapter found the assay to perform within the objectives and have met the expected outcomes with no major re-work on the final assay construction. The majority of the re-work was completed during the Design and Development stage of the prototype kit that took approximately five years to complete. The lengthy development time of the assay was due to a range of factors that included problems with stabilities, background, diagnostic accuracy and other performance issues caused by poor performing raw materials and buffers. A prototype version of the kit was released for the purpose of a multi-centre evaluation for the Global Programme to Eliminate LF participating organisations (see Chapter 7).

The first multi-centre trial was conducted in 2008 by six participating centres organised by the GAELF, Task Force for Child Survival and Development. The participants were CDC, Smith Collage and Washington University in the USA, JCU in Australia, Institute of Malarde in French Polynesia and WHO in Ghana. The trial centres across the globe received kits for evaluation directly shipped with ice pack packaging from Cellabs Pty. Ltd. in Brookvale, NSW, Australia where the prototype kits were manufactured as a batch. The feedback from the trials provided an abundance of information on the performance, stability and usability of the ELISA that helped in improving the assay design before the final version was released.

Some of the issues raised include stability problems experienced with kits sent to Tahiti, French Polynesia and Washington University, USA. It was reported that kits sent to Washington University have lost some kit activity after a cool room breakdown. The kits were subjected to temperatures as high as 40^{o}C over the weekend. Re-testing these kits demonstrated a reduced reactivity that does not meet specification. Kits sent to French Polynesia also reported kits not meeting specification. After investigations, it was determined that the kit stability was affected by inadequate cool room storage for the recommended storage temperature. The project managers cannot confirm whether the kits were stored at 2-8^{o}C from the time the kits were received and the time they were used.

The reported information was invaluable during the standardisation stage. It ensured extensive experimentation to determine that the conjugate and the antigen-coated plates were not stable enough to withstand temperatures outside the recommended storage temperature of 2-8^{o}C. New formulations of buffers and methods for the anti-IgG4 conjugate and the antigen-coated plates were developed and tested for stability using accelerated method by subjecting at 37^{o}C until a sharp reduction of reactivity was seen. After promising results seen in accelerated stability, the long process of validating the final format for stability commenced in 2010 and concluded in 2011. As seen with the results (Section 3.4.5), the kit stability has improved since the prototype kit and verified stable under the prescribed 12 months shelf life of the kit.

A typical diagnostic performance study includes a range of well-characterised samples of diseases including those that may be cross-reactive such as other helminthic infections and other filarial infections that are closely related. The antigen characterisation work by Chandrashekar et al., (1994, 1995) included a full range of control samples from brugian and bancroftian filariasis, *Loa loa*, *S.mansoni, L.loa, Mansonella perstans, Ascaris lumbricoides, Strongyloides stercoralis, Ancylostoma duodenale, Hymenolepis nana Drucunculus medinensis*, *Enterobious vermicularis and Onchocerca volvolus.* A limited number of samples of other diseases were used in verification of cross-reactivity in this chapter due to the constraints of procuring human plasma or rare serum samples of diseases caused by other nematodes and other tropical, mosquito-transmitted diseases and filarial parasites.

Clearly, the biggest challenge facing the validation studies was the procurement of well-characterised samples that can be used for specific tests. A majority of the samples used in these studies were collected specifically for other studies. Excess samples have been kindly shared and donated to help develop the Bm14 Ab ELISA. Today, the ethical clearances required to procure clinical samples has become a long process of approvals, documentation and paperwork. Applications and requests do not guarantee approvals. There is also the difficulty in the procurement of samples for the purpose of commercialisation. An option to consider is to outsource validation studies, though, this process adds to the cost of product development and should be carefully considered. The Bm14 ELISA has also been independently evaluated which adds to product quality assurance.

The general elements of commercialisation process can also be integrated with development stages of an IVD medical device by design and careful consideration of regulatory requirements. Besides validation studies, some understanding of the basic elements of regulatory requirement is also important to ensure that the Essential Requirements are addressed. The Essential Requirements and preparation for regulatory certification for TGA are covered in detail in Chapter 5.

CHAPTER 4

DEVELOPMENT OF AN ANTIBODY DETECTION RAPID DIAGNOSTIC TEST BASED ON THE Bm14 RECOMBINANT ANTIGEN

4.1 INTRODUCTION

In the last decade, the advent of new and state-of-the-art technologies has revolutionised immunodiagnostics, paving the way to a new level of diagnostics approach. Rapid diagnostic tests (RDT) at the point-of-care (POC), in the home, clinic and the field has become the choice because of the many advantages it offers. POC diagnostics integrated with state-of-the-art information technology (IT) has significantly improved the process of fieldwork. Results can be obtained and populated within minutes by a hand-held device connected wirelessly to a central computer located thousands of kilometres away. One of the most commonly used POC diagnostic devices is the lateral flow. Home pregnancy tests are the most common lateral flow RDT in the market, the first RDT to be FDA approved in 1977 for home use ("A History of Pregnancy Testing", 2015).

Today, RDT has become a standard diagnostic tool across many disciplines but its role in the elimination of neglected and tropical disease diagnostics has made a considerable impact. RDTs provide speed in obtaining results, and do not require highly trained technicians, power supply or laboratory equipment and infrastructure like other assays. RDTs are relatively cheap, adaptable to IT data collection applications and are easy to use. The technology has replaced the labour intensive and difficult task of collecting field venous blood for immunodiagnostic testing with an easy, non-invasive fingerprick blood sampling on the spot. RDT is convenient for high-volume field testing and was first introduced to tropical medicine, for use in malaria eradication programmes in the mid-1990s (Frost & Reich, 2009). Specific recommendations of RDT were documented in the *New Perspectives in Malaria Diagnosis* WHO report *(*Malaria, R.B. & WHO, 2000). RDT was recommended as an alternative to the gold standard blood film method that is laborious, requiring laboratory equipment and highly trained microscopists.

For the LF elimination programmes, the role of RDT has become essential after the GPELF multi-centre trial in 2008-2009 (Gass et al., 2012). The multi-centre trial evaluated seven different diagnostic tests including the Bm14 ELISA, for use as tools for checking the resurgence of transmission after mass drug administration (MDA). At the conclusion of the multi-country trials, the RDT (Alere, Binax-NOW formerly ICT Binax) card test for *W.bancrofti* antigen was recommended as the primary diagnostic tool for fieldwork. The Og4C3 antigen ELISA, faring better with accuracy, was to be used for laboratory-based testing.

In 2009 – 2011, a Transmission Assessment Survey (TAS) was conducted across 11 countries to determine a suitable assessment method using RDTs for surveillance (Chu et al., 2013). The Alere ICT BinaxNow card test was used for the filarial antigen of *W.bancrofti* and the BmR1 recombinant antigen-based RDTs for antibody detection used Pan LF for *W.bancrofti* and Brugia Rapid for *Brugia* spp. The challenging TAS programme has identified some issues with the application of RDT in programmatic situations and these *lessons learnt* can be used to make decisions in the future. The study identified the need for complimentary RDT antibody tests based on the available recombinant antigens such as Bm14, Bm33 and Bm123 that can be useful for future TAS programmes.

The great need for diagnostic tools was discussed by leading researchers at the start of GPELF in early 2000s (Lammie et al., 2004; Melrose et al. 2004; Weil & Ramzy 2007). To date, this need has not been satisfactory fulfilled to warrant a call for improved diagnostic tools, as identified by the first TAS. The recombinant antigen Bm14 is important in the development of antibody detection tests, and it has proven to perform well in the Bm14 ELISA format. The Bm14 recombinant antigen (Bm14 rAg) was well characterised and thoroughly evaluated (Chandrashekar et al., 1994,1995) for LF antibody detection. It is specific for both *W.bancrofti* and *Brugia* spp. and useful for elimination programmes where it does not overlap with onchocerciasis.

RDTs are based on the same immunochromatographic principles. A mobile liquid phase containing the target sample analyte and specific binding protein labelled with wine-coloured colloidal particles such as gold nanoparticles, called the conjugate. The solid phase, a porous material, nitrocellulose membrane or polymer allows liquid to move by capillary action. The solid phase is immobilised with the capture protein such as antibody (Ab) or antigen (Ag) referred to as the test line. A liquid phase migrates

through the solid phase for immunoreactions, the specific binding of the target sample analyte at the test line resulting in the appearance of a coloured line. It also includes a second line called the control line that functions to validate each test. The test can be constructed in different formats such as a dipstick, a flow through or lateral flow. The strip may be constructed without a housing, or inside a housing with a sample well for delivering the sample analyte, and a window to view the results. Certain principles and methods of RDT have been successfully patented in the US from late 1980 to 1990s (Appendix 6), the majority currently held by a US company, Alere (formerly Inverness). Although some patents have lapsed in the past few years, others may still pose some restrictions to the commercial application of the technology in Australia.

The development of the Bm14 dipstick assay uses the same principle in a simple and efficient format. A solid phase of nitrocellulose membrane strip is scored with a capture Bm14 Ag line followed by a second control line using a rabbit anti-mouse IgG Antibody. In the mobile phase, monoclonal anti-human IgG4 Ab conjugated to gold nanoparticles at the Fab' domain end binds to form a complex with the Ab present in blood or serum samples. The complex moves upwards or across the nitrocellulose strip by capillary action, towards the other end of the strip. Development of a coloured line on the strip occurs as the conjugate-sample Ab complex binds specifically with the immobilised Bm14 rAg line. If IgG4 LF Ab is not present in the sample, no binding occurs at the Bm14 rAg test line. The control line becomes visible because the conjugated antibody raised in mouse binds specifically to the immobilised anti-mouse IgG control line. A result with one line indicates a valid negative test and a result with two visible lines indicate a valid positive test.

Bm14 recombinant application in an RDT format has never been explored but the successful development of rAg Bm14-based ELISA gives the opportunity to apply the knowledge gained to the development of a useful RDT. The objective of this chapter is to develop a lateral flow dipstick using the Bm14 rAg and produce a functional RDT that can be used in the field and as an alternative to the Bm14 ELISA. Follow-up optimisation and evaluation studies will be required to realise its potential as a useful tool for the elimination programmes as more countries enter the TAS stages of elimination programmes.

4.2 AIMS

The work in this chapter aims to outline the development of a rapid test based on a recombinant antigen Bm14 and to:

1. Describe industry-based scientific methods and steps on how to develop a RDT that can be used as a template for the future development of similar tests.

2. Convert the Bm14 ELISA into a RDT format and ensuring the performance characteristics achieved in the ELISA is consistent.

3. Construct a robust, reliable and high-quality lateral flow dipstick for detecting brugian and bancroftian filariasis using blood or serum samples. The dipstick version is a field-based alternative to Bm14 ELISA, for use in elimination programmes.

4.3 MATERIALS AND METHODS

4.3.1 General Development Methods

4.3.1.1 Two-step Dipstick Method

For evaluation of dipstick material of construction, the Bm14 dipstick used a two-step method where the first reaction occurs by adding the sample and the liquid conjugate in a sample well to allow binding. The second step was to add the prepared membrane strip into the well to allow the bound sample-conjugate to move up the nitrocellulose strip and react with the immobilised antigen line. This method was the quick and most practical method for experimentation because the format allows less variable factors that can affect the evaluation of reagent and its interaction with the materials (Fig 4.1(a)). The two-step method is impractical and awkward because of the need to dispense two liquid components. Double handling of strips makes it difficult to handle in field conditions. Therefore, this format will only be used for development studies.

4.3.1.2 One-step Dipstick Method

The one-step dipstick (Figure 4.1(b)) was assembled on a vinyl card with a sample pad and conjugate pad fixed with the conjugate at one end of the test strip. The nitrocellulose membrane was affixed to the middle of the strip and the adsorbent pad at the other end to wick up the sample from the membrane. The sample was added to the sample pad and the dipstick placed in a plastic well containing the buffer. The dipstick was left for three to five minutes or as soon as a visible test and control line is seen. This dipstick format is ideal for the final product because of its functionality and ease of use for field conditions.

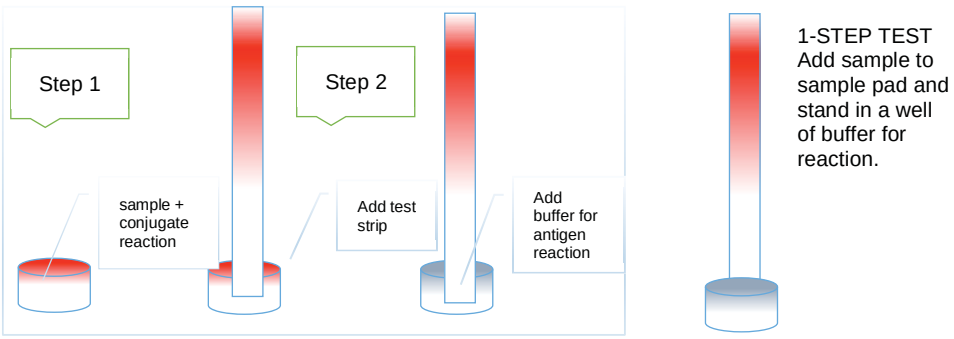


Figure 4.1: Schematic diagram of two dipstick methods used. (a) Two-step dipstick method involves awkward handling of test strips. (b) One-step dipstick method is more convenient with less test strip handling. Sample is added on the pad before standing in a well of buffer.

4.3.1.3 Constructing the Dipstick

Individual test strips were assembled for the purpose of evaluating materials of construction described below in Section 4.3.3, as it conserves antigen than if whole cards were used. Using a very fine watercolour brush, rAg Bm14 was painted on individual strips with a very light single stroke technique. This improvised technique proved to be effective while ensuring that the membrane was not damaged on application.

Figure 4.2 shows a schematic diagram of the different materials that make up a dipstick. To construct the dipstick, a vinyl backing card with an adhesive side is used to hold nitrocellulose membrane and provide structure. This is followed by the application of a conjugate pad, sample pad, blood filter pad (if this is being used), adsorbent pad and the application of overlaminates for protection.

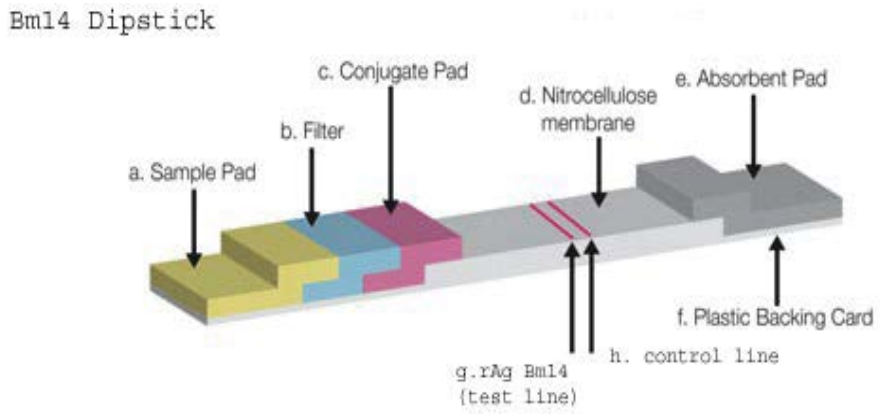


Figure 4.2 The construction of the Bm14 Dipstick format. A schematic illustration of a RDT test strip showing how each component is layered for a lateral flow dipstick. The sample moves from the left to the right of the strip until the test and control lines are visible. In order of assembly: (f) backing card; (d) nitrocellulose membrane; (g) test line; (h) control line; (c) conjugate pad; (b) optional filter pad and; (a) sample pad.

4.3.2 Samples and controls

A reference positive blood sample was prepared by freshly mixing four parts of a non-endemic negative blood (NegDD) with six parts of a highly positive *W.bancrofti* serum sample (Wb-09). This was used as a reference sample due to the lack of a positive blood sample with enough volume to last the course of development.

Coded reference negative samples of blood and serum were collected by venepuncture with verbal consent from the staff at Cellabs Pty Ltd, Sydney, NSW, Australia. All the samples were from individuals born in Australia and never lived

outside of Australia, or born overseas in country non-endemic for LF and without any history of exposure to LF. These samples were confirmed negative for LF using Bm14 ELISA.

A reference panel of 14 matching blood and serum samples from endemic Papua New Guinea (PNG) donated by Dr Gary Weil (Washington University, St Louis, MO, USA) for use with the Bm14 were used as internal validation samples. These samples were also tested using the Bm14 ELISA to determine the approximate antibody concentration that can be used as a reference for test line intensity. Appendix 7 shows a table for these reference samples.

4.3.3 Evaluation of Construction Materials

Materials for use with the dipstick were chosen by experimentation. Commercially available materials from reputable manufacturers such as Whatman, Millipore or Schuell & Schuell (S&S) were procured as samples. These were evaluated within the respective expiry dates or within three years from received date where no expiry date was specified. The experimentations were performed between 2002 and 2005. The following sections describe the experiments performed to determine the most suitable material for a Bm14 dipstick, followed by the results to ensure the flow of information rather than including findings in the results section.

4.3.3.1 Solid Phase

4.3.3.1.1 Nitrocellulose Membrane

The nitrocellulose membrane was chosen as the solid porous medium for the Bm14 dipstick because some degree of robustness is required. Dipsticks were not encased in plastic housing like those in cassette formats because dipsticks require a stronger material compared to paper-based solid media. Performance criteria for a suitable membrane are;

(a) flow rate to allow complete clearing of the sample within a maximum time of five minutes;
(b) defined test and control lines after reaction;

(c) good clearing and no background or shadows on the membrane after the reaction has taken place.

Other considerations were cost, availability, packaging formats and compatibility with the current equipment for rapid tests (refer to Section 4.3.4 for equipment descriptions).

Bm14 rAg test and control lines were applied on the membrane by hand using a very fine watercolour brush. The membrane was air-dried for a minimum of 4 hours at room temperature and assembled for a two-step dipstick method (Fig 4.1a). An adsorbent pad for sample wicking was attached to the top end of the strip. The roughly constructed dipsticks were used to run a positive sample and a negative blood sample to examine the interaction between the membrane and immobilised test and control lines and the sample. Table 4.1 shows a list of nitrocellulose membranes evaluated.

Table 4.1. List of nitrocellulose membranes evaluated.

Name	Catalogue Number	Manufacturer Details
HiFlow HF135 HiFlow HF180 HiFlow HF240 HiFlow Plus	SHF1350405 SHF1800405 SHF2400405 SHF1800405	Millipore Corporation, MA, USA
Pall Predator	Cat No. 80598	Pall Australia, VIC, Australia
Whatman 5um Purabind	Purabind A-FP	Whatman International, Kent, England
Unisart CN140	1UN14AR050025	Sartorius Stedim Australia Pty Ltd, VIC, Australia

Pall Predator was laminated-ready on one side making it a robustly constructed material. However, this was found not suitable due to some bubbling or spreading of antigen when using the striping machine. Unisart membranes performed well with clearing, though, test and control lines appear weaker as compared to the Millipore membranes. The Purabind had a lot of inter-lot variation and these inconsistencies were not acceptable. Millipore membranes were individually tested but the Millipore HF240 with a flow rate at 240 seconds/4cm was chosen as the most suitable for the dipstick. The slow rate allows the binding of antibodies and complexing at the test line. This

characteristic leaves a stronger coloured test and control lines with clearing of the sample across the membrane completed within 3 minutes.

4.3.3.1.2 Plastic Backing Material

Membranes were attached to a plastic backing card using the Biodot 0.01 Matte/Matte polyvinyl (PV) 60mm x 300mm cards (Part No. GL-187 S/0 40537, Biodot, CA, USA) to give the nitrocellulose membrane a solid support. Backing cards have pressure sensitive glue to hold the materials such as membrane, conjugate pad, sample pads and adsorbent pads and can be assembled lengthways in three sections. The top adhesive section for adsorbent pad is back-split 30mm. The middle adhesive section for the membrane is split at 25mm and the sample pad and conjugate pad adhesive section at the bottom is back-split at 25mm (Figure 4.3). Assembled cards were carefully cut to strips of 5mm thickness and labelled at the back to ensure that the membrane can be identified. The backing cards do not contribute to the immunoreaction of the dipstick. Therefore, no backing material evaluation was done. Made-to-order G&L backing cards 80mm x 300mm, 0.10 white matte vinyl laminated on one side with GL-187 acrylic pressure sensitive adhesive with back splits from G&L (Part No. GL41612, G&L Precision Die Cutting, CA, USA) was used for the final stages of the project.

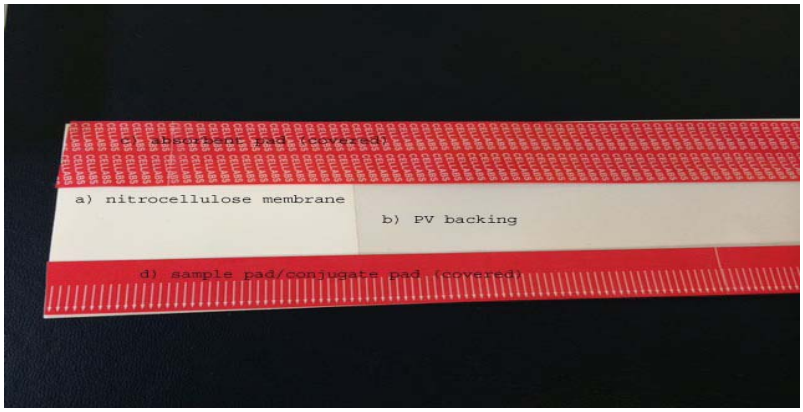


Figure 4.3: **Pre-cut nitrocellulose membrane card.** Card assembled in three sections: **(a)** a sheet of nitrocellulose membrane; **(b)** the PV backing material; **(c)** thin PVC layer to protect adsorbent pad assembly; **(d)** thin PVC layer covering the sample and conjugate layers assembled underneath.

4.3.3.2 Sample Pad

A sample pad (Fig 4.2 (a)) is a material made of filter paper or glass fibre. The main function of the sample pad was to absorb the sample and separate or trap particulate matter or other non-specific components without affecting the sample analyte. Sample pads or sample filters may be used in conjunction with a sample wick such as those of the Cytosep 1662 (Table 4.2) but for reasons of economy, a sample pad that will perform both functions of sample absorption and filtration is the ideal material. Performance criteria for a sample pad involve evaluating the efficient absorbance of the sample through the sample pad while trapping irrelevant proteins and particles, allowing sample analyte through to migrate towards the conjugate pad.

Table 4.2: List of evaluated sample pad for the Bm14 dipstick

	Function	Catalogue No.	Manufacturer
Cytosep 1662	Sample pad Sample filter	1662	Gelman Sciences,
Whatman LF Whatman VF1 Whatman GF/F Whatman GF/AVA Whatman BCF180	Blood separator	F487-14	Whatman International, Kent, England
Whatman Fusion 5	Sample pad Sample filter	Fusion 5	Whatman International, Kent, England
Pall A/D Glass Fibre Pall SP300 Pall 300GF Pall SP300GR	Sample pad	S80347 SP300 S00GF SP300GR	Pall Australia, VIC, Australia

A reference positive blood sample (Ref+veSB) was used to investigate the performance of each sample pad. After adding 30uL of the blood sample on duplicate dipsticks of each sample pad, the dipsticks were left to stand in plastic wells containing 60uL of clearing buffer. After 5 minutes, differences in performance were observed with different sample pads regardless of the

manufacturer. Observations were noted on the amount of blood retained on the sample pad and the ability to release a clean sample analyte through to the membrane, free of red or brown discolouration. Control line reactivity was good for all the sample pads except for the Whatman sample pads that do not exhibit good clearing compared to the Pall SP300GF.

Figure 4.4 shows an image of the differences in material interactions for different sample pads. In a separate test not shown in the image, Fusion 5 pad completely traps the sample, seen as false negative with the Ref+veSB blood sample. Removing the sample pad from the test strip and re-testing resulted in a visible test line. Fusion 5 sample pad is deemed unsuitable for the assay.

Figure 4.4: Sample pad functionality test results. Whatman, Pall and S&S sample pads varies in degrees of clearing efficiencies as observed on the membrane after the wicking of blood samples.

4.3.3.3 Conjugate Pad Evaluation

Conjugate pads are made of paper or glass fibre material to accommodate the impregnation of gold conjugate without affecting the labelled antibody and the release of a gold conjugate with minimal retention. The material properties must be of high quality to enable a controlled release of gold conjugate after the binding and complexing of specific IgG4 Ab to the conjugated detector Ab when

the sample passes through the conjugate pad layer. The location of conjugate pad in relation to the sample pad and membrane construction is clearly shown in the schematic illustration in previous Figure 4.2. The reaction at this part of the test strip happens in a few seconds as the liquid phase is wicked to the nitrocellulose membrane where the capillary action quickly moves the liquid phase towards the test line.

A short list of the different brands of conjugate pads evaluated for the Bm14 dipstick is listed in Table 4.3. The pads were received in square sheets and were carefully cut to 5 x 5 mm squares as conjugate pads to cover the width of the test strip being constructed. Conjugate pads were soaked in gold conjugate at 3uL/pad in a small weigh boat and dried at room temperature overnight. The next day, the dry pads were assembled ready for testing.

Table 4.3: List of conjugate pads gold labelled IgG4 detector Ab.

Name	Catalogue Number	Manufacturer
Millipore GF	GFCP203000	Millipore
Accuwick Ultra Pall Type A/D Glass Fibre	PRO730 S80347	Pall Australia, Lane Cove, NSW, Australia

Accuwick Ultra performed well with single test strips but Pall Type A/D is too thick to release efficiently conjugates without the need for an additional buffer for a complete release to occur. Millipore GF glass fibre proved to be the most suitable material because it allows the sample to interact with the pad before release onto the membrane. A visible test and control line can be seen significantly clearer when compared to the lines from Accuwick glass fibres.

4.3.3.4 Sample Adsorbent Pad

Sample adsorbent pads used for dipsticks are filter papers with good wicking properties. These are commercially available in different thickness, material composition and adsorbent properties (Figure 4.5). The adsorbent pad functions to wick the liquid phase to avoid any backflow on the nitrocellulose membrane. Its location in relation to the membrane in the strip is shown in a schematic illustration in Figure 4.2 above. The speed at which samples are

wicked up from the membrane is a factor to consider when choosing a suitable adsorbent pad for use and thickness is not necessarily a better choice for a test.

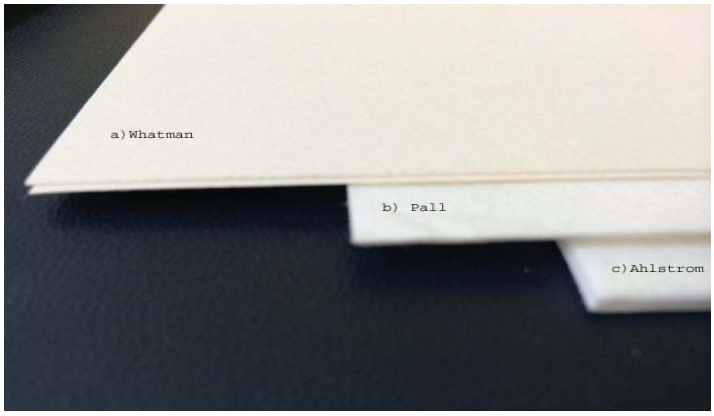


Figure 4.5: Different types of absorbent pad evaluated. (a) Whatman, (b) Pall and (c) Alhstrom with different thickness.

Backflow of the liquid phase can cause background, seen as shadows against the white nitrocellulose membrane or a total backflow of dark brown sample covering the entire membrane. A Backflow effect (Figure 4.6) can be observed if the absorbent pad does not have a good wicking property. The inefficiency of adsorbent pads to wick also contributes to clearing problems resulting in conjugate partially clearing the test lines. Clearing problems affect the visibility of test line if the sample contains a very small, but detectable antibody that produces a weak, positive test line.

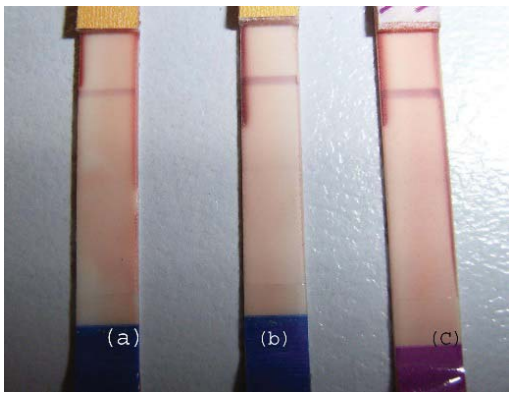


Figure 4.6: Effects of strip cutting. The Backflow effects caused by wicking problems **(a)** irregular white patches due to backflow of sample; **(b)** sample backflow partially covering membrane and; **(c)** backflow covering entire membrane. Strips also exhibit the trapping of liquid phase on the sides of the membrane seen as dark red areas due to the use of a regular guillotine cutter.

To determine the adsorbent pad for use in the dipstick, a range of available adsorbent pads (Table 4.4) were cut to size and carefully assembled at the top end of the cards. After cutting 5mm individual strips from the cards, blood sample Ref+veSB was used to test wicking performance.

Table 4.4: List of adsorbent pad materials evaluated

Name	Catalogue Number	Manufacturer
S&S 903 S&S 470	10535097 10539796	Schuell & Schuell, Whatman Group, Dassel, Germany
Whatman Chr17 Whatman CF7	Standard 17 CF7	Whatman Asia Pacific Pte Ltd., Victoria, Australia
Accuwick Type 133 Accuwick Type 113	S70008 S7 0007	Pall Australia, Lane Cove, NSW, Australia
Ahlstrom 204	Grade 204	Ahlstrom, PA, USA

The S&S absorbent paper has good wicking properties and solid construction but inconsistencies were seen with S&S 407 when wicking different samples of blood. Whatman Ch17 and S&S 903 performed well by helping clear the sample off the membrane with S&S 903 showing superior wicking properties as seen on the amount of sample wicked on the pad (see Figure 4.7).

Figure 4.7: Wicking ability of different absorbent materials. Note the amount of blood sample wicked by the absorbent pads. 20uL of sample was tested with 3 drops of buffer is wicked at different efficiencies.

4.3.3.5 Other materials for RDTs

4.3.3.5.1 Clearing Buffer

A clearing buffer composed of 0.1M Tris based buffer with Triton-X was used to wash up residual reagent and analyte from the sample as the liquid phase travels towards the other end of the strip. Adding a detergent component to the buffer helps to minimise sample component from sticking to materials and causing assay problems. The buffer was packaged in a small dropper bottle for convenience with each drop approximately 20uL when measured using a calibrated pipette. For the sample to efficiently clear the length of the dipstick while ensuring the migration of liquid phase through the test and control lines, six drops of buffer equivalent to 120uL was used.

4.3.3.5.2 Dipstick Accessories

The test uses a sample well for adding samples and buffers before wicking onto the test strip. A sample well made of any non-binding plastic such as 96-well culture plate or a dilution block that can be mounted on a base for stability is suitable (Figure 4.8). Variations can be used by the end-user if the test is being used in the field and a small test tube or similar is required to avoid test strips being blown away by the wind or dropping to the ground.

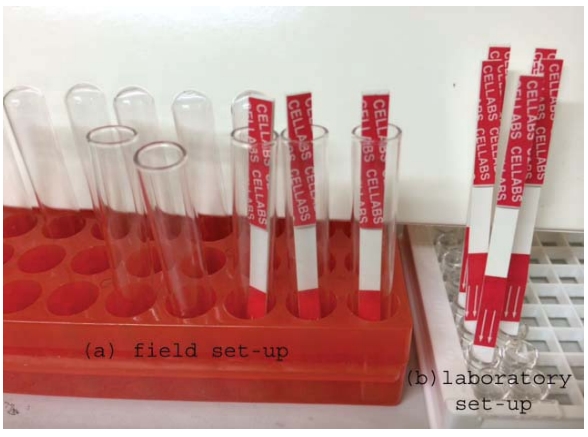


Figure 4.8: Materials for use as a strip holder. (a) mini test tubes provide a stable container for test strips when testing in the field; (b) simple ELISA wells or culture wells can be used to hold test strips on the laboratory bench.

To protect the internal construction of the dipstick, a thin strip of plastic sheet overlaminate was required at both the sample end and the top adsorbent end of the strips. A made-to-order 0.002mm white polyester with acrylic pressure sensitive adhesive and release liner with 0.001mm clear overlaminate film (Cat.no. GL-41610, G&L Precision Die Cutting Inc., CA, USA), was used. These were cut to the desired measurements and applied on the constructed cards before cutting to individual strips.

4.3.4 Rapid Diagnostics Machinery

4.3.4.1 Reagent dispensing machinery

Test and control lines needed to be defined, straight and lightly applied to ensure reagent concentration was consistent while avoiding contact with the membrane. To achieve this, the dispensing machine (also called striping machine) uses pressurised air to apply reagent lines without damaging or disturbing the integrity of the delicate membrane surface (Figure 4.9). The dispensing machine used (Isoflow Reagent Dispensing System, Version 1.54, Imagene Technology Inc., NH, USA) was a compact machine at 36cm width x 66cm length footprint requiring minimum space for operation. A simple handheld device connected to the machine controls the striping movement during a small tank with pressurised air, automatically utilised during striping. Figure 4.9 shows the dispensing machine used for the development and scale-up manufacture of test strips.

The machine parameters for speed and flow pressure operation was completely dependent on the amount of reagent applied to a standard 60 x 30cm card factoring in the speed, flow and air pressure of a single striping movement. Parameters were determined by experimentation (Section 4.3.6).

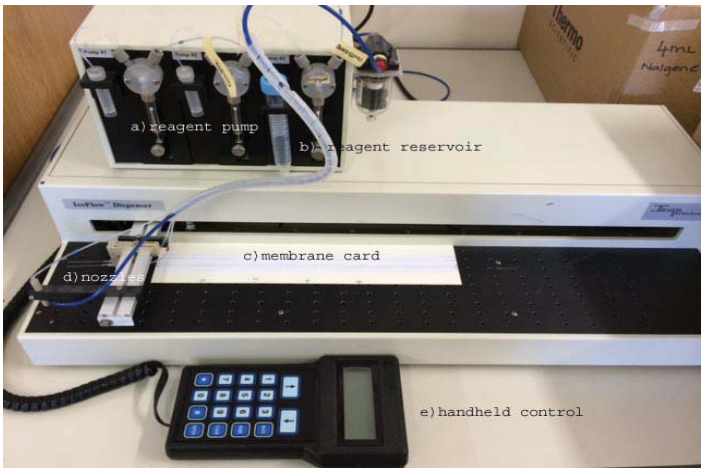


Figure 4.9: An image of the Imagine striping machine. Automatic application of rAg and control lines on nitrocellulose membrane is consistent and controlled using a dispensing machine.

4.3.4.2 Guillotine machine for strip cutting

A strip cutter machine is important machinery required in the production and development of a RDT because of the ease and accuracy in cutting through the membrane. The early evaluations used a manual paper cutter as these need to be used in small amounts to conserve reagents. Regular paper cutters do not have the precision and the correct blade pressure, thickness and speed required to cut through nitrocellulose membrane without damaging the sides. Damaged strips can cause the capillary action to move to one side only or causing the appearance of bubbles in the membrane. A previous image (Figure 4.6) shows problems with membranes strips cut using a general guillotine cutter. The liquid phase is trapped at the blunt cut edges of the membrane strip, seen as dark areas on both sides of the strip. This imperfection affects the liquid phase from flowing evenly on the membrane surface as it flows and accumulates at the edges, preventing proper wicking and clearing of the strip.

A strip cutting machine (Index Guillotine Cutter, Model I, A-Point Technologies, NJ, USA) was used to produce accurate card cuts between 3, 4 and 5 mm strip widths. For maximum rigidity, a 5mm strip was chosen as the standard width for

use. The machine has a small 31cm width x 46cm length footprint, a simple computerised control without the need for computer and output suitable for low to medium throughput.

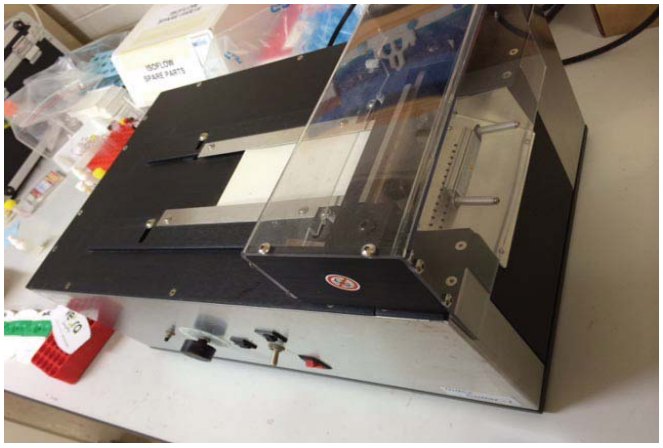


Figure 4.10: A strip-cutting machine. Individual test strips are cut efficiently to accurate sizes (+/-0.15) width using an automated cutting machine with optional cutting speeds.

4.3.5 Immunoreagent for Dipstick

4.3.5.1 IgG4 Antibody gold labelling

Anti-IgG4 Ab HP6023 used as the labelled detector antibody in the Bm14 ELISA (Chapter 3) was used for gold conjugation in this section. A colloidal gold (Au) nanoparticle of approximately 40nm Au sol was used to label the detector Ab. Au conjugate initially used for early evaluations for construction materials was custom made (Alchemy, Ireland) by sending a small coded volume of HP6023 purified antibody for gold conjugation. The Au conjugate functions well in pilot tests but due to the cost of offshore custom production, a direct gold labelling method was developed in the laboratory. Colloidal gold sol production and conjugation for use in the dipstick reagent optimisation was used, based on published methods described and as adapted by Oliver (1999). The method was available laboratory equipment to produce 40nm gold sol conjugated to the HP6023 antibody.

4.3.5.2 Control Line

The control test line used a Rabbit anti-mouse IgG (Silenus, Victoria, Australia). The concentration for coating was determined by striping a starting concentration of 2uL/mL on a single strip and reducing the concentration two-fold to determine the concentration that will give a line colour intensity comparable to the test line. This process was done for each batch to ensure the line intensity matched that of the test line when a reference sample was tested. The purpose of a control line was to indicate the validity of a test.

4.3.6 Preparing the Dipsticks for Functionality Tests

4.3.6.1 Antigen Concentration

A crude method of immobilising the rAg using a very fine watercolour paintbrush was initially used as the most practical way of striping low numbers of individual strips while conserving the rAg during development. Scale-up production of dipsticks required the optimal rAg Bm14 concentration immobilised on 80mm x 300mm cards using the dispensing machine. To determine the optimal rAg for striping, half cards with 3uL/strip conjugate were used to apply 4mg/mL, 3mg/mL, 2mg/mL and 1mg/mL. 20uL of blood was used, followed by six drops of buffer.

The resulting strips for the antigen concentration tests show no distinct visible test lines on 1mg/mL and 2mg/mL. A slightly visible test line on 3mg/mL was seen, and a good solid visible test line appears comparable to the control line (4mg/mL concentration). At this concentration, it is estimated that 2ug of antigen is used per 5mm strip (40uL/100mm card = 0.4uL/1.0mm therefore 2uL of antigen per 5mm test strip).

To dispense antigen at the required concentration, the dispensing machine parameters were adjusted to a dispense rate of 0.1 and aspirate rate of 10. The standard syringe size 100, inlet volume 40 and outlet volume of 60 were used.

4.3.6.2 Conjugate concentration

Three sets of 5mm test strips with 6.4ug/strip antigen lines were constructed by using a 5mm x 5mm conjugate pad soaked with 1uL/ strip 2uL/strip and 3uL/strip of the gold conjugate. The test strips were performed using 20uL volume blood sample per test strip followed by six drops of buffer.

The resulting evaluation shows the test line from 1uL/strips as a very faint pink line. Both 2uL/strip and 3uL/strips were comparable indicating that the increased conjugate volume per strip does not improve the line intensity. The optimal volume of conjugate per strip is therefore determined at 2uL per test strip (0.4uL/mm). Parameters for the striping machine were therefore adjusted to a dispensing rate of 0.8 and aspirate rate of 8 using the standard syringe size of 100, with tube inlet volume of 40 and outlet volume of 60.

Machine aspiration action caused spluttering of tiny aerosols of conjugate onto the membrane that resulted in purple spots that interfered with the test. Determining a parameter for conjugate application on a strip of conjugate pad required working out a balanced aspiration rate and dispense speed to apply accurately the conjugate concentration.

4.3.6.3 Sample and Buffer Volume

To determine the minimum sample volume that can be used to get a clear and solid visual reading from the test line, 5uL, 10uL and 15uL volumes of blood and 5uL, 10uL serum sample was used. The higher amount of antibody was found in serum as compared to blood. There was no need to look at higher volumes for samples using the serum. Test strips with a combination of 6.4ug/strip antigen, 2uL/strip conjugate and a 7ug/uL anti-human IgG control line were used. Sample MP1 with matching blood (ELISA OD 1.0) and serum samples (ELISA OD 1.3) was tested.

Blood sample results on the dipstick showed no difference with the intensities of the test line. This indicates a minimum of 5uL blood (approximately equivalent to a small drop of blood from a finger prick) can be used to get a visible signal on the constructed dipstick. Figure 4. 11 below shows the test line does not

show any significant difference in the intensity of test line using different volumes of samples. This indicates that test line intensity is dependent on the optimisation of dipstick characteristics. Immunological reactions of labelled antibody, sample analyte and antigen needs to reach the maximum binding that will give a clear visible result on the membrane.

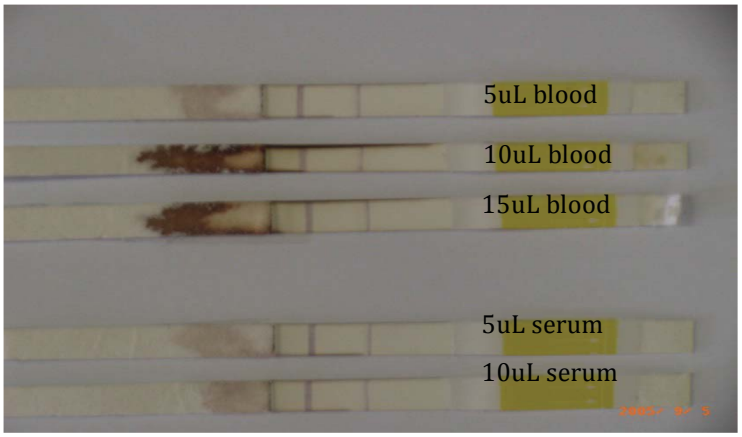


Figure 4.11: Sample volume evaluation. The image shows how the visual intensity of test line is not affected by sample volume less than 5uL of blood or serum.

Although the minimum sample required as observed in the experiment is 5uL of blood or serum, the amount of blood for use in the test is determined at 20uL based on simulations of finger-prick blood. Figure 4.12 shows the size of 20uL blood from a finger-prick sample. Accurate volumes of finger-prick blood cannot be guaranteed in field situations and realistically, will not be measured by a pipette before delivering sample on the sample pad. Using a larger volume of sample will ensure that the dipstick is constructed to cope with the maximum sample without causing any assay problems.

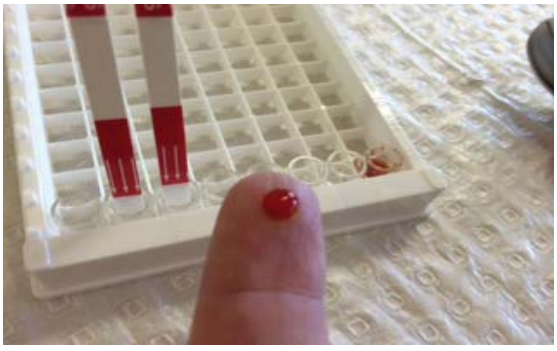


Figure 4.12: Size of a 20uL fingerpick blood sample. An overflow of blood does not cause any signal interference on the test line of the Bm14 dipstick

4.4 RESULTS

4.4.1 Constructing the one-step dipstick

The final construction of the dipstick uses a G&L 80mm x 300mm vinyl backing card (Section 4.3.3.1.2). This is assembled by first attaching a 25mm x 300mm Millipore HF240 nitrocellulose membrane on the middle adhesive section of the card. The 4mg/mL rAg Bm14 test line was dispensed at a pre-determined reagent dispenser parameter (Section 4.3.6.1), and dried for a minimum of 4 hours at room temperature in a controlled and enclosed room. The bottom section of the card was assembled with a 5mm x 800mm conjugate pad, overlapping by 2mm to ensure that contact is made with the membrane surface (Figure 4.13). A 40nm gold conjugated anti-IgG4 MAb HP6023 was sprayed on the surface of the conjugate pad strip using a pre-determined parameter for the dispensing machine (Section 4.3.6.2). The card was dried overnight at room temperature in a controlled and enclosed room. The next day, a 5mm x 300mm strip of sample pad was assembled by layering on top of the conjugate pad with a 2mm overlap to ensure contact between the sample pad and the conjugate pad. The top section of the card was assembled using a 30mm x 800mm Whatman Chr17 filter paper as the adsorbent pad before overlaminates were applied to both top and bottom sections to complete the card construction. The card was cut into 5mm thick dipsticks using the Guillotine cutting machine.

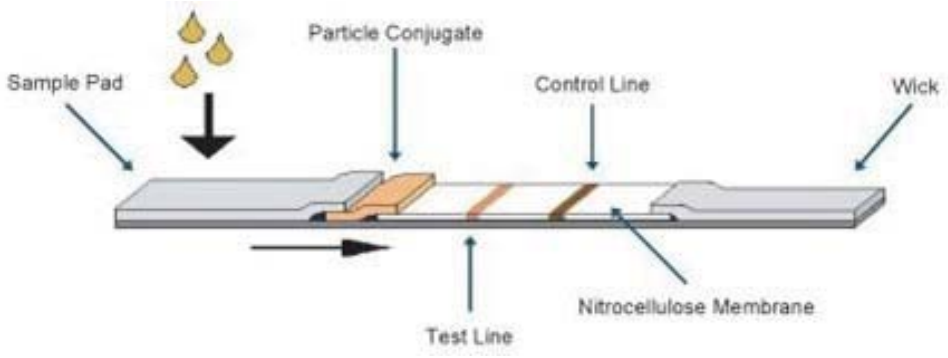
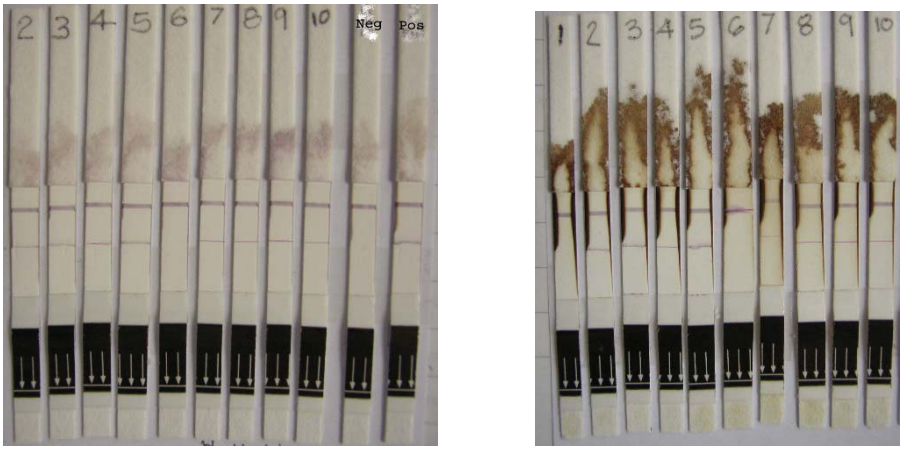


Figure 4.13: A schematic diagram of the assembled materials. The overlap ensures the migration of the liquid phase through the test strip from left to right.

4.4.2 Prototype Functionality Test – Proof of Concept

The panel of reference blood and matching serum samples (DSS series) of known antibody reactivity based on the Bm14 ELISA were tested using the prototype Bm14 dipsticks. Serum samples DSS2 to DSS10 (Figure 4.14a) were positive except for DSS6 found negative by the Bm14 dipstick. These results were confirmed by the ELISA OD values, a 100% concordance. The matching blood samples DSS1 to DSS10 (Figure 4.14b) were all positive by Bm14 dipstick except for DSS6 (ELISA OD 0.198) found negative by the Bm14 dipstick. The results were confirmed by the ELISA OD values, a 100% concordance. This functionality test for the Bm14 dipstick is satisfactory though further evaluations on a larger sample in the field or laboratory in multi-centre trials are required. Further adjustments and improvement on antibody detection limits and define test performance can be applied once evaluation feedback is received.



(a) **(b)**

Figure 4.14: Functionality test. (a) 10uL positive serum samples were used in each strip with 8 out of 9 positive samples detected, a concordance of 100% with ELISA. **(b)** 20uL of positive blood samples were used in each strip with 8 out of 10 detected, a concordance of 100% with ELISA. Controls were not done for blood.

4.5 DISCUSSION

All methods and procedures made use of the widely available literature and followed crude techniques in an effort to conserve valuable reagents and make use of available laboratory equipment and infrastructure. Production of RDT is carried out in controlled humidity environment to ensure that the nitrocellulose membrane is not affected by variable processing conditions. There was a lack of a humidity-controlled room for the project however care was taken to keep a constant temperature between 18^{o}C to 22^{o}C in an enclosed room especially used for RDT work. Future follow-up projects may need to look at improving RDT assembly by providing a humidity-controlled room. The final materials used to produce the prototype dipsticks came from a batch of supplier samples provided for the purpose of evaluation. Other plastic components such as backing cards and overlaminates were made-to-order as the supplier samples were not functional for card assembly. Future batches of dipsticks for multi-centre evaluations will require new materials with long expiry dates, longer than the expected 24 months for the assembled dipsticks. Although the 24-month stability has not been formally studied in this chapter, it has been observed that the prototype dipsticks assembled in 2005 were still reactive when tested four years later.

One of the constraints of this project was the lack of a local supply for RDT materials. There are only a few suppliers of RDT material in Australia because of a few RDT manufacturers. The majority of the RDT sold in Australia are imported from the US, UK, China or India. The reagent dispensing machine or striping machine and automatic guillotine cutter were ordered from specialised RDT equipment manufacturers in the US. RDT materials of construction had to be imported from the US and UK, which takes an average of 4 weeks. This scenario adds to the cost of materials and the ready supply. Therefore, some considerations were made when choosing materials. For example, evaluations for adsorbent pad in Section 4.3.3.4 observed S&S903 and Whatman Char17 performing well but because Schuell & Schuell do not have an Australian branch, Whatman Char17 was chosen. Although RDT manufacturing has been around for many years, it is a technology not commonly found in Australian biotechnology companies. With the low-cost and moderately uncomplicated equipment needed for a medium scale-up as demonstrated in this chapter, there should be more local RDT manufacturing.

Sample positive reference Pos+veSB was a normal blood from sample NegDD, of non-endemic origin spiked with 40% positive bancroftian serum Wb-09. This was done because of the lack of the positive blood sample with enough volume that can be used as a reference for the entire development project. Preparing the sample this way ensures a consistent antibody level that can also be compared to the equivalent antibody level in serum, taking note that, there is approximately 40% of serum in the blood. The final protocol determined the use of a 20uL blood sample as a conservative volume. Therefore, the Ref+veSB is equivalent to 10uL of serum per test, a 40/60 ratio of serum and blood. Because the rAg concentration has been set to a higher end of the optimal concentration, it ensures that smaller sized fingerprick samples, less than the determined 10uL optimal sample volume, can still be detected if positive, the results in Section 4.3.6.3 showing that approximately 5uL of blood does not affect the line signal as compared to using a 15uL blood sample. In the final protocol, 20uL of the sample was used for good measure to allow for material inconsistencies that may not have been observed during the development. In an optimised assay, the desirable volume would be 10uL of fingerprick blood. Optimisation of dipstick parameters was still required to validate the current prototype protocol. Not included in the optimisation of sample volume study is the detailed determination of the minimum volume of blood sample required to detect the weak positive samples that react around OD 0.2 – OD0.5 by ELISA. All the samples tested in the functionality test in Section 4.4.3 were reacting more than OD1.0 so a new study will require a larger panel of samples with matching blood and serum. This study will give a better idea of the antibody detection limit for the dipstick, using the ELISA OD value results as a reference in determination. A characterised RDT assay is beneficial especially for the surveillance or TAS studies because the approximate antibody detection limit is known.

The dipstick format was chosen although it would be most ideal to develop a lateral flow cassette form that solves the handling inconveniences of a dipstick. The reason for this is due to the patents that restrict the commercialisation of RDT lateral flow cassette formats in Australia. Some of the RDT patents cover different aspects of the immunochromatographic assay, shown in Appendix 6 as of August 2014.

Expanding the number of samples in realistic testing conditions such as multi-laboratory studies, field, POC or clinic settings will help make improvements to the current Bm14 RDT through feedback and performance findings. The performance of

prototype dipstick as observed in the results Section 4.4.3 works very well as seen in the functionality tests that serve as proof of principle for follow-up prototype evaluations. Further optimisation studies and new adjustments to the dipstick construction parameters will be required after prototype evaluations. Other studies that need to be completed for the RDT are cross-reactivity, sensitivity, specificity and stability, which are all integral assay performance characteristics.

CHAPTER 5

QUALITY ASSURANCE AND REGULATORY REQUIREMENTS OF A DIAGNOSTIC TEST: BRINGING A RESEARCH PRODUCT TO MARKET

5.1 INTRODUCTION

The tests used to analyse medical samples such as blood, serum, tissues or urine of human origin are classified as an *in-vitro diagnostic* medical device (IVD). The term IVD is used for any medical device or part of a medical device such as individual reagent, control material or instrument that is used together in a kit (Mannonen & Riikonen, 2006). IVDs have a specific intended purpose that needs to be regulated for quality and ensure the performance meets the intended purpose.

In Australia, any diagnostic test that is marketed must be legally registered with the Australian Therapeutics Goods Administration (TGA), an entity that regulates IVDs under the Department of Health. TGA registration of IVDs enlists each diagnostic test in an Australian Register of Therapeutic Goods (ARTG). This database contains information about the medical device or medicine, including formulations and manufacturer details. The purpose of regulation is to ensure that IVDs manufactured and distributed for use in Australia are of the highest quality and safety standards to protect public health or the health of individuals.

IVDs can be used in a laboratory or clinical setting, point of care (POC) or the home (TGA, 2010). Rapid diagnostic tests (RDTs), polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) used in the LF elimination programmes are examples of IVD. The definition of an IVD is detailed in its scope and excludes other classes of diagnostic tests not within the TGA definition. IVD classification needs to be confirmed before commencing development or import to determine the viability of developing a test for commercial purposes, due to the high cost of registration of IVDs in Australia.

Prior to 2014, the Australian regulations for IVDs did not include laboratory-developed tests (LDTs) used for routine clinical laboratories and research only diagnostic tests such as the Tropbio Og4C3 Antigen ELISA. These tests were not optimised or standardised for a specific purpose and were widely used without any evidence of product performance verification and validation. Without optimisation, standardisation and validation of product performance, the quality and reliability of a test is not quality assured. The implications of a research-grade diagnostic test for use in elimination programmes can sometimes be overlooked. Although these provide a viable option for disease elimination because of the low cost for large-scale testing, the usfulness of a standardised and regulated test is far more practical (Bergquist, Johansen & Utzinger, 2009). Parameters of a research-grade kit that are not fully qualified and validated have the potential to fail, making them unreliable and often can mislead critical programmatic decision making. A quality assured and validated diagnostic test is the key to a useful and reliable tool for any application. It is, therefore, crucial to develop diagnostic kits that are useful for the purpose of elimination programmes and make these commercially available.

TGA has recently implemented regulatory reforms in 2010 based on the globally harmonised regulations. The new regulation does not take into account the end-user application because anything that is classified IVD must be regulated therefore, “Research Use Only” products are no longer excluded as they have been prior to 2010. The new regulations were fully implemented with a three-year transition period that saw full implementation by July 2014. The revised Regulations for IVD 2002 (TGA, 2012) retained some elements of the Therapeutic Goods Act 1989 (referred to from here onward as the “Act”). The Regulations were specifically for IVD while adopting components that have been modelled and harmonised with the Global Harmonisation Task Force (GHTF) on medical devices as a way forward in regulatory harmonisation and convergence. GHTF was a voluntary group formed in 1992 with five member countries, European Union, United States, Canada, Australia and Japan. GHTF adjourned in 2012 and was succeeded by the International Medical Device Regulators Forum (IMDR) whose volunteer members are representatives from global regulating agencies. The main aim was to harmonise regulatory processes of member countries and simplify processes. This means that if a common IVD framework was to be used, regulatory certifications of IVDs are acceptable among the member countries as evidence of compliance with the harmonised regulation. Australia was the first to

implement the new global IVD regulatory format as recommended by the GHTF/IMDR, and the rest of the member states are expected to follow.

The harmonised regulatory framework is expected to improve the availability of IVDs for both export and import. However, the new harmonised framework means tougher regulatory processes and cost. Small scale or start-up Australian manufacturers may be forced out of business due to the added cost of managing regulatory affairs. Tests that are not covered in the old regulation such as laboratory developed tests (LDT) for clinical laboratories and distribution companies selling IVDs manufactured outside Australia are now subject to the new regulation. Users of LDTs are now required to undergo Conformity Assessments adding to the cost of running a clinical laboratory (Favoloro et al., 2011a, 2011b).

Regulatory affairs have since become a large part of diagnostic test development and manufacture and are a growing sector in its own. It is a challenging time for the life sciences because of the demanding regulatory requirements that face the industry. First, there is an exhaustive amount of paperwork and legislation to comply with and second, there is a significant amount of ongoing compliance paperwork for as long as a product is produced and supplied. Universities in the US have since offered degrees and postgraduate studies in Regulatory Science and recently, Australian universities have followed suit, due to the demand and need to create these new graduate programs to deal with the specific regulatory issues in the sciences.

Regulatory compliance can be a complicated and very confusing undertaking and non-compliance to the requirements of the legislation can potentially pose roadblocks to product commercialisation. It can delay or prevent access to these important tools for scientific research and diagnostic laboratories. Development of novel diagnostic tools can take many years from conception to realisation therefore it is logical to include regulatory considerations when designing and developing a diagnostic test. It is therefore important for the scientists who develop some of the novel and useful IVDs to have some understanding of the basic IVD regulatory requirements. Applied in the planning and development of a product, these essential principles of safety and quality are imperative to the successful commercialisation process of a diagnostic tool.

This chapter encompasses the requirements and procedures for registering a diagnostic test with the TGA. These are methods and principles that are applied to the

registration of the Bm14 IgG4 Antibody CELISA test for Lymphatic Filariasis. It provides the basic quality assurance and regulatory requirements for consideration when developing a diagnostic test for the researcher. The information provided in this chapter was gathered from industry know-how and experience. The application of a quality system required of an IVD for inclusion in the ARTG and regulatory process for a commercial-ready Bm14 Antibody CELISA can be used as a template.

For detailed information on the TGA regulations and specific requirements, refer to the Therapeutic Goods (Medical Devices) Regulations 2002 (TGA, 2012) and the Australian Regulatory Guidance for Medical Devices (ARGMD) documents (TGA, 2011b).

5.2 AIMS

The aim of this chapter is to demonstrate the process of commercialising a research and development (R&D) product by conforming to local IVD legislation and in particular:

1. To provide important considerations and guidance on the basic regulatory requirements for scientific developers of IVD in Australia;

2. To demonstrate how a quality management system (QMS) was prepared for the Bm14 Filariasis Antibody ELISA. How the IVD legislative requirements are met for a commercial-ready product is covered and;

3. To provide a template or guidance on how to bring forward a completed R&D IVD product to market that meets requirements of local legislation and allow it to be marketed in Australia and overseas without restrictions.

5.3 METHODS

5.3.1 Compliance with the Regulations

The Regulations 2002 was an amended document made under the Act for medical devices that take into consideration the recommendations from the GHTF for a globally harmonised medical device regulatory framework. The Regulations and

Chapter four of the Act cover all the elements required of the Bm14 ELISA IVD for inclusion in the ARTG. The following sections discuss how the Bm14 ELISA QMS process incorporates these key elements of the Regulations into its design and manufacturing processes.

5.3.1.1 Essential Principles

Compliance to standards of quality and safety for the manufacture of quality IVD medical devices was demonstrated by complying with the 15 TGA Essential Principles (TGA, 2014b). The first six were general principles applicable to IVD medical devices. It mainly focuses on quality and safety to ensure that the use of the device will not compromise public health or the health of an individual (TGA, 2012). The other nine Essential Principles focus on principles applicable to a specific kind of device, and its design and construction. Therefore, some of these principles do not apply to the Bm14 ELISA.

To provide evidence of conformance to the Essential Principles, a "Medical Devices Principles Checklist" was used as a guide in gathering all the information in one document summary. This checklist provides a short description of the principles. A column was included where the device manufacturer provides information as to the procedures used. The standards are used (international standards ISO or Australian and New Zealand standards) and other normative documents applied to conform to each set of the principle that applies, were also listed. Acceptable standards to be applied were dictated by TGA and can be found in a set of documents called the Standards Order (TGA, 2014a). The Essential Principles checklist simplifies the task of gathering information about the device. This was mainly designed to assist during the Conformity Assessment (Section 5.2.3) of a new IVD for inclusion in the ARTG (Section 5.2.4). The Bm14 ELISA design, development, manufacturing processes and the quality management system integrates the Essential Principles demonstrated using the TGA Essential Principles Checklist (TGA, 2014b).

5.3.1.2 Conformity Assessment

Conformity Assessment (CA) is the process of determining whether an IVD and its manufacturing process conform to the quality and safety required by the therapeutic goods legislation. There were some CA processes applicable to different classes of IVD depending on the kind and the purpose of the IVD. The assessment was made by the TGA for Australian manufacturers who produce IVDs under Class 2, Class 3, Class 4 and Class 4 In-house (refer to Section 5.2.3 for classification rules). CA certification from other appropriate regulatory bodies (Notified Body) may be accepted by TGA as evidence of conformity. This is a commonly used route to compliance for IVDs manufactured outside Australia that fall under the Australian IVD classes for CA. Assessments were based on a risk level, therefore Class 1 IVD intended for exports only, does not undergo CA assessment, however, a Technical File (refer to Section 5.3.1.3) needs to be maintained and made accessible by the IVD manufacturer.

For the Bm14 ELISA, an application for CA was made under Class 2 “Part 1 Full Quality Assurance Procedures” including the design, production, packaging, labelling and quality control. To demonstrate that the Bm14 ELISA meets all the requirements for a CA as claimed, a Technical File was prepared. This compiled file may be used as evidence of conformity and presented to the assessors during audits.

5.3.1.3 The Technical File

A Technical File is a compilation of documents that outline the evidence of conformity to the required legislation, a requirement for registering low-risk IVDs under Class 1 and CA applications for medium to high-risk IVDs. The index of the Technical File documentation provided to the TGA for Bm14 CA application is shown in Appendix 8. All products registered in the ARTG require a Technical File to ensure that persons who are not familiar with the product and QMS, can easily navigate through the steps taken to conform with regulations during an assessment.

The Technical File held the bulk of QMS details and information. Supporting documents and regulatory overview were used as the first reference point for QMS inspections. In cases where an adverse event has occurred relating to the IVD, the technical file was submitted to TGA for CA assessment.

5.3.1.4 Classification Rules of IVD

The TGA regulatory framework has risk-based classification rules that were mainly used for identifying IVDs for listing or certification. The purpose of classification into different categories was to determine the level of risk the device poses to public health. In IVD terms, this risk is the probability of a device giving the incorrect result that can affect the diagnosis, treatment or health management of an individual. It also takes into account adverse events that may inadvertently affect the health of the general public (TGA, 2011a).

There are four classes of IVD that were categorised based on the potential risk to public health with Class 1 IVD being the least and Class 4 IVD having the highest risk. The classification rules appear in TGA (2012). The Bm14 ELISA was categorised under Rule 1.3, Class 3 IVD, for the detection of agents posing a moderate public health risk or high personal risk. Although the category was a Class 3 IVD, a higher level of classification can take precedence over this category. The filarial tests were classified under Class 2 devices (Section 5.3.1.5) of The Global Medical Device Nomenclature (GMDN) which was used by the TGA as a way of harmonising with global regulatory bodies. The method of determining the GMDN code for filariasis assays appear below in Section 5.3.1.5.

5.3.1.5 Global IVD Nomenclature Code for Filariasis

As part of the global harmonisation of regulatory processes, GMDN codes were used for the purpose of identifying devices for the global regulatory process. This can be useful for a test intended for use around the globe. Terminologies used in the coding system are explained in detail in the international standard ISO15225 Medical Devices - Quality Management - Medical Devices Nomenclature data structure (ISO, 2010). The codes were used in Australia to identify a type of registered device in the ARTG or to describe collectively a "kind of device". This means that all "kinds of the device" can undergo one assessment, under one registration number thus, simplifying the process of registration. To simplify this concept, we take “filariasis” as an example of a “kind of device” classified under a collective term which can be used for all

kinds of test such as the Bm14 Antibody CELISA, Bm14 dipsitick RDT or Og4C3 Filariasis Antigen ELISA.

The first step in determining a collective term is to identify the “kind of device” for the test. Level 1 collective term for the filariasis tests can be categorised under CT701 Infectious Disease IVDs, however because these tests were intended to be used in Australia, a higher category was required. “Export only” IVDs are classified under the L1 collective term. Level 2 collective term for the filariasis tests fall under L2 CT356 Parasitic Infectious Diseases IVDs as the preferred term. The coding was arranged in a hierarchical structure starting from the first four letters that stands for general terms, followed by three numbers of more specific classification. Bm14 under Level 2 Collective Term was classified based on IVDs of “the same kind” with characteristics typical of those in Clinical Chemistry and Infectious Diseases. The advantage of using the collective term means that a manufacturer can register the filariasis tests under one collective term and have one assessment and pay one fee.

Determining the correct collective term using the GMDN can be confusing due to the assay falling under different categories as “the same kind” and does not have the same purpose as the European Diagnostic Manufacturers Association (EDMA) Global In Vitro Diagnostic (GIVD). This is also commonly used in the greater European area to identify IVDs. The EDMA classification of the Bm14 filariasis assays is 15 05 10 90 (EDMA, 2005).

5.3.1.6 Design and Development Elements

In-built elements of design and development for Bm14 ELISA are a requirement inspected during TGA on-site audits to ensure conformity to IVD legislation on Design and Development. To demonstrate these elements, a file folder was prepared for the important aspects of product development for the Bm14 ELISA from customer requirements to valid scientific methods, raw materials, concepts and the outputs. Appendix 9 outlines the Bm14 ELISA Design, Development and Commercialisation file folder.

5.3.1.7 Inclusion in the ARTG

All IVDs manufactured in Australia can only be legally supplied if they were registered in the ARTG. The inclusion of IVD in the ARTG can only be made by a sponsor, an Australian manufacturer or a distributor of imported IVDs for supply in Australia. Depending on the Class of IVD, the sponsor makes the application with TGA through an appropriate conformity route, a CA (Section 5.3.1.2) or a Technical File Review (Section 5.3.1.3). Once the Bm14 ELISA had gone through the process of CA and successfully approved by TGA, it was added in the ARTG under a “same kind” system (L2 CT356). The IVD manufacturer needs to maintain some responsibilities that included the management, implementation and monitoring of the QMS, post-marketing surveillance, and other regulatory responsibilities. These were checked periodically by TGA through a site inspection or audit, a process taking an average of two days, every 12 -24 months for as long as the IVD was listed on the ARTG. At the time of writing, the Bm14 Filariasis Antibody ELISA had passed the CA for inclusion in the ARTG and listed in 2014.

5.3.2 Quality Systems of IVD Commercialisation

5.3.2.1 Good Manufacturing Practices (GMP)

The term GMP is used by manufacturers internationally to refer to a model for a quality management system that complies with standards and local legislation. GMP ensures a safe and high-quality IVD and incorporates a set of principles in the development and manufacture of IVD.

In Australia, TGA uses the term QMS, superceding the term GMP to describe IVD compliance to regulations since the introduction of the new TGA Framework of 2014 which was applied to the Bm14 ELISA. QMS was a specific GMP model appropriate for the manufacture of IVD. It was argued that TGA assesses the quality of IVD manufacture based on the implementation of the Quality Management System (QMS). Assessments are also based on the compliance with IVD Regulations and ISO13485, therefore, these should be referenced instead of GMP. Despite this, the term GMP is still used throughout TGA documentation to describe quality standards in manufacturing.

5.3.2.2 IVD Manufacturing Standard ISO13485

The International Standard for an In-vitro Medical Device (ISO13485) is a universal set of instructions and guidelines that outline the requirements of setting up a Quality Management System. The Standard provided information on the acceptable processes for the Bm14 Antibody CELISA manufacture. It included the flow and traceability of all information from raw material to the finished product, required documentation, writing methods, procedures and the required evidence of safety and quality when kit batches were produced.

TGA used the Standard and other normative documents to assess the Bm14 ELISA application for CA and issued an ISO certificate to confirm the assessment using ISO13485. The certification became integral as the evidence of quality assurance for each batch produced. The Bm14 ELISA was designed for use mainly for the elimination programmes for LF therefore it was important to have the development and manufacturing processes validated and certified by TGA and CE Marking using an international manufacturing standard.

5.3.2.3 A Quality Management System

QMS is a set of processes that give structure and direction to achieving a quality policy and objectives in terms of manufacturing diagnostic kits. The QMS was structured to ensure traceability and accountability of all materials, methods and processes for every batch of Bm14 ELISA produced.

To set-up the QMS for the Bm14 manufacturing process, a QMS manual (Appendix 10) was prepared by using the sections of the ISO13485 (Appendix 11) as a template. By using the standard sections as a template, all manufacturing processes, quality and safety requirements and the quality policies and objectives can be addressed by following the guidelines. Once implemented, the QMS was consequently used as evidence for a manufacturing process that follows quality and safety as required by the standard. Audits and assessments for quality manufacturing of the Bm14 ELISA by the TGA used ISO13485 to verify that the processes consistently follow methods under the QMS. The QMS was used as evidence of compliance for

the European Union (EU) In vitro diagnostic medical device EC 98/79/EC (CE Marking). The Bm14 ELISA was included in a CE Marking certification, required by global partners who will use the kit for epidemiological or clinical purposes within the EU countries and states. This process was beyond the scope of this chapter and not covered but mentioned for additional information.

5.3.4 Important Requirements of the New IVD Framework

5.3.4.1 A Vigilance System

In addition to the Regulation and ISO13485 compliance, IVDs listed in the ARTG were required to implement a Vigilance System. A Vigilance System includes adverse event reporting procedures and the associated post-market reports, advisory notices and recall procedures. The Vigilance System was first adopted by the CE Marking certification body as recommended by the GHTF.

The principle of a Vigilance System was to shift to the manufacturer the accountability and responsibility of acting, monitoring and reporting of all adverse events or near-adverse events that have occurred with the use of IVDs. IVD manufacturers are thus required to handle vigilance when assessing risks and potential risks associated with the IVD. Vigilance is a post-market requirement that includes reporting to TGA possible adverse events or near-adverse events that are reported by end-users within a specified time-frame. Vigilance includes the monitoring of the safety of IVDs placed in the market. Manufacturers were to report to TGA any problems or potential problems that were known or become known that may affect the integrity of an IVD placed in the market. An adverse event is defined as a case where a person dies directly as a result of the use of an IVD. A near adverse event is a case where a person's health is significantly affected as a direct result of the use of an IVD. The definition of adverse event applied to the use of Bm14 ELISA can be ambiguous because the assay does not come into contact with the patient. Risk assessments performed on the Bm14 ELISA (Appendix 12) and its use confirm that the major risk applies when used in a clinical setting. A misdiagnosis due to the incorrect preparation of the patient sample may be a potential risk that can lead to a near adverse event where a patient is not treated accordingly because of a false negative result.

It is the responsibility of the IVD manufacturer to report an adverse event or near adverse events as well as monitoring the market for reported cases involving other similar products. Data gathered about other adverse events were to be used as “lessons learnt” and considered when assessing the safe use of IVDs.

5.3.4.2 Post-Market Surveillance

Post-market Surveillance is an element of a quality system that monitors the integrity of a test that has been listed in the TGA ARTG. The majority of the Post-market Surveillance elements that were required for a QMS encompassing the Bm14 ELISA were built into the QMS design. Quality monitoring was integrated with the QMS Non-conformity log, customer complaints, vigilance reports relating to the test and vigilance reports relating to other similar tests in the market. The concept of monitoring was to gather the information that can be used to address or foresee any potential risks or problems relating to the use of the test.

5.3.4.3 Risk Management of IVDs

Risk assessments for the Bm14 Antibody CELISA were completed by following international standards, Elimination or Reduction of Risk of Infection Related to IVD Reagents (EN13641) for the design and manufacture of IVD. Medical Devices – Application of Risk Management to Medical Devices (ISO14971) Annex C and H applicable to IVDs were also included in the Risk Assessment reports. A failure mode effects and analysis (FMEA) was a method used to determine any potential risks found with the manufacturing process and post-market state of the filariasis tests. It is used to renew 18-month cycles of reports and to keep track of the action results for improving the potential risks that were found.

5.3.4 Traversing the Australian IVD Regulatory Framework

The TGA website provided all the information required to take a newly developed IVD such as the Bm14 ELISA, from the research prototype to a TGA registered

commercial product manufactured by the ISO13485 Standard. The point of reference used to traverse the numerous TGA website pages for IVD regulation was the Guidance Documents. A list of these important documents that supports the process of conformity to IVD regulation required for commercialisation appears in TGA (2013).

5.4 RESULTS

In 2010, the Bm14 ELISA was included in a QMS that met all the requirements of the current TGA legislation of IVD as discussed in sections in the above methods. This was included in a current ARTG listing as a commercialised “Diagnostic Kit”. The QMS encompasses the processes of a system to an international standard ISO13485 (British Standard, 2012) that allowed the test to be legally supplied as an IVD in Australia, and globally for its intended use. In 2014, a new TGA framework for IVDs called the Conformity Assessment took effect. The Bm14 ELISA manufactured under the 2010 QMS was submitted for Conformity Assessment and successfully approved for inclusion in the ARTG Indentifier 231975 as a commercialised kit under GMDN code L2 CT356 Parasitic Infectious Diseases in 2014 (Appendix 14).

The process of manufacturing under a QMS ISO13485 allowed the transition of the Bm14 CELISA kit to be CE Marked through the route to compliance, Annex III EU 98/79 EC for self-declared products (Figure 5.1).

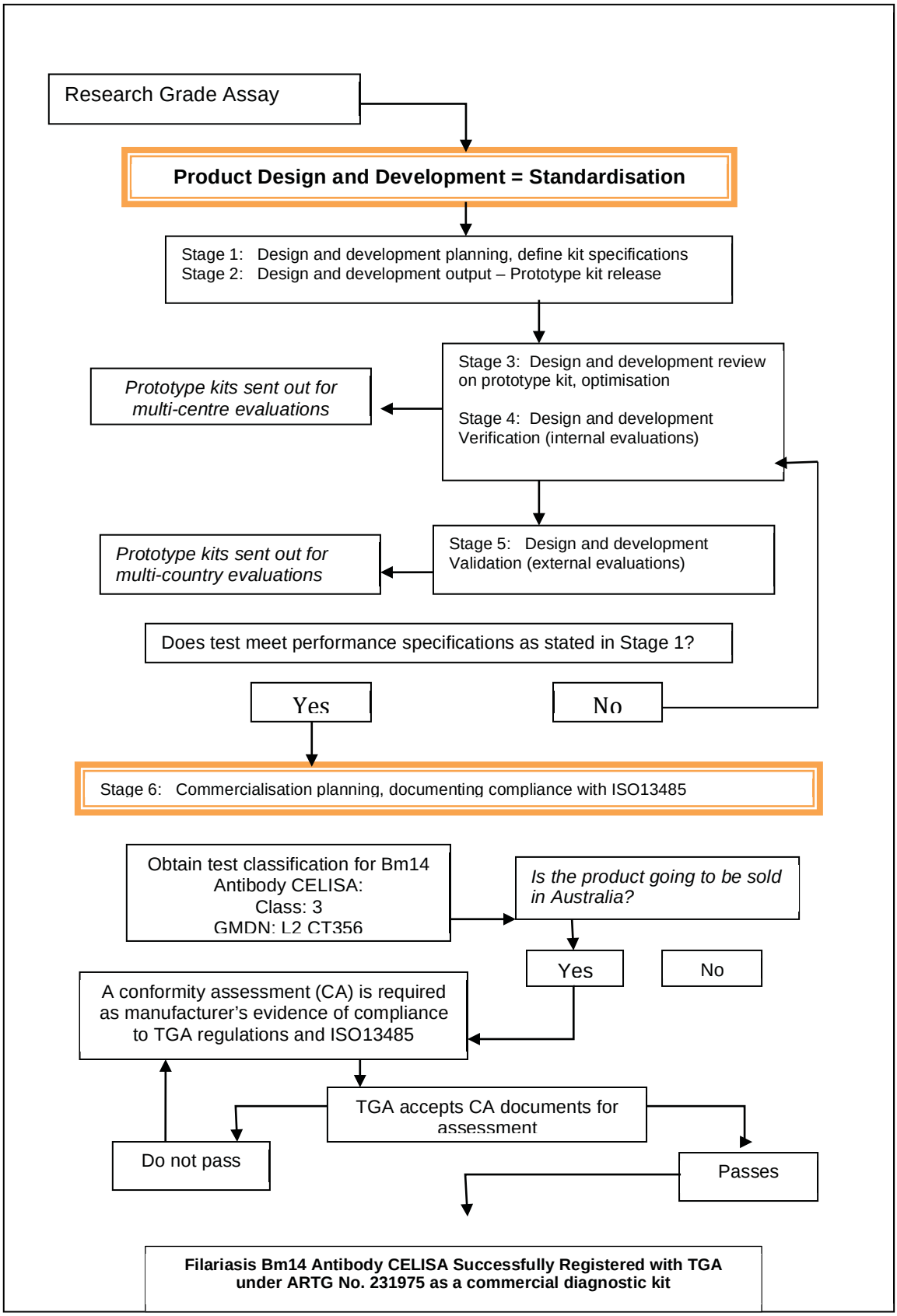


Figure 5.1: Flow chart of the development, standardisation and commercialisation.

Figure 5.2: The quality controlled (QC) Bm14 Antibody CELISA kit. A 5-plate ELISA kit containing pre-packaged buffers and reagent.

5.5 DISCUSSION

The process of conforming to regulatory IVD legislation was necessary to move a research product to commercialisation. This process can become a difficult and time-consuming task for the regular R&D scientist whose job is to develop important diagnostic tests. Regulatory affairs are a task for quality managers who need to have a good understanding of the science behind the product as well as the requirements of the Regulations to successfully commercialise a product. As learnt from the process of commercialising the Bm14 ELISA, it was equally important for R&D scientists to be informed about regulatory elements required of a test under development. From the design, development and validation phases of a newly developed test for human diagnostics as seen in Chapter 2 and 3, addressing quality requirements and other considerations is key to a successful commercialisation.

The requirements outlined in this chapter were those considered to be important for the commercialisation phase. A large part of the commercialisation phase of the Bm14 ELISA development was spent writing methods for all the steps of assay manufacture. Included methods were receipt of product, processing and manufacturing of the kit until it is ready for release and supply to the customer. This commercialisation

requirement was not thoroughly discussed in this chapter. Instead, comments on the important principles of the regulatory requirements were made. A QMS can be easily modelled using the Index of ISO13485 standard (Appendix 11).

The application for CA under the new TGA Framework 2014 took more than 12 months to complete, and this was due to all the Australian IVD products being transitioned from the old legislation since 2010. This new harmonised legislation may have advantages because regulatory assessments were based on the international standard ISO13485. This means that countries imposing the use of ISO certified diagnostic kits for import will accept the Bm14 assays without any registration issues.

The TGA certification for the Bm14 ELISA means that the assay can be used in Australia for clinical use. The CE Marking certification means that the countries in the EU, as well as other countries that only accept a CE Marked product for use in clinical setting, can legally use the kit in their respective countries. In the research setting for both Australia and globally, ISO certifications are not required. Assay certification is evidence that an assay has undergone a high-quality standard of manufacturing and a standardisation process, therefore, the uniformity, quality and integrity of a kit is assured. This is crucial to elimination and control programmes because studies being conducted need to use reliable tests to give the correct results to steer programme management decisions. Elimination and control programmes and their on-going monitoring and surveillance are costly, and programme success is dependent on the availability of reliable, efficient and cost-effective diagnostic tools.

CHAPTER 6

FILARIASIS-SPECIFIC IgG1 AND IgG4 ANTIBODY RESPONSE IN A HIGH-ENDEMIC COMMUNITY

6.1 INTRODUCTION

Parasitic infections in humans induce an immunological response against the immunogen as a means of clearing pathogens and controlling the onset of disease. The first line of defence following the penetration of the skin barrier by an invader is innate immunity; a non-specific reaction with an arsenal of white blood cells and phagocytes to attack invaders and natural killer cells to remove infected cells. Most infections can be cleared efficiently by antigen-presenting cells of the innate system called dendritic cells without the involvement of other immune mechanisms (Babu & Nutman, 2012). In the case of persistent infections such as large helminthic parasites (Maizels et al., 1993), the adaptive immune system is activated because a prolonged innate immune reaction can be damaging to healthy cells. The adaptive immune mechanisms are slower and function to expel the invader specifically by responding with the proliferation of T-cells and B-cells. In LF infections, the T cell type 2 (TH2) response is involved in the proliferation of cytokines IL-4, IL-5, IL-9, IL-10, IL-13. The most prominent antibody response is of subclasses IgG1, IgG4 and IgE. IgE has been observed to be present for a long time after the clearance of infection but it is a non- specific response. It is dependent on IL4 and IL13 and said to be related to the activation of long-lived memory B cells. Subclass IgG4 is dependent on the proliferation of IL4 and IL10 cytokines and the most dominant subclass in the presence of mf (Babu & Nutman, 2012).

The response of IgG antibody subclasses has been observed to vary depending on disease pathology. The early studies of Ottesen et al., (1985) noted the corresponding dominance of subclasses to clinical forms of the disease. In the presence of high mf loads in individuals living in endemic areas, the prominent IgG4 and an IgE response have been observed (Ottesen et al., 1985; Lal & Ottesen, 1985; Maizels et al., 2000; Garraud et al., 1995; King et al., 1993; Haarbrink et al.,

1999; Terhell et al., 1996, 2000, 2002; Neilsen et al., 2002 and Hussain et al., 1987). Induced by mf, IgG4 levels can reach as high as 95% of the total IgG in infected patients (Ottesen et al., 1985). These findings resulted in IgG4 being recognised and widely accepted as a surrogate marker for active infection. In contrast, elephantiasis patients who do not have mf have pronounced levels of IgG1, IgG2 and IgG3 (Kurniawan et al., 1993; Rajan & Gundlapalli, 1997). This reduction in IgG4 may signal the clearing of mf as the individual progresses to advanced stages of the disease (Bal et al., 2003). In lymphoedema patients, the increased levels of IgG3 antibody and a decline in IgG4 has been observed (Neilsen et al., 2002).

Although antibody subclass IgG4 is strongly associated with active infection and the presence of mf, there are also studies to suggest that IgG1 may have a role in early detection. The study by Melrose et al., (2000) of non-immune expatriate mine workers in Papua New Guinea (PNG) found an elevated filarial IgG1 response in those who were exposed. The IgG1 response increased in levels the longer the workers were exposed outside in the environment. The same cannot be said for those who worked inside offices, suggesting that the first group who worked outside were exposed to filariasis. A study by Frances et al., (2008), observing Australian defence personnel deployed to Timor-Leste also found a higher IgG1 prevalence in those who were exposed. A significant number of seroconversions were of IgG1 (49/907) compared to IgG4 (1/944). It suggests that an IgG1 response is associated with incoming L_3 exposure because many of these seroconverters clear infection rather than developing the disease.

If IgG4 is a specific response against mf and adult worm antigens, perhaps, there is a distinct switch of antibody subclasses from L_3-specific IgG1 and as the disease progresses, IgG4 is presented. This means that the identification of a specific IgG1 against Bm14 recombinant antigen may be more suitable in detecting ongoing transmission for PCT assessments if it correlates to L_3 exposure. A study by Kurniwan-Atmadja et al. (1998) investigated the antibody response of reactivity of somatic antigen extracts from different parasite life stages to find which antibody subclass is a response against the infective larvae (L_3) surface antigen. It was found that L_3 antigen extract is strongly reactive with IgG1 rather than IgG4, suggesting that IgG1 has a role in early detection of exposure (Njenga et al., 2007). The study has been followed up from the work of Day, Gregory & Maizels (1991)

where IgG antibody responses were observed in age-specific immunity studies of young children exposed to L_3. By using L_3 antigens to determine antibody reactivity, IgG1 is seen in the youngest group. Although both studies presented very interesting findings with respect to IgG1 and associated early filarial exposure, the antigens used were from crude parasite antigens. These antigens as described previously are said to be cross-reactive. A more recent study using the recombinant Bm14 antigen supports the theory that IgG1 appears early. An age- specific study in Kenya by Njenga et al., (2007) found an L_3-specific IgG1 response with children in the age group 4 to11. Again, this is suggestive of L_3 exposure. The rise of IgG4 subclass seen in the older age group is indicative of a subclass switch from IgG1 to IgG4 that occurs as infection progresses, a sign possibly related to pathological changes.

Even though subclass IgG1 is known to represent antibody response to microbial infections (Adjobimey & Hoerauf, 2010), it is also the first IgG antibody to be mounted in a microbial infection after the IgM response. Therefore, if it is true that LF-specific IgG1 represents incoming L_3 antibody response, its use as a marker for infection is more appropriate than IgG4. The early detection of antibodies to a recent infection in a sentinel group is key to marking the ongoing transmission or the resurgence of infection in the monitoring and surveillance stages of LF elimination programmes. Because antibody response is detectable earlier than the changes in mf status or antigenaemia, it is a powerful indicator for immunological markers of infection.

The early estimates of the average period it takes before active infection is detected based on microfilariae is about 5 to 8 years based on the results of seven independent studies as reviewed earlier by Bundy et al.,(1991). This estimated average time of pre-patent stage may now be outdated because of the use of improved antibody tests with higher sensitivity and specificity. More recently, Supali et al. (2004) found anti-filarial IgG4 in the age group 3 – 5. Studies by Terhell et al. (2000) also confirm that anti-filarial IgG4 can be detected in babies as young as 18 months old.

There is no doubt that the current availability of improved species-specific recombinant antigens used in LF diagnostics can provide accurate results. The

availability of a standardised Bm14 recombinant antigen (Bm14 rAg) ELISA makes it possible to investigate the antibody response of IgG1 and IgG4 subclass. Bm14 is one of the most promising antigens for antibody detection that is non-reactive with *Strongyloides* (Melrose 2004), the availability in standardised ELISA format has made it possible for new investigations into the relevance of IgG1 and IgG4 responses.

How can the use of available tools for detection of markers of LF infection, specifically the antibody subclass IgG1 and IgG4, be of any use in the detection of early exposure to LF? This chapter will investigate the role of Bm14-specific IgG1 and IgG4 subclasses in a set of serum samples using the standardised, recombinant-based Bm14 CELISA antibody test. It will also include the use of the new version of Og4C3 ELISA to detect circulating filarial antigen (CFA). Investigation of IgG1 and IgG4 antibody responses will focus on the youngest age group as they are the ideal sentinel group for determining whether the transmission is still ongoing. This investigation will also aim to observe if there is a point at which the IgG1 response 'switches' to the IgG4 response as current knowledge of LF immunology suggests. This subclass switching is believed to occur somewhere during the pre-patent phase of infection. The switching from IgG1 to IgG4 can provide information on whether there is a cut-off time to distinguish IgG1 exposure and the indication of active infection by IgG4. If there is a significant IgG1 response to incoming L_3 exposure, it may serve as an early marker for infection compared to IgG4.

A set of serum samples from individuals living in Lovangai, sub-locality of Metvoe, New Ireland Province of Papua New Guinea was used in this study. The province of New Ireland can be considered highly endemic. This was based on a survey for a professional doctorate research thesis by Reeve (2010), showing CFA Og4C3 ELISA prevalence at 55.5% and mf prevalence at 15.5%. This is also backed by a survey of two neighbouring sub-localities with results of 44.9% at Namanatai and 55.5% at Kavieng CFA prevalence by Og4C3 ELISA (Graves et al. 2013). This community has not undergone PCT at the time the samples were taken (W. Melrose, personal communication 11th May 2015).

6.1 AIM

This chapter will investigate the use of antigen and antibody markers as predictors of early LF infection and how these markers can be used to assist the end-point determination and monitoring stages of LF elimination programmes.

6.2 MATERIALS AND METHOD

6.2.1 Serum Samples for Investigational Purposes

Serum samples collected from 192 residents of LF endemic sub-locality of Metvoe, Lovangai, New Ireland, PNG (NI sample set) were used for this project. These were provided by Dr Wayne Melrose, (College of Medicine, Veterinary and Biological Sciences, James Cook University) for the purpose of determining the prominence of IgG1 and IgG4 on different age profiles for this thesis. The serum samples were characterised previously for circulating filarial antigen (CFA) using Tropbio Og4C3 ELISA (Tropbio, Townsville, QLD, Australia) and ICT BinaxNOW Filariasis card test (Alere, Orlando, FL, USA) and mf status, but no information on clinical symptoms was provided.

6.2.2 Determination of IgG4 Status

To determine the IgG4 status, 192 samples were tested using the standardised Bm14 ELISA kit following the manufacturer's instructions for use (IFU). The kit positive control was used to construct a standard curve for use as assay reference and control during batch testing. Results were read using an ELISA plate reader (Molecular Devices Vmax, BiosStrategy, Vic, Australia) at dual wavelength 450/620nm. Results interpretation used raw OD values as a direct proportional measure of antibody with optical density (OD) 0.3 (OD 0.2 + OD 0.1) cut-off value (COV). A "grey zone" or borderline was defined as those equivocal samples reading between OD 0.3 and OD0.35 for IgG1 and IgG4. The upper limit of detection for the ELISA reader was considered to be OD 3.0+. Over OD 3.0, the spectrophotometer reading becomes less accurate. Equivocal samples were re-

tested three times, and two out of three readings over the COV were required to designate these samples as positive.

6.2.3 Determination of IgG1 Status

To measure IgG1 for the NI sample set, the same batch of Bm14 ELISA in Section 6.2.2 was used but replacing the anti-IgG4 peroxidase HRP conjugate. A commercially available anti-IgG1 clone 8c/6-39 monoclonal antibody conjugated to peroxidase HRP (Cat No. MP003, Binding Site, In-vitro, Victoria, Australia) was used. The anti-IgG1 conjugate was diluted at 1:4000 using the Bm14 ELISA kit conjugate diluent and following the method as per kit IFU. The interpretation of OD results uses the same method as the IgG4 ELISA (Section 6.2.2).

An IgG1 positive control for use in constructing the standard IgG1 curve was devised by pre-screening 30 random samples from the NI serum sample set and pooling a small volume from 6 samples. This sample pool gave a reaction of OD 2.

6.2.4 Determination of Filarial Antigen Status

6.2.4.1 Standardisation of the Og4C3 ELISA

The NI sample set previously characterised for circulating filarial antigen (CFA) status was based on the original Tropbio Og4C3 ELISA. The interpretation of results for Og4C3 ELISA CFA uses the kit recommended method of arbitrary values assigned to levels of CFA or “titre groups” on a standard curve devised by More & Copeman (1990). This method was appropriate for CFA positive (CFA+) approximation. It is, however, not practical if the assay was not standardised because batch-to-batch inconsistencies also affect results. A borderline result can either be considered negative or positive depending on whether the kit batch standard curve meets the “typical standard curve” specification. To ensure that the standard curve specification was consistently met for each batch of kits, production of a batch of kits must meet standards of a quality system. Standardisation and optimisation of the Og4C3 ELISA were undertaken in 2013 with the support of the manufacturer of the kit, Cellabs Pty Ltd, NSW,

Australia. The aim was to improve the kit and bring the assay in line with current ELISA methods, and produce these under a controlled quality management system (QMS) similar to the methods applied for Bm14 ELISA in Chapters 3 and 4. The newly standardised and optimised assay was presented as the “Tropbio Og4C3 ELISA Mark II”, made available in January 2014.

6.2.4.2 Determining and Validating the Use of a Cut-Off Value

The newly standardised and optimised Tropbio Og4C3 ELISA Mark II was used to test the CFA status of the sample set. The IFU method (Appendix 4) was followed but the interpretation of results made use of the absolute raw OD ELISA values to allow comparisons with IgG levels. To determine the OD cut- off value (COV) for CFA levels, 152 non-endemic serum samples from Australian residents was used to calculate the negative mean OD +3SD to give COV = OD 0.2. This COV also corresponds to “titre group 1” or equivalent to the interpretation that all samples less than ten antigen units do not have CFA. This rationale holds true because standard 1 contains only the diluent “blank” while standard 2 contains the lowest detectable antigen in the set of standards 1 to 7 for the kit. Arbitrary antigen units categorise equivocal sample between Standard 2 (corresponding to 32) and Standard 3 (corresponding to 128) however, using “equivocal” based on arbitrary antigen unit values means that antigen is detected but in low amounts. To be as accurate as possible in this study, low amounts were considered relevant in detecting the early rise of CFA in the patent stage.

The addition of a substrate chromagen in ELISA develops a colour complex that is directly proportional to the amount of antigen and antibody binding that is present in each well. The resulting OD value is the measure used to determine absolute reactivity for determining IgG1, IgG4 and CFA. Cut-off values were therefore carefully considered to ensure the most accurate determination of status for each sample tested.

6.2.5 OD Values Interpretation

Table 6.1 shows the specifications used for each test. An OD of > 3.0 is considered over the limit of plate reader detection.

Table 6.1 ELISA specification and interpretation table.

Result	IgG1 ELISA	IgG4 ELISA	Og4C3 ELISA
Negative Reference	< OD 0.25	< OD 0.25	< OD 0.2
Positive Reference	>OD 0.4	>OD 0.4	>OD 0.4
Borderline	OD 0.30-0.35	OD 0.30-0.35	OD 0.2-0.3
Cut-off	< OD 0.3	< OD 0.3	< OD 0.2

6.2.6 Data Analysis

Statistical analysis of results used IBM SPSS Statistics version 22 software and Microsoft Excel 2013 Data Analysis function. Excel was used for graphical presentation. The use of statistics was applied to data that can be converted to percentages such as the analysis of prevalence. Data interpretation was mainly based on graphical representation because there was no quantitative method available to compare the intensities of the ELISA tests used.

Data was arranged and presented as a whole without categorising into CFA status. The data was further divided into CFA positive and CFA negative groups to make comparisons on the antibody subclass IgG1 and IgG4. The data was analysed in sections as a whole followed by the stage of infection as CFA negative group (CFA) and CFA positive group (CFA+). The data was also analysed by age groups and finally by the youngest age group.

6.3 RESULTS

6.3.1 Description of the New Ireland Sample Set

Age frequency distribution for the 192 samples appears asymmetrically skewed to the right with data centred on those aged 6-10 (Fig. 6.1), with the youngest age being 4 and the oldest 60.

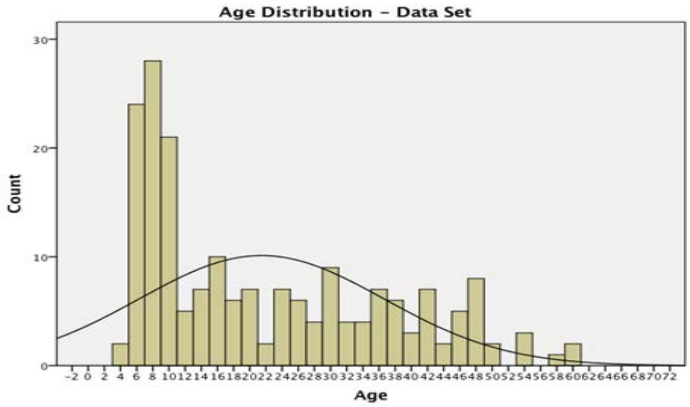


Figure 6.1: Age distribution of 192 serum samples

All 192 samples were tested for CFA using the Tropbio Og4C4 ELISA (Fig. 6.2), Bm14 IgG1 ELISA (Fig. 6.3), Bm14 IgG4 CELISA (Fig.6.4) and mf counts (Fig. 6.5). The results were plotted in a bar graph to give an overview of the sample set.

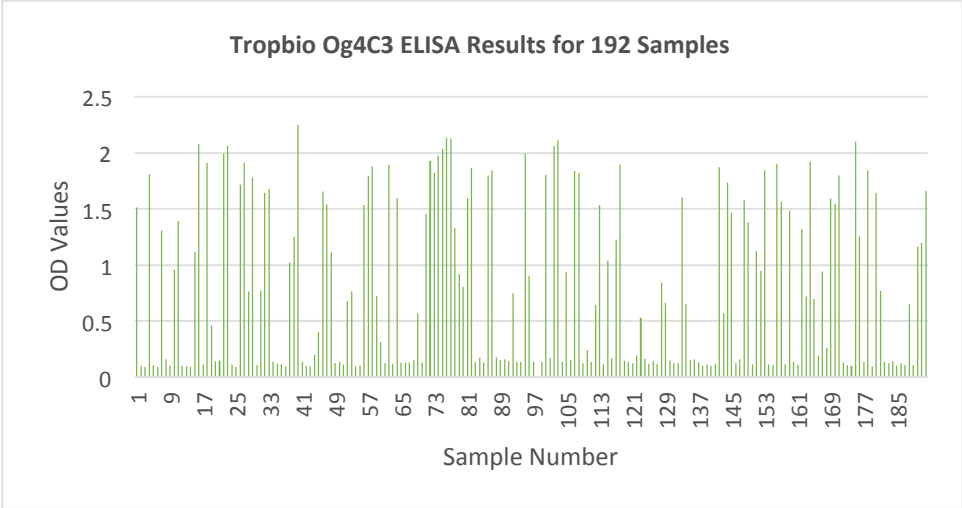


Figure 6.2: Og4CFA Antigen ELISA results of 192 samples tested. The mean OD was 0.078 using a cut-off value of OD 0.2. There were 99 CFA positive samples out of 192 (51.5%).

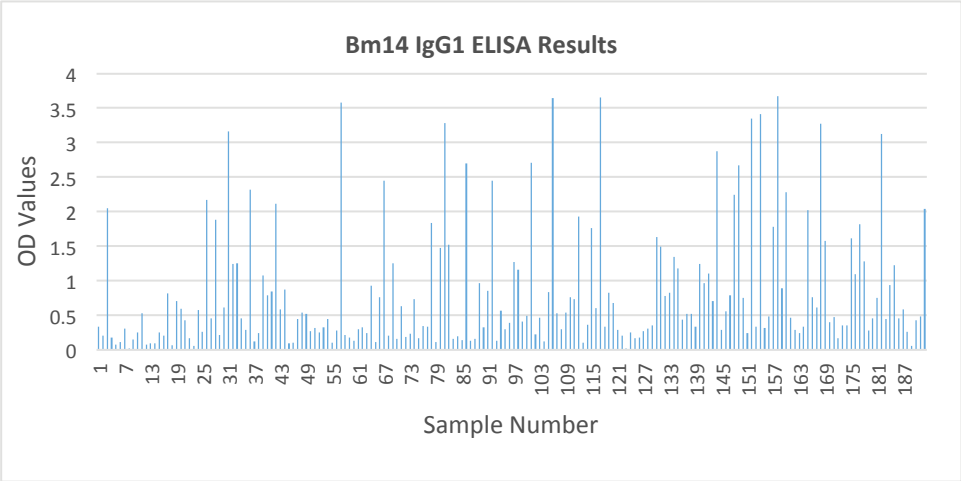


Figure 6.3: Bm14 IgG1 ELISA Results. The mean was OD 0.83 using a cut-off value of OD 0.3. There were 126 IgG1 positive samples out of 192 (65.6%).

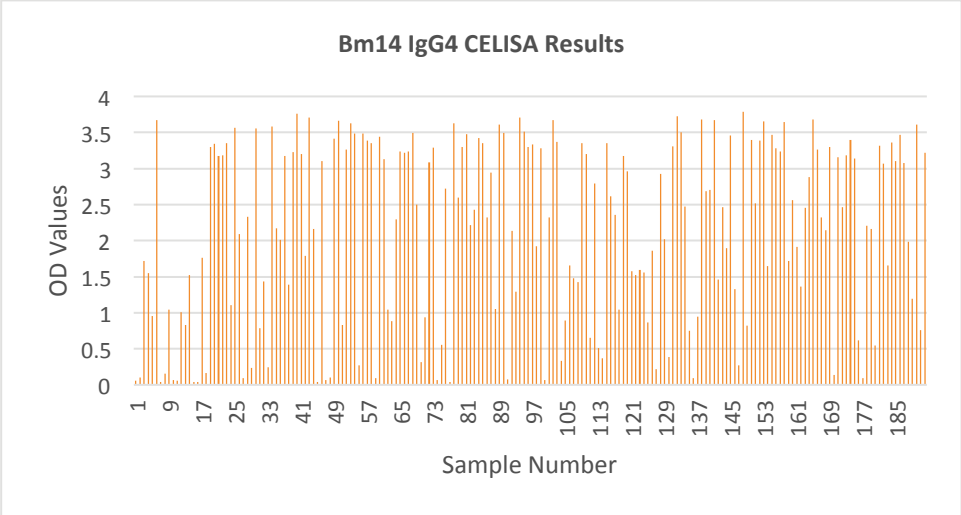


Figure 6.4: Bm14 IgG4 CELISA Results. The mean was OD 2.16 using a cut-off value of OD 0.3. There were 166 IgG4 positive out of 192 (86.5%).

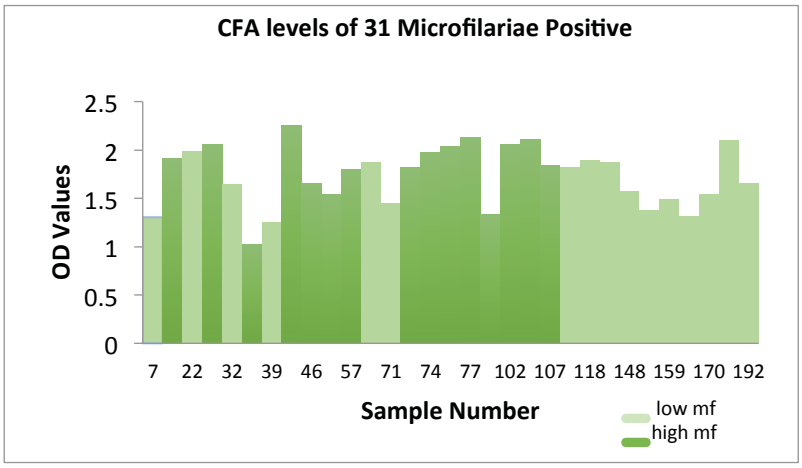
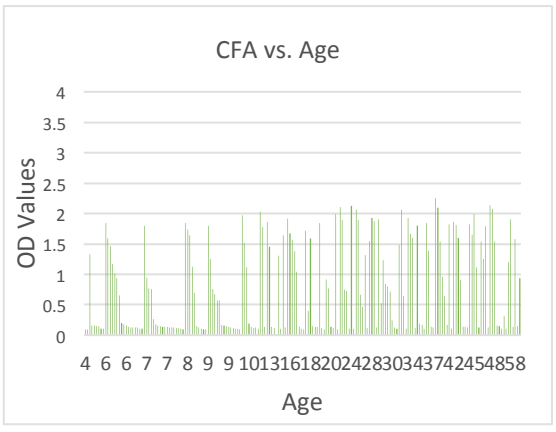


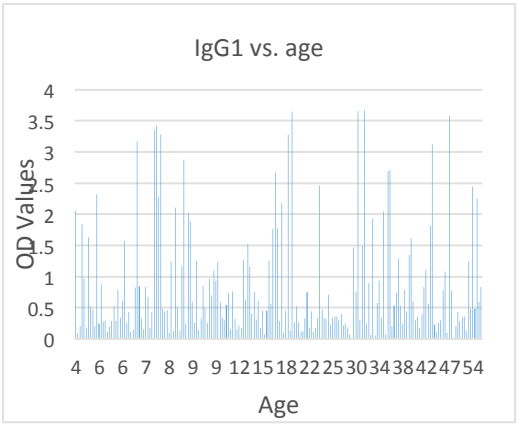
Figure 6.5: Mf positive Results. The 31 Mf positive samples (16.1%) are shown as levels of CFA in a graph. The light green columns had mf counts of 1-100 and the dark green columns had mf counts >100.

6.3.2 Investigating Relationships by Age

Data was arranged according to age to investigate if there are trends or correlations seen from the youngest to the oldest age. The graph of CFA against age (Fig. 6.6a), IgG1 against age (Fig. 6.6b) and IgG4 against age (Fig. 6.5c) shows no observed correlation with age and therefore does not give any useful information in determining early exposure. However, the IgG4 response is observed to be dominant in the whole samples set.



(a)



(b)

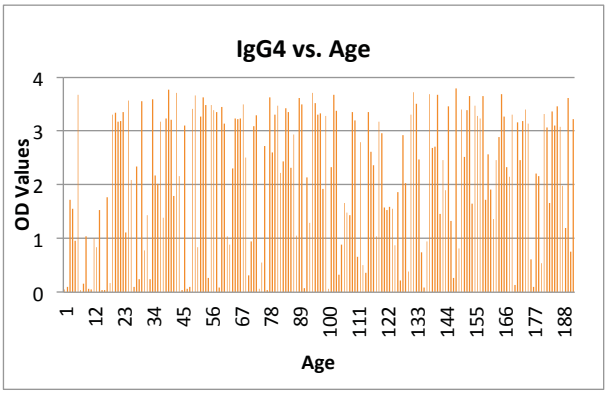
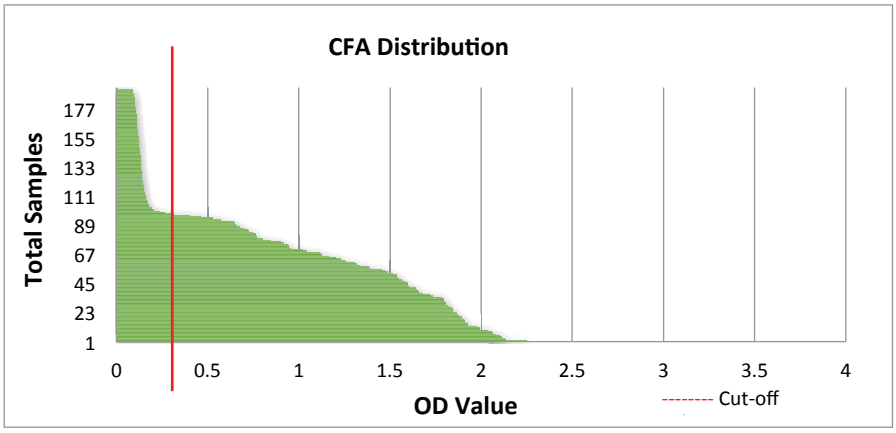


Figure 6.6: The results of three markers arranged by age. The graphs for: (a) CFA vs age; (b) IgG1 vs age and; (c) IgG4 vs age show no observed trends or correlations of reactivity with age.

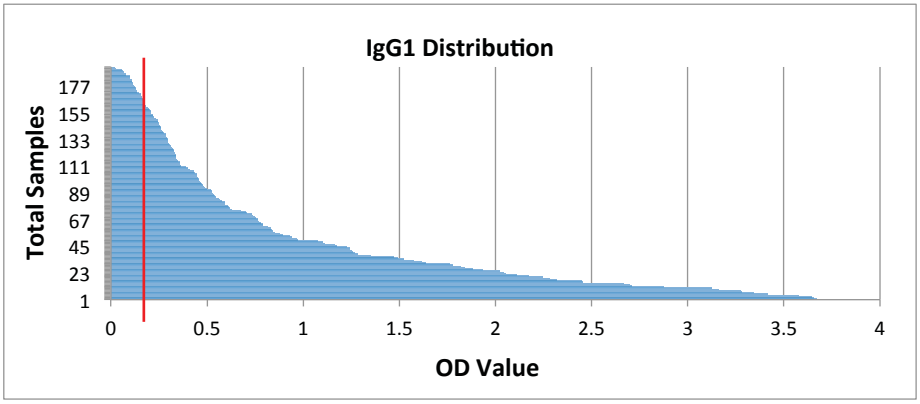
(c)

6.3.3 Investigating Relationships by Infection Stage

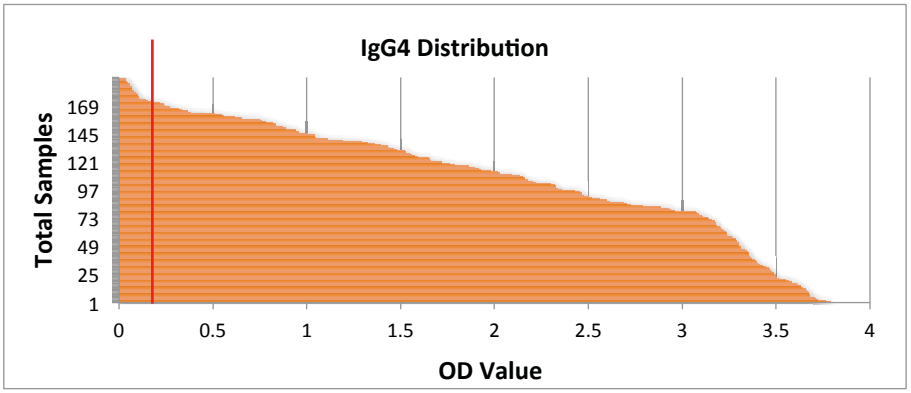
The data was divided into two groups according to infection stage (CFA negative group and the CFA positive group). Figure 6.7 shows the CFA distribution graph ranked by OD Value. The red line indicates the cut-off for all negative reactors and the positive reactors at OD 0.2. Figure 6.7c shows antibody reactivity with a dominant IgG4 compared to IgG1 (Fig.6.7b). The graphs show a high numbers of IgG4 compared to IgG1.



(a)



(b)

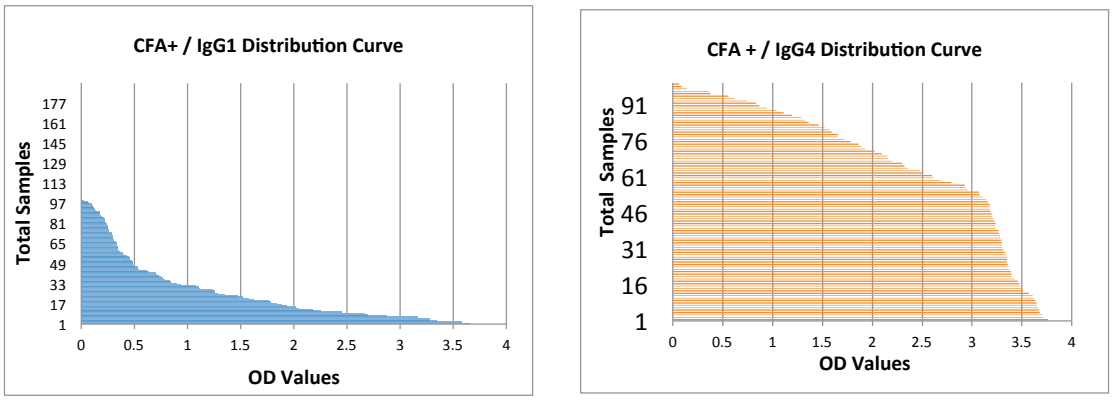


(c)

Figure 6.7: Distribution curves for LF markers. (a) CFA, (b) IgG1 and (c) IgG4 curves show the distribution in OD values that can be used for comparison.

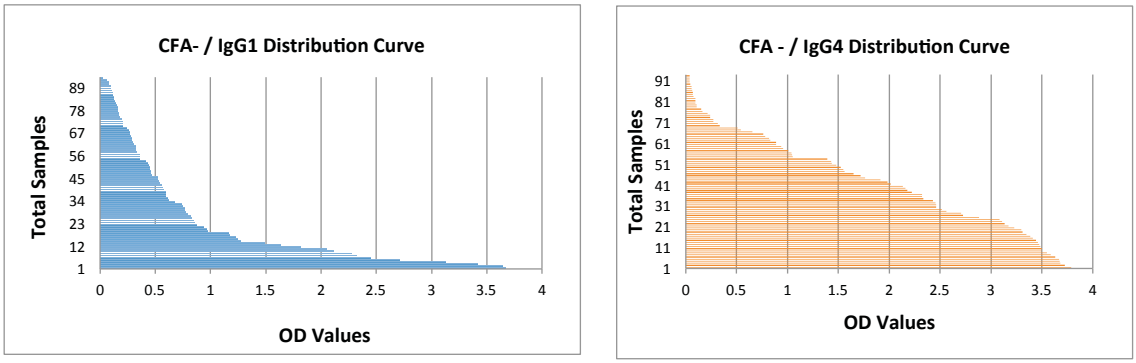
To describe the antibody (IgG1 and IgG4) reactivity for each stage of infection, the two groups were plotted separately in a bar graph for comparison. The CFA positive (active infection) group shows a generally low IgG1 (Fig. 6.8a) compared to a dominant IgG4 (Fig. 6.8b) reactivity. This picture is similar to the CFA negative group (Fig. 6.9) showing relatively similar low IgG1 (Fig.6.9a)

reactivity patterns as those in the CFA positive group but the IgG4 reactivity for those CFA positive are distinctly higher.



(a) **(b)**

Figure 6.8: CFA Positive showing antibody distribution. (a) IgG1 curve showing a less dominant reactivity compared to (b) IgG4 curve showing a more dominant IgG4 reactivity.



(a) (b)

Figure 6.9: CFA negative distribution curves. (a) IgG1 curve showing a less dominant reactivity compared to (b) IgG4 dominant reactivity.

6.3.4 Correlations with Positive Results

This section will describe the results of data analysis based on five age groups to show the age profile of the variables mf, CFA and antibody (IgG1 and IgG4). The whole data was expressed as percent positive for each age group to give an overview. Table 6.2 gives a summary of the results for use in graphs and comparisons.

Table 6.2: Summary of positive test results in percentage by age-age groups

Age Group	oCFA +	% CFA	IgG1+	% IgG1	IgG4+	% IgG4	Mf +	% Mf	Total
4-10	27	36.00	50	66.67	51	68.00	4	3.3	75
11-20	17	48.57	22	62.86	32	91.43	5	14.8	35
21-30	21	75.00	16	57.14	26	92.86	9	32.1	28
31-40	15	62.50	18	75.00	23	95.83	6	25.0	24
>40	19	63.33	20	66.67	28	93.33	7	23.3	30
Total	99	51.56	126	65.63	160	83.33	31	16.1	192

Figure 6.10 shows the age profile of the variables as a percentage of positive results. All the mf positive samples were also CFA positive, showing a strong correlation (R=0.99). The correlation of CFA prevalence to the IgG1 response was negative although the IgG4 response was correlated (R=0.805). There was no correlation between the prevalence of IgG1 and IgG4.

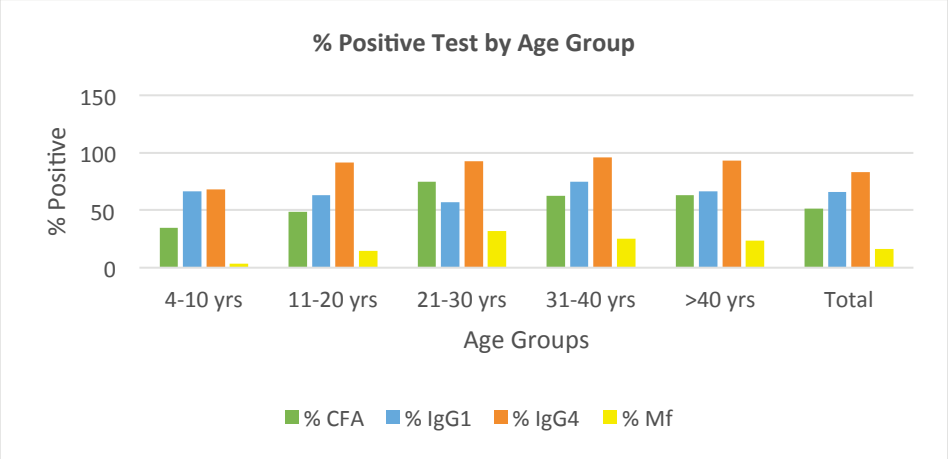


Figure 6.10: Bar graph for the % positive outcomes. Mf, CFA and antibody IgG1 and IgG4 for all the five age groups.

6.3.5 Investigating Age Profile (CFA+ and CFA-)

This section will show the results for samples that tested positive for IgG1 and IgG4 for both the CFA positive infection stage and the CFA negative infection stage. Table 6.3 shows the summary of results in % positive for each antibody subclasses categorised by infection stage.

Table 6.3 Summary of CFA+ and CFA- for IgG1 and IgG4 for each age group.

Age Group	Total	CFA+	CFA+ %	CFA +		CFA-	CFA- %	CFA -	
				IgG1+	IgG4+			IgG1+	IgG4+
4-10	75	26	34.66	77% 20/26	73% 19/26	49	65%	40% 30/49	65% 32/49
11-20	35	17	48.57	82% 12/17	94% 16/17	18	51%	56% 10/18	83% 15/18
21-30	28	20	71.43	50% 10/20	95% 19/20	8	29%	75% 6/8	88%
31-40	24	15	62.5	80% 12/15	100% 15/15	9	38%	67% 6/9	89% 8/9
41-60	30	19	63.3	53% 10/19	95% 18/19	11	37	91% 10/11	91% 10/11
Total	192	99	52%	65% 64/99	88% 87/99	Total: 93		67% 62/93	77% 72/93

The active infection group and the corresponding % positive for IgG1 and IgG4 were plotted to show the age profile (Fig. 6.11). The CFA rate is not correlated with the IgG1 antibody rates although the correlation of CFA and IgG4 is strong (R=0.833). There was no linear antibody rate across the age groups and no correlation between the IgG1 and IgG4 across age groups.

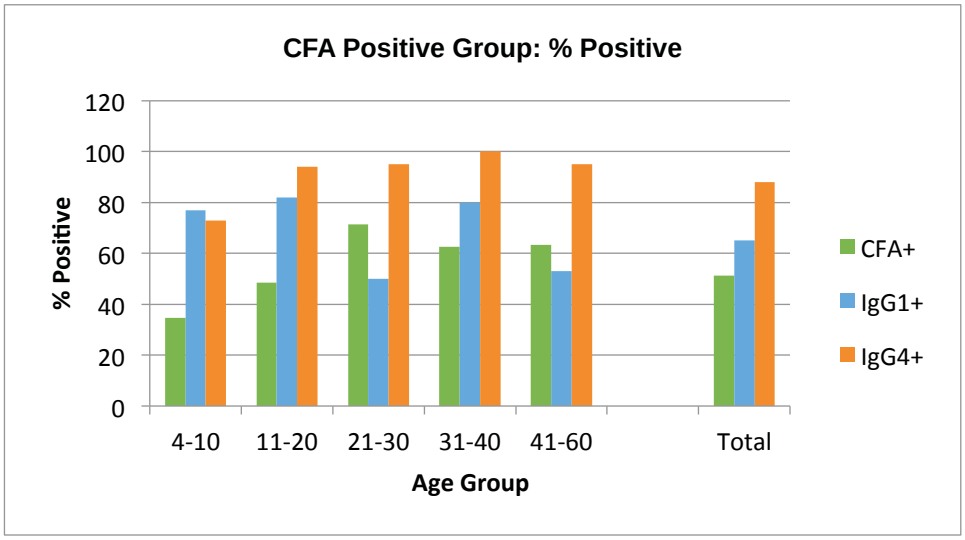


Figure 6.11: Age profile of active infection group. The bar graph shows the percentage of positive results for CFA and antibody subclasses (IgG1 and IgG4) by age group.

The CFA negative group and the corresponding % positive for IgG1 and IgG4 were plotted to show the age profile (Fig. 6.11). The antibody rates of both subclasses show a strong correlation across age groups (R=0.882). Both antibodies were linear with IgG1 slightly more linear (R=0.93, p=0.023) than IgG4 (R=0.87, p=0.058).

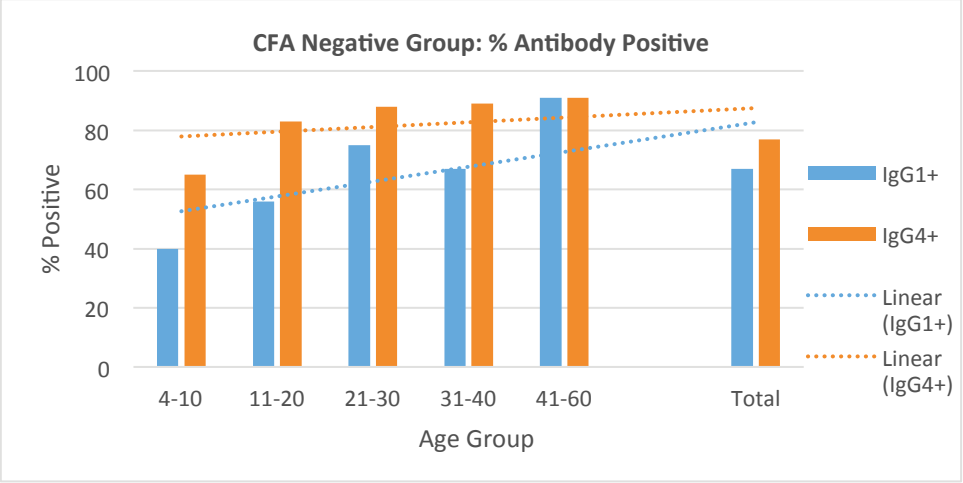


Figure 6.12: Age profile of CFA Negative group. The bar graph shows the % positive rates of the antibody subclasses IgG1 and IgG4.

6.3.6 Antibody Reactivity of the Youngest Age Group

This section will discuss the findings for the youngest age group to investigate if IgG1 is a marker that best represents early exposure as compared to IgG4 subclass. The antibody responses were investigated as a group and further separated according to the two stages of infection (CFA+ and CFA-) for investigation. The results are shown in separate sections.

There were 75 samples in the youngest age group 4-10 (a), with 46 males and 29 females. There were four mf+ of which 3 are males and 1 is female. The youngest age with mf and CFA was a 5 year old male who was also positive for both IgG1 and IgG4. There were 26 samples that were CFA positive (34%).

6.3.6.1 Children in the CFA Positive Group

The 26 samples in the infected group were plotted on a bar graph to show the results for all the markers (Fig.6.13).

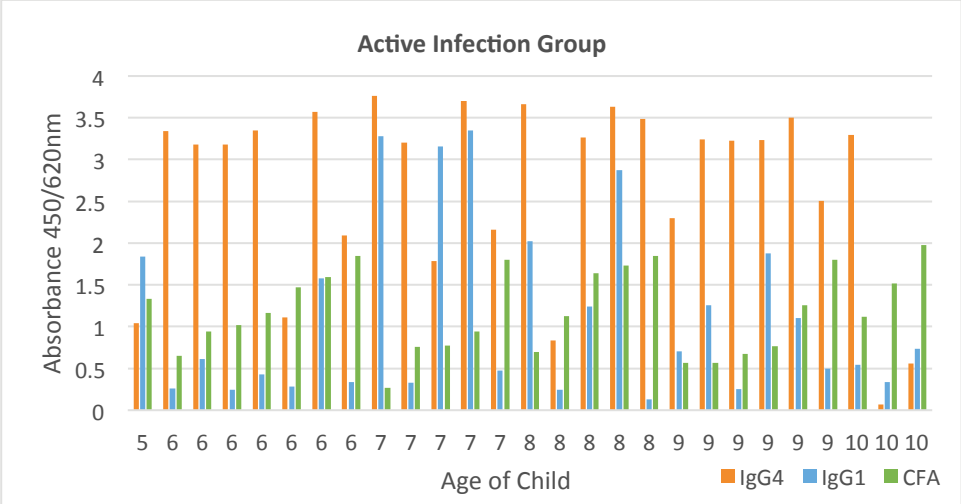
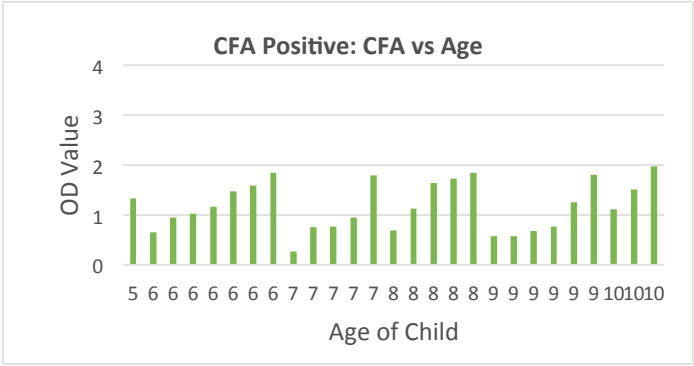
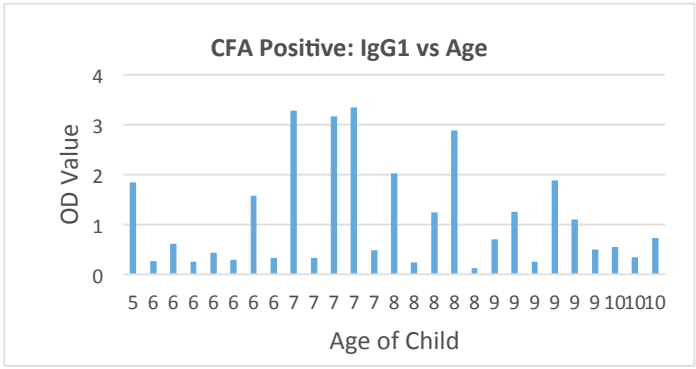


Figure 6.13: Graph for CFA Positive Children. The OD levels of antibody and the corresponding CFA levels are shown to give an overview. There were no trends observed.

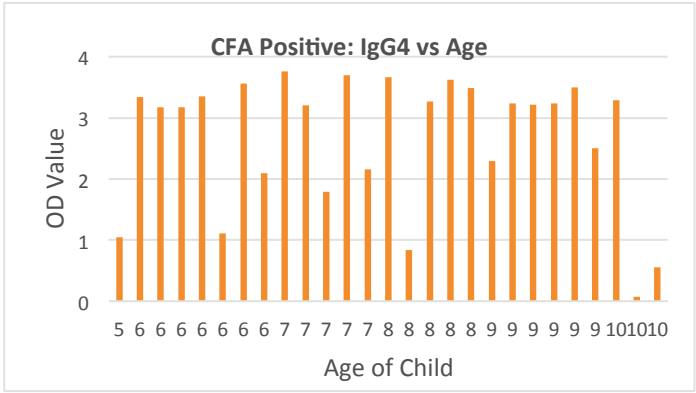
The youngest group was arranged by age to observe any correlations (Fig. 6.14). No age correlations can be observed with CFA, IgG1 and IgG4.



(a)



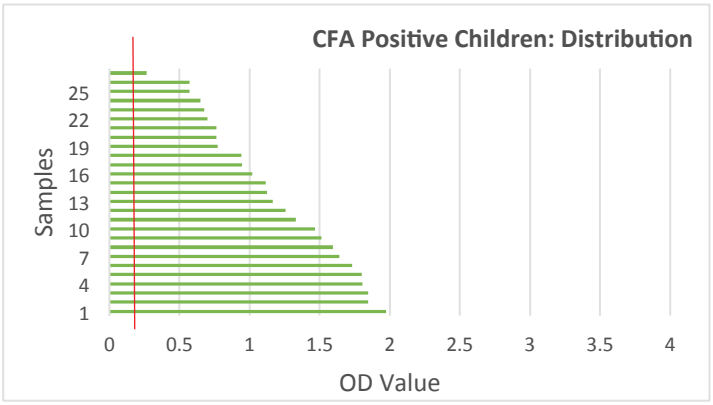
(b)



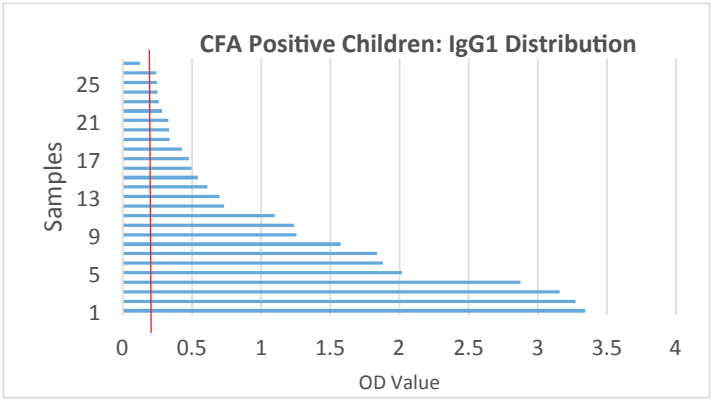
(c)

Figure 6.14: CFA positive graphs of results arranged by age. No correlations seen for: (a) CFA results arranged by age; (b) IgG1 results arranged by age and; (c) IgG4 results arranged by age.

The youngest group was shown as a distribution curve for each marker (Fig. 6.15). IgG4 is observed to be dominant as observed earlier.



(a)



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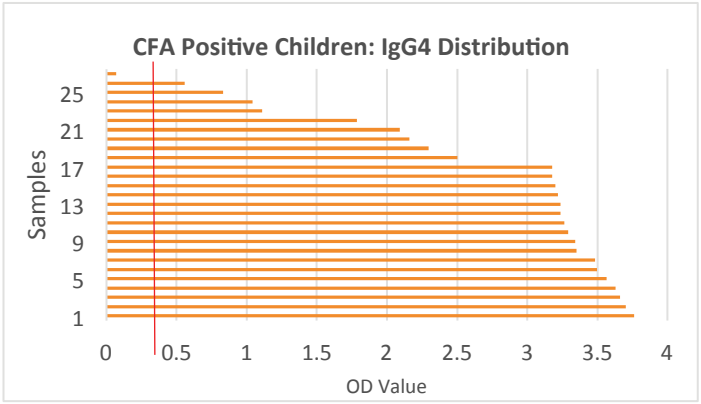


(c)

Figure 6.15: Distribution curves for CFA positive children. (a) CFA, (b) IgG1 and; (c) IgG4.

6.3.6.2 Children in the CFA Negative Group

The antibody results of the children who were CFA negative is shown in a column graph to give an overview of reactivity (Fig. 6.16). No correlations can be observed between the IgG1 and IgG4 subclasses. The result was categorised and graphed separately as CFA negative and CFA positive (Fig. 6.17). Arranging the result according to age did not show any correlations.

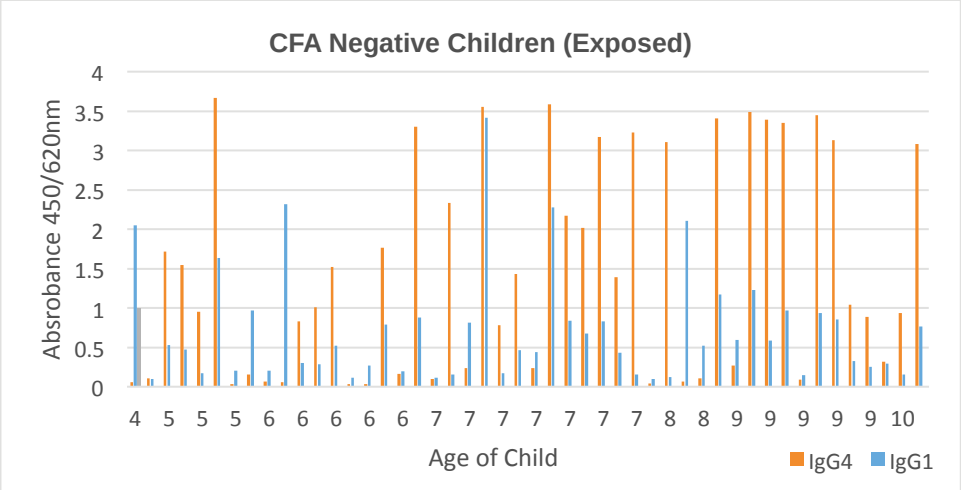
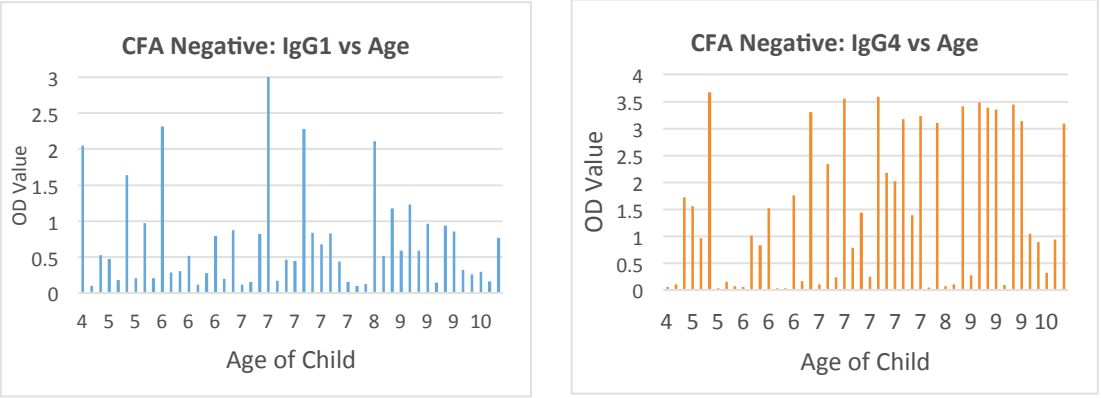


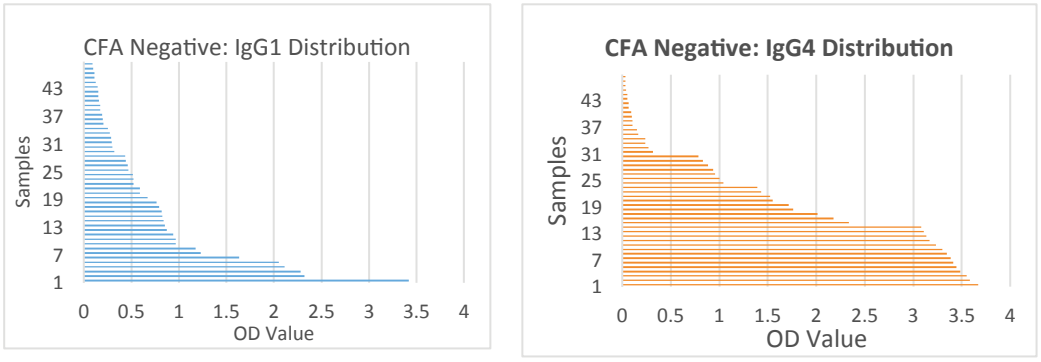
Figure 6.16: Graph for CFA Negative Children. The OD levels of antibody and are shown to give an overview. There were no observed correlations.



(a) **(b)**

Figure 6.17: Graphs for CFA negative children by age. (a) CFA positive vs age and (b) CFA negative vs age.

The results for antibody subclasses in children were plotted on a distribution curve, separately by infection stage (Fig. 6.18) to observe the antibody reactivity. It shows the consistently dominant IgG4 compared to IgG1 for the children who are not infected.



(a) **(b)**

Figure 6.18: Distribution curve for CFA negative children. (a) IgG1 distribution and (b) IgG4 distribution showing a more dominant IgG4 response.

6.4 DISCUSSION

The New Ireland samples used for investigations in this chapter were provided with sample numbers, mf test results and Og4C3 CFA results. No clinical background information or methods of sampling available for the sample set. The CFA status was confirmed by using the new format Tropbio Og4C3 Filariasis Ag ELISA Mark II and those that were positive were classified as infected. All the CFA negative samples including those that tested negative for antibody were considered exposed because being antibody negative does not necessarily mean the individual is not exposed. There has been no PCT in New Ireland, PNG where the samples were collected and there is a high prevalence seen based on OgGC4 CFA. Since there are no detailed description of New Ireleand LF prevalence, these samples are not taken as a representative of the population rather, the

results and observations were used to demonstrate how antigen and antibody IgG1 and IgG4 assist in the end-point and monitoring stages of a PCT programme. The mf result for the whole data was not analysed in detail because of the complex variables in the study. This information was used to help in defining the samples for data interpretation.

To make sense of the results, the approach was to look at the data as a whole and observe any correlations by a graphical display. The whole data was first arranged by sample number as a general overview, followed by arranging the results by age and by the distribution of OD results. The whole data was divided by the stage of infection: 1) CFA negative and 2) CFA positive, and observing these two groups according to sample number, age and distribution. This approach was done because OD values are semi-quantitative and cannot be used to get an accurate indication of the intensity of antibody response due to the lack of quantification methods for each subclass. There is an opportunity to improve on this research if a method of quantification can be developed. More work is needed in this area.

The graphical interpretations describe the data and shows that there is no correlation of CFA, IgG1 or IgG4 by age however, when the results were converted to percent positive for age groups, gave some interesting information. Percent positive data shows that there is a strong positive correlation of mf and CFA in each age group. The number of individuals developing mf is correlated to the number of persons who are positive for CFA, a correlation also observed in age-specific prevalence studies by Njenga et al., (2007) in Kenya, although in this study, the prevalence of CFA for each age group was correlated to IgG4 rather than IgG1 correlations in Njenga's study. The age group of 21-30 years old shows the highest prevalence of CFA and mf but the number of IgG1 positives are the lowest in this age group. There may be other factors at play such as the adaptive immune mechanism that lowers or blocks the IgG1 response where infection rate is the highest. Since IgG4 is strongly correlated with CFA and in high rates, it is a more appropriate marker for active infection than IgG1.

The CFA negative group shows that both antibodies are correlated across age groups. IgG1 equally represents exposure because of an observed increase as the age increases with a strong linearity while IgG4 is linear and correlated

strongly with infection. The IgG1 linearity observed here do not corroborate with the results Njena et al., (2007) where the age-specific IgG1 was shown to be higher in children than in adults. The observed linearity of IgG1 in CFA negative group is not observed in the CFA positive group. This observation confirms that the IgG1 rises and as the age increases, many also seroconvert to IgG1 because of prolonged exposure.

The presence of IgG1 also confirms the findings of Melrose et. al., (2000) on IgG1 exposure of expatriate workers in PNG and the findings of Frances et al., (2008) on the elevated IgG1 levels of exposed defence personnel in Timor-Leste. These studies, however, used either crude antigen extracts from *Dirofilaria immitis* or in the study of Frances et al., (2008), both of *D.immitis* antigen for screening and antigen from adult worm extracts of *B. malayi*. The results in this study do not, however, support the findings of Kurniwan-Atmadja et al., (1998) of a dominant IgG1 response over IgG4 in early filarial exposure.

The youngest age group was of interest because it is at this age that individuals are exposed to infection for the first time as opposed to re-infection in older ages. It is also interesting to make some observations at this age group because school-aged children are used as the sentinel group for monitoring of resurgence of infection after the implementation of PCT. The graphical output of data consistently showed a dominant IgG4 even in the youngest age group who did not have CFA. Both antibody classes IgG1 and IgG4 were present and both were high in % positivity. Both antibody responses may overlap because there was no definitive class switching observed where the IgG1 decreases and the IgG4 increases as a response to active infection. It is possible that because Metvoe has never received any drug intervention and the transmission is intense, the seroconversion is vigorous and antibody switching may not be observed. In the study by Terhell et al. (2000) of the paediatric population, it was found that IgG4 in the highly endemic community rises very quickly, and may be the case being observed here. The subclass switching may be better observed in a low transmission setting or a post PCT area.

The presence of both, IgG1 and IgG4 throughout the age groups does not correlate with the general antibody response knowledge found in the literature. The conceptual antibody response for infection assumes an IgG1 response that appears following incoming L_3 infection and as it wanes, a subclass switching

occurs with IgG4 that becomes dominant in the patent stages of infection. The IgG4 is said to wane, and mf becomes undetectable during advanced stages of infection (elephantiasis). Compared to the results of the experiments, it has been observed that IgG1 and (more prominently) IgG4 were present and detectable by the Bm14 ELISA across the age groups for those exposed (CFA-/Ab+) and those with active infection (CFA+). The antibody response, either IgG1 or IgG4 is confirmed in this experiment to be an excellent marker for exposure that can be used for young children in a sentinel group. It is expected that the youngest children born after the implementation of PCT will be free of infection and the use of antibody assay to confirm that the PCT programme has been effective in eliminating the transmission of infection, a vital part of a monitoring program.

Since IgG1 was not found to be significantly correlated to early exposure to L_3 compared to IgG4, further investigations can include studies of cohort from a low transmission area or sentinel. Also, because the available ELISA methods do not include quantification of antibodies, the studies can be improved by the additional development of a suitable antibody quantification method to investigate the IgG1 and IgG4 in greater detail.

CHAPTER 7

SEROLOGICAL APPLICATIONS AND EVALUATION STUDIES USING THE FILARIASIS ANTIBODY BM14 ELISA

7.1 INTRODUCTION

The World Health Assembly in 1997 (WHA50.29) identified lymphatic filariasis (LF) as a global public health problem that can be eliminated. The World Health Organisation (WHO) in 2000 formed the GPELF to support this cause. The GPELF aims were to: (1) stop the spread of infection by interrupting transmission and; (2) reduce the suffering of those who are affected. The strategy to eliminate was to interrupt the transmission of infection using a single dose of a combination of drugs over a period of five or more years to the entire at-risk populations (WHO, 2013a). The repeated MDA or PCT is said to be effective in killing the parasite and reducing the prevalence of mf and antigen in an implementation unit (IU). The rationale was to decrease mf and filarial antigen prevalence to very low levels where transmission can no longer be sustained (Bockarie et al., 2009; Rebollo & Bockarie, 2013). After nearly 15 years of elimination programmes, there has been a significant progress, partly, due to the phenomenal support of drug companies GlaxoSmithKine (GSK), Merck & Co. Inc., and Eisai (Ichimori et al., 2014; Ottesent et al., 2008). Their long-term donations of anti-filarial drugs made a difference to the elimination efforts.

In May 2013, the Sixty-sixth World Health Assembly (WHA66.12) met to agree and adopt some public health measures aimed at improving greater health benefits for all (WHO 2012). One of the 24 resolutions and five decisions in the WHA66.12 includes the acknowledgement of the 17 neglected tropical diseases (NTDs) that remain a public health burden, afflicting those in the poorest of communities. The five decisions of the assembly focused on the continued implementation of control, elimination and eradication and also its integration and access to intervention programmes aimed at accelerating NTD elimination efforts. Integral to the decisions was the move to reach the targets proactively as outlined in the WHO document, Roadmap for Implementation (WHO, 2012). The document outlines the strategies to accelerate the

efforts to control, eliminate and eradicate a list of neglected tropical diseases of which includes LF. By 2017, nearly all of the 81 LF endemic countries that are currently undergoing rounds of PCT should be close to meeting the requirements to stop and move to the surveillance phase of elimination. Those countries that need to start mapping or currently in the early phases of elimination would enter intervention programmes towards the target year 2020. It is expected that all 81 LF endemic countries will be either declared free of LF or be in the final stages of being declared LF-free by the end of the year 2020 (WHO, 2012).

Elimination programmes, most notably for helminth infections, need to assess the diagnostic approach to address specific needs at different stages of elimination, and require useful diagnostic tools suitable for each of the phases of elimination. As discussed by Weil & Ramzy (2007), mapping and planning requires diagnostic tools such as those that test for microfilariae, LF antigen, antibody and parasite DNA to define endemicity. These diagnostic tools help managers make decisions on where PCT programmes should be implemented by confirming whether active transmission exists in target communities. The next phase involves monitoring of PCT progress after implementation and assessing when PCT can be stopped. The end-point is established if an implementation unit (IU) is below the prescribed threshold of microfilariae or antigen prevalence and if not met, a continued PCT is to be administered. The final phase is the post-PCT surveillance programmes that aim to detect if the resurgence of transmission has occurred after the successful completion of rounds of PCT. It was agreed at the beginning of the GPELF elimination programmes that the antibody test would be used to detect the resurgence of infection in sentinel groups, and parasite DNA testing to detect any mosquito infection rates.

More than ten years have passed since the WHA meeting in 1997, with the elimination efforts having achieved a great deal of work. It is clear that the accelerated WHO neglected tropical disease (NTD) programmes (WHA66.12) acknowledged the urgency to address the objectives of the previous LF initiatives in 1997 (WHA.5029). In LF elimination where the year 2020 target is only a few years away, a multicentre study was conducted from 2007 – 2010. The study evaluated available diagnostic tools that can be used to define the end-point of PCT programmes (Gass et al., 2012). Six different countries evaluated seven different diagnostic tools, the mf detection, PCR, Tropbio Og4C3 ELISA, ICT BinaxNOW card test and, filarial antibody Bm14 ELISA, PanLF RDT and Urine SXP. In conclusion, the chosen diagnostic test for use

in defining end-points of PCT was the antigen ICT BinaxNOW card test for *W.bancrofti* and antibody BmR1-based rapid test (Pan-LF and Brugia Rapid) for *B.malayi*. Despite the lack of sensitivity of antigen tests in areas of low mf and antigen prevalence, antigen testing was recommended as the method for assessing transmission in IUs. The current WHO method for assessing transmission (WHO, 2011) uses these RDTs to detect antigen in bancroftian filariasis and antibody in brugian filariasis areas.

As discussed by Chu et al. (2013) in the transmission assessment surveys (TAS), antibody tests such as Bm14 ELISA were deemed more suitable for post-intervention surveillance studies. Antibodies to LF can potentially be a powerful indicator of the resurgence of infection in sentinel groups.

To address the need for a reliable antibody assay, standardisation work for the recombinant antigen (rAg) Bm14-based antibody ELISA commenced in the year 2002. The research-grade assay was developed by Dr Gary Weil's group at the School of Medicine, Washington University in St Louis, MO, USA (WU). The project was carried out at the manufacturing laboratories of Cellabs Pty Ltd (Sydney, Australia) from the process of re-design, standardisation and concluding with the commercialisation. The first batch of prototype ELISA kits was distributed for multicentre trials in 2007, attracting a variety of feedback on the quality, performance characteristics and general aspects of the Bm14 Antibody CELISA. The sharing of experience and information on the use of the kit in field conditions and laboratory setting resulted in overall assay improvement by a continued optimisation process that extended towards the year 2009. By the year 2010, the assay was commercialised and successfully registered in 2014 as an IVD International Standard (ISO13485) compliant product manufactured in Australia.

The objective of this chapter is to summarise the application of the Bm14 Antibody CELISA. The development of the Bm14 ELISA was not straightforward as it involved the feedback and demands of a dynamic global elimination initiative. Evaluation results and feedback of investigations that used the Bm14 ELISA as early as 2006 through to 2014 publications are covered.

7.2 AIM

The aim of this chapter is to summarise the important investigations and evaluations that have used the Bm14 ELISA as a detection tool for LF antibody. This demonstrates the applicability of a standardised assay available as a commercial product for LF elimination programmes, epidemiological research studies and clinical diagnostic use.

7.3 METHOD

Laboratory books were reviewed for any notes relating to product development and design changes from the year 2002 through to 2010, and other additional notes up to the year 2014. Computer files, emails and product development files for the Bm14 ELISA standardisation at Cellabs Pty Ltd were reviewed to include all informal product performance and evaluation results and discussion received from evaluating centres and other evaluating laboratories outside of GAELF. These were summarised for presentation.

A search using PubMed, Ovid and ProQuest databases accessed at James Cook University journal search library was done using key words “lymphatic filariasis Bm14 ELISA”, “Bm14 ELISA”, “LF antibody ELISA”, “filariasis CELISA” and “lymphatic filariasis elimination”. The search was extended using the Internet search engine, Google Scholar for the same keywords to find other publications.

7.4 RESULTS

7.4.1 Prototype Kit Evaluations

7.4.1.1 Prototype Evaluation November 2006

External evaluation using a small number of shared serum samples and titration by four different testing laboratories. The purpose of this evaluation was to test the prototype version of the Bm14 ELISA and determine if the performance characteristics are acceptable for use in a larger multi-centre trial. No available

details were given as to the conditions of testing, however, the kit instructions for use (IFU) were followed to perform the test. The laboratories used one batch of kits produced as batch KF303 with a cut-off value (COV) OD 0.2. Four laboratories used the kit to evaluate inter-laboratory reproducibility of the prototype kit namely: Cellabs, Sydney, Australia, the manufacturing laboratory (Lab A); Washington University School of Medicine (WU), St Louis, MO, USA (Lab B); Dr Pat Lammie at the Centers for Disease Control (CDC) lab (Lab C) and; Dr Tom Nutman laboratories (Lab D).

Figure 7.1 below shows a graph comparing the results of the trials at the four different laboratories. The discordant results were seen with the positive control recommended testing dilution at 1:100 with Lab A results reaching as high as OD 3.0. None of the other laboratories achieved a positive control result higher than OD 2.0. Test samples (n=5) shared between Lab A and Lab B showed a similar result ($t=0.119$, $p=0.908$) although the positive control tested at 1:800 was slightly lower in Lab B. Test results from both laboratories were inconclusive as the positive control results did not meet specification (OD> 1.5).

These results prompted the questions of kit stability by investigators. However, invalid positive control results were seen in Labs C and D, and agreement of results for Lab A and B, suggesting that kit stability was not an issue. There were discordant results between the testing laboratories including kit storage conditions that affected the kit stability. ELISA assay conditions such as assay time, temperature, sample dilutions and equipment performance were varied.

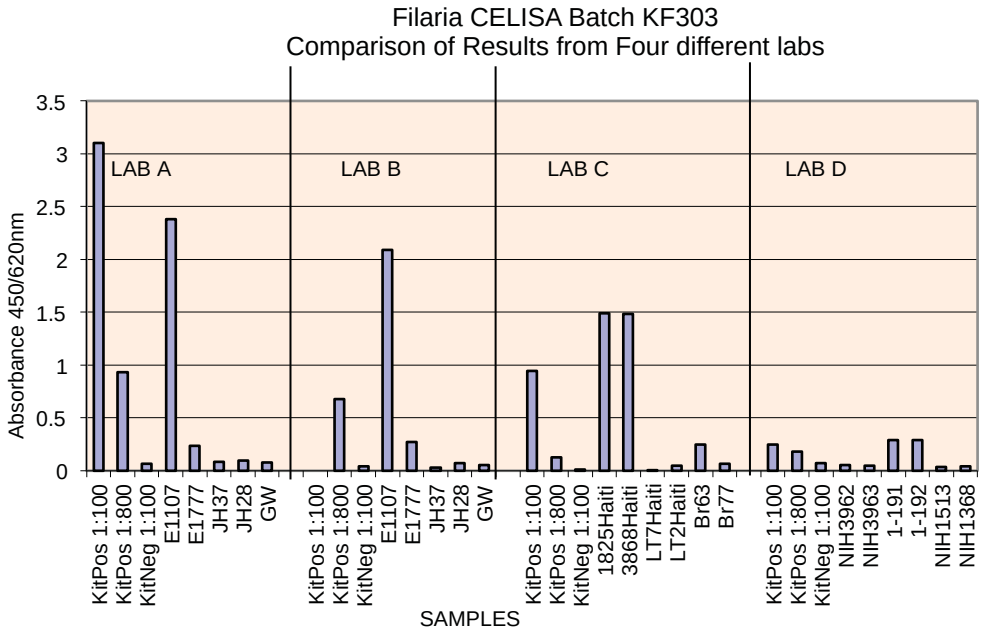


Figure 7.1: Multi-centre evaluation results of prototype kit version batch KF303. Lab A: Cellabs manufacturing laboratories, Sydney, Australia; Lab B: Washington University, MO, USA; Lab C: CDC laboratories, GA, USA and; Lab D: Dr Nutman Lab

7.4.1.2 Independent Multi-Centre Trials 2006

An independent multi-centre trial was conducted by WU involving four laboratories using a shared panel of coded serum samples. The purpose of this multi-centre trial was to evaluate the Bm14 ELISA independently, outside the GPELF group. These samples were a mix of brugian and bancroftian filariasis and other helminthic infections of strongyloidiasis and ascariasis. Samples from the non-endemic USA and samples from the serum bank of the testing laboratories were also reported. The testing laboratories in the study were; Washington University, St Louis, MO, USA (WU); Centers for Disease Control and Prevention, Atlanta, GA, USA (CDC); Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany (BNI) and; James Cook University, Townsville, QLD, Australia (JCU). The study was conducted by Dr Gary Weil's group at WU, and the results initially presented as a poster (Appendix 13) at the 57th

Annual Meeting of American Society of Tropical Medicine and Hygiene (ASTMH) conference in November 2008 and the final results published by Weil et al. (2011). A summary of the study results (Table 7.1) shows the sensitivity and specificity outcomes.

Table 7.1: Summary of sensitivity and specificity. Results from an independent multi-centre trial conducted using the prototype kit version prior to the Gates/GAELF multi-country evaluations.

Samples	Positive	Sensitivity	Samples	Positive	Specificity
B.malayi	32/35	91%	Non-endemic	0/20	100%
B.timori			Strongyloides	4/51	92.2%
W.bancrofti	96/98	98%	Ascaris	0/30	100%
			Rheumatoid factor	0/6	100%
Filter paper blood eluates vs serum/plasma	16/16	100% concordance	Onchocercariasis	19/25	76%
			M.perstans/ M.strepotcerca	*22/24	66.7% *area with some onchocerciasis
			M.perstans	*3/12	

7.4.2 Gates Foundation - GAELF Multi-centre Evaluation of Diagnostic Assays

7.4.2.1 The Gates/GAELF Multi-centre Evaluations

In July 2007, the Gates Foundation provided support to conduct a multi-centre evaluation with funds from the Taskforce for Child Development (now called Taskforce for Global Health) on behalf of GAELF. One of the objectives was to determine the most appropriate programmatic tools for LF community assessments entering PCT, the end-point assessments to determine when to stop intervention and, the continuing surveillance of resurging transmission (GAELF, 2007). The multi-centre trials commenced in the later months of the year 2007 through to 2009 to include six participating countries at five testing laboratories. Four different batches of Bm14 ELISA kits were sent out to meet the different testing times and logistic requirements of the five testing

laboratories. The kits were used in IUs believed to have low residual infection rates. Kits were shipped via international courier (Federal Express, Sydney, Australia) from the manufacturing laboratory at Cellabs Pty Ltd, Sydney, New South Wales, Australia in thick cardboard boxes with gel ice-packs and took an average of four days to reach the testing laboratories. Each laboratory was advised to store the kits at 2-8^{o}C upon arrival. Results for this multicentre evaluation appears in the final journal publication by Gass et al., (2012).

Table 7.2 summarises the batches of kits and feedback as reported by the primary investigators to the Bm14 ELISA manufacturing laboratory. The following sections (7.4.2.2 – 7.4.2.4) summarises results and feedback shared by the group.

Table 7.2: Summary of Bm14 ELISA kit batches for evaluation

Country	Testing Laboratory	Kits sent and Batch	Comments
Zanzibar	Smith College	4 x KF307 8 x KF308 8 x KF311	No shared results received by manufacturing laboratory.
Haiti	CDC	10 x KF307 2 x KF307 6 x KF308	Kit Positive control for KF308 was variable. Internal positive control validated the assay.
French Polynesia	Institute Louis Malarde (ILM)	4 x KF307 4 x KF308	April 2008 testing at ILM found that kit positive controls did not meet specifications at >2.0 OD. Control results were variable at 0.8-1.1 OD Sample buffer and conjugate diluent were found to be contaminated with fungal growth. Replacement buffers were sent.
Sri Lanka Malaysia Indonesia	Washington University (WU)	10 x KF307	Kits were found to be stable beyond the 12-month shelf life. Background problems with the use of filter paper blood spots with 2009 kits caused false positives. Results not used.
Ghana	WHO, Accra (Noguchi Lab)	4 x KF307 10 x KF308	No shared results received by manufacturing laboratory.
Tuvalu Fiji	JCU/WU	8 x KF307 4 x KF308	Background problems with the use of filter paper blood spots caused false positive results. Results not used.
India	-	-	Cancelled

7.4.2.2 Tahiti Post-intervention Studies

During the multi-centre studies, two batches of kits were sent to Institute Louis Malarde (ILM), Tahiti, French Polynesia, for evaluation under the supervision of Dr Catherine Plitchart. It was reported that bottles of conjugate diluent and sample diluent from the first batch of kits were contaminated. Records show that the contamination was due to fungal growth in the buffers containing a small percentage of calf serum. This contamination was confirmed by inspecting the retention stock stored at 2-8^{o}C at the manufacturing lab. Following the report, experiments were conducted to address this observation and find an alternative formulation and methods of dispensing that will prevent the introduction of contaminants. Standard operating procedures (SOPs) were implemented to improve the preparation of buffers. The SOP included filtering the buffers with 0.2um size filters and adding 1% Bronidox®L (Cognis, BASF, Australia), a microbial solution inhibiting bacterial growth and enzyme activity. No reported cases of buffer contamination have been received since the buffer modification.

In April 2008, results of studies with serum samples from Tahiti were shared for comment with the manufacturing laboratory. Figure 7. 2a are results of serum testing from Tahiti by Dr Plitchart. Figure 7.2b shows the standard curve in three plates.

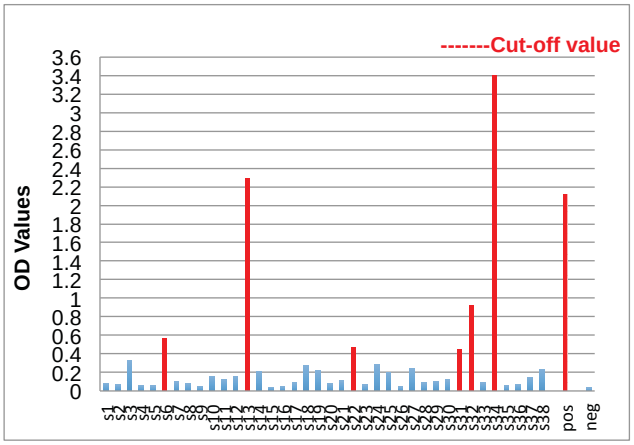


Figure 7.2a: Results of serum samples from Tahiti. Chart shows six IgG4 positive samples at a cut-off of OD 0.4, courtesy of Dr Catherine Plichart, ILM, French Polynesia

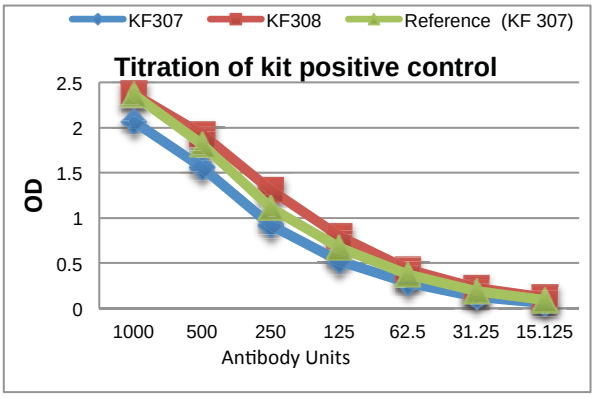


Figure 7.2b: Results of kit batch positive control. Titration of positive control for the Bm14 assays to demonstrate the validity of results in Fig 7.2a.

The shared result in Fig. 7.2b above demonstrates a valid ELISA according to kit specifications (>OD2.0 at 1:100 dilution). The positive results in 7.2a indicate an IgG4 response to exposure and mark the presence of transmission, although further studies in this IU is required to verify the findings. No other communications and follow-up were received and no publications were written on this study. This short evaluation report from an IU in Tahiti demonstrates an effective application of the Bm14 ELISA in surveillance programmes to detect the resurgence of infection.

7.4.2.3 CDC Bm14 ELISA Testing Results

The CDC testing laboratory tested filter paper blood spot samples from Haiti under the supervision of Dr Patrick Lammie. The testing procedures at the laboratory followed the kit IFU and used the kit positive control (PC) at a preferred dilution of 1:200 as the laboratory's preferred dilution, instead of the kit recommended a dilution of 1:100. An additional internal positive control (IC) was also used. Both controls were included in each plate of testing to verify assay conditions and are shown below (Fig. 7.3). Some results were shared with the manufacturing laboratory for comment. Upon analysis, the control OD results were found to be variable on different plates using kit batch KF307. The result varied between OD 1.5 and OD2.5 (blue dots), compared to result for batch KF308 having more consistent results ranging between OD 2.0 and OD

2.5 (grey dots). The IC tested consistently at one dilution, between OD1.0 and OD1.5, suggesting assay batch-to-batch consistency and validity (orange and yellow).

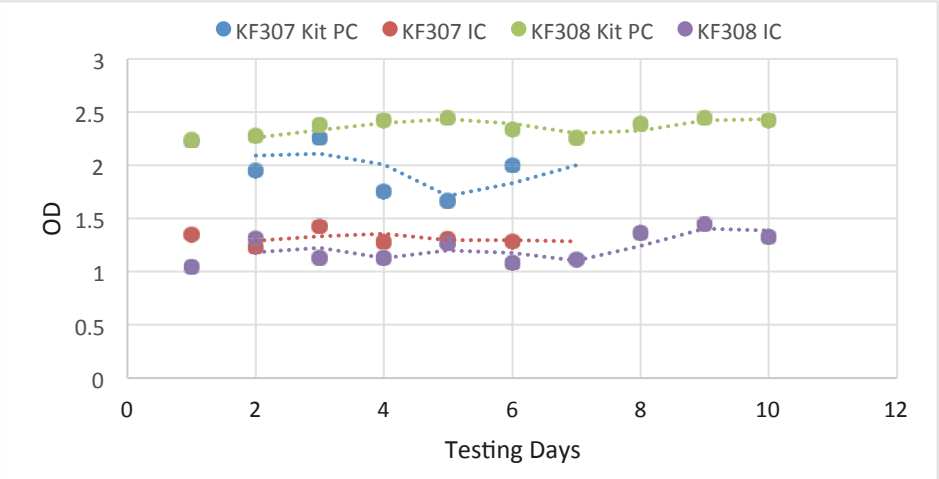


Figure 7. 3: Testing results for CDC testing laboratory. Positive control (PC) results for Bm14 ELISA for (a) batch KF307 showing a variation of OD between 1.5-2.5 at a dilution of 1:200 and; (b) a more consistent results for batch KF308 with higher ODs between 2.0-2.5. Internal control (IC) was used as and additional multi-centre control.

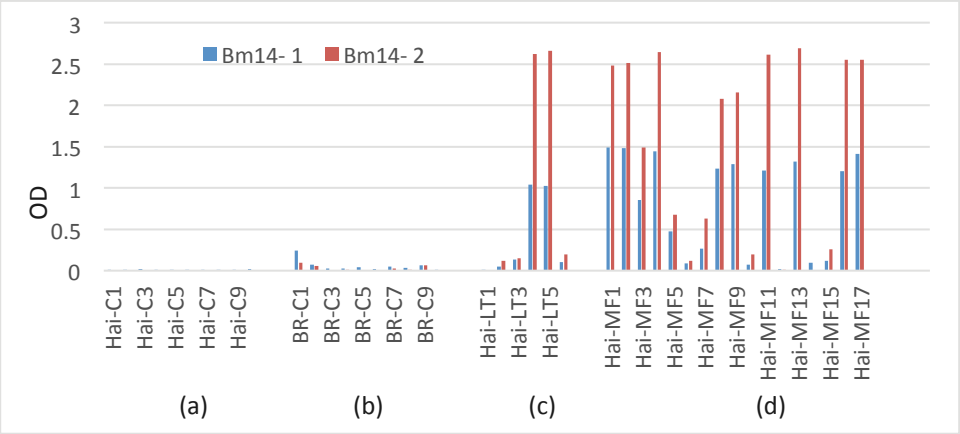


Figure 7.4: CDC results courtesy of Dr Patrick Lammie. **(a)** Haiti samples mf (-); **(b)** Brazil samples mf (-) and strongyloides (+); **(c)** Haiti samples mf (-) with lymphoedema and; Haiti samples mf(+).

Results in Figure 7.4 graph shows a comparison of the research-grade assay using Bm14 antigen (Bm14-1) and the standardised Bm14 ELISA (Bm14-2). The difference between the reactivity is significant. The less sensitive research-grade assay (blue) misses samples detected as positive using the standardised assay (red).

7.4.2.4 Washington University Results

Filter paper blood spots from Sri Lanka and also from Tuvalu were tested at the laboratory in WU under the supervision of Dr Gary Weil. The Bm14 ELISA procedure in the instructions for use (IFU) was not followed. Filter paper blood spots were used as samples instead of serum and the protocol was amended to use 2 hours incubation time for samples. The samples used in this survey were from an area with several rounds of PCT and approximately 0.3% mf positive (mf+) and 3.5% antigen rates (1.3% ICT-antigen rates) but 70% antibody positive by the Bm14 ELISA. It was expected that the survey results would reflect low mf rates. However, the unexpected high antibody rates have apparently cast doubt over the results, which, consequently questioned the kit performance. It also reported that these results applied to a few study sites in Sri Lanka and Tuvalu but not all. Results shared in July 2009 were deemed untenable and were not used.

Figure 7.5 below shows results from an investigation at the WU testing laboratory into the variations seen with the prototype kit in 2008 compared with the kits received in 2009, using filter paper blood spots as the source of serum.

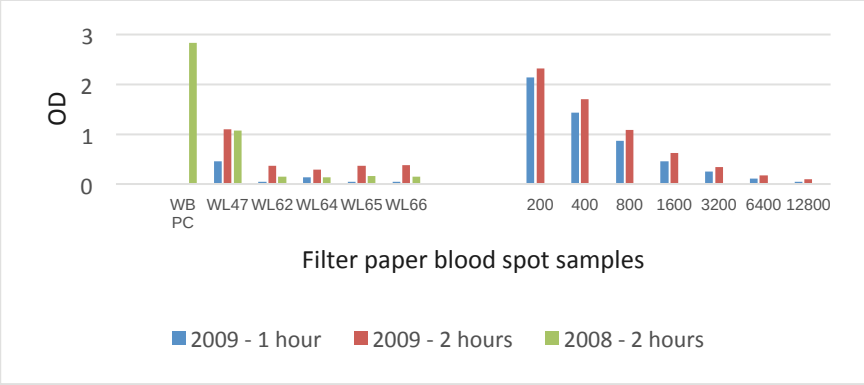


Figure 7.5: Results from Dr Gary Weil at WU. Comparison of results from prototype kits and optimised GMP kits released in 2009, courtesy of Dr Gary Weil at WU.

7.4.3 WHO LF Support Centre, James Cook University (JCU)

7.4.3.1 Thesis for the degree of Doctor of Philosophy: Lymphatic Filariasis Elimination: Residual Endemicity, Spatial Clustering and Future Surveillance Using the New Filariasis CELISA Diagnostic Assay.

The thesis research work is by Joseph (2010) from the LF Support Centre, School of Public Health, Tropical Medicine and Rehabilitation Science, JCU. It made use of the Bm14 ELISA as the antibody detection tool to address the LF elimination needs in the Pacific Islands, particularly Samoa. This important research work has demonstrated the usefulness of the Bm14 ELISA in the field for LF transmission assessment, PCT end-point determination and surveillance. Four journal publications from 2010 – 2011 resulted from this thesis are summarised in the sections below.

7.4.3.2 First evidence of spatial clustering and transmission assessments in Samoa.

A study of five villages in Samoa was conducted to determine areas of residual foci for the purpose of determining transmission patterns and clustering that would help in making decisions about ongoing elimination programmes. This study was conducted in May 2008 before the seventh round of PCT in the two islands of Samoa. Because such an assessment requires a sensitive diagnostic tool to detect exposure to infection, the Bm14 ELISA was chosen as the primary tool for antibody detection. Finger-prick blood samples from school-aged children under ten years of age were used because it was expected that these sentinel children would not be exposed to infection if the PCT programme had been successful. The study successfully demonstrated special clustering of infection by identifying hotspots of antibody positive children. Following further verification studies, the hotspots detected were linked to the presence of microfilariae positive individuals in households within proximity to each other. For further details on this interesting study, a journal publication is available by Joseph et al., (2010).

In the same study, the five villages were assessed for LF transmission since the 1999 MDA rounds. This study demonstrates the importance of using complimentary, sensitive, reliable diagnostic tools for LF elimination assessments such as the use of the antibody test along with mf and circulating filarial antigen (CFA) detection tools. Children less than 9 years old, born before the interventions were included in the assessments. Five villages were found to have varying mf+ and CFA positive prevalence with different degrees of antibody prevalence, including those children less than 9 years old born before the MDA. The results of this study were published by Joseph et al. (2011a).

7.4.3.3 The use of serum from filter paper blood spots for testing in the Bm14 ELISA

Blood spot collection using filter paper discs (Cat. No. 05-002-12, Tropbio, Australia) was adopted by the GAELP elimination programme as the source for test serum in the Bm14 ELISA. Blood spot eluates from filter paper discs is a very convenient method of field sample collection compared to venous blood collection. It provides many advantages, however, the stability of antibodies on filter paper have not been validated for use with the Bm14 Antibody CELISA. Background problems have been observed with negative samples resulting in a high percentage of false negatives. Because the thesis work by Joseph required the use of serum from filter paper blood spot eluates, its application in a field setting in Samoa has been studied in great detail by Joseph & Melrose (2010). The sharing of results and blood spot samples between the end-user laboratory and the manufacturing laboratory for the purpose of improving kit performance has been important in the development and standardisation work for the Bm14 ELISA.

7.4.3.4 Application of antibody testing using Bm14 ELISA in the Elimination Programmes of the South Pacific.

Three countries, Tonga, Vanuatu and Samoa under the Pacific Island Countries and Territories (PICT) elimination programmes used the Bm14 ELISA as an antibody diagnostic tool for surveillance studies. The strategy was to use child transmission surveys (CTS) by CFA, followed up by mf detection to determine if the transmission is ongoing. It has also been identified that mf and CFA

detection tools become less sensitive as the transmission rates are lower at the surveillance stages of LF elimination programmes therefore antibody tests have become a reliable indicator for areas with residual endemicity. The study found Vanuatu and Tonga sentinel children to be negative for CFA with antibody prevalence at 6% and 6.3% respectively. Samoa had mf and CFA positive children with antibody prevalence as high as 30.7% requiring further investigations. Details of this study can be found in the published journal article by Joseph et al. (2011b)

7.4.4 Epidemiology Work in West Africa by Dr Simonsen's group

Following the standardisation work of the Bm14 ELISA in 2009, the kits were made available for commercial use outside of the GAELF elimination programmes. Dr Paul E Simonsen and his group at the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark (UoC) used the Bm14 ELISA to assess the LF endemic areas in West Africa. Ongoing studies are being conducted using the Bm14 ELISA as the marker for exposure to transmission and the published journal articles of the studies before December 2014 are listed in Table 7.3.

Table 7.3 Published investigational work by Dr Simonsen et al.

Study site	Purpose of study	Publication
Metropolis of Dar es Salaam, Tanzania	Epidemiological study to assess LF for planning, implementation and control	Mwakitalu et al. (2013a)
Luangwa District, South-East Zambia	Epidemiological study to assess LF for planning, implementation and control	Shawa et al. (2013)
Tanga, Tanzania	Assessment of LF disease and transmission after seven rounds of MDA.	Mwakitalu et al.(2013b)
Tanga, Tanzania	A detailed study on the effects of six rounds of MDA as implemented by the National Lymphatic Filariasis Elimination Programme (NLFEP) in north-eastern Tanzania.	Simonsen et al. (2013)

7.4.5 Surveillance Studies After Five Years of PCT in Menoufiya, Governorate, Egypt.

In February 2012, a transmission assessment survey (TAS) was conducted in three sentinel villages in the district of Menoufiya Governorate. Serum samples from children ages 6-7 years old, born after five rounds of PCT were tested using the Bm14 ELISA to determine antibody exposure status. This was used as the indicator to determine whether the transmission was ongoing in these villages expected to have interrupted LF transmission. The study found no antigen positive children using the ICT/BinaxNow rapid card tests but found variable antibody prevalence of 9%, 1.9% and 0.2% in the three sentinel sites. The results may also indicate the low sensitivity of antigen tests in these post-PCT areas where mf and antigen prevalence are at very low levels. These results suggest that residual transmission is present, however, this needs to be validated, perhaps determining whether antibody positive individuals are also positive for mf. Further studies using a comprehensive method may help in finding potential hot-spots.

7.4.6 A comprehensive TAS Study in Sri Lanka

After six years of PCT, Rao et al., (2014) conducted a TAS in eleven evaluation units (EUs) between 2011 and 2013. The comprehensive study followed the WHO TAS recommendations (WHO, 2010) and additional antibody testing using the Bm14 Antibody CELISA. Mf rates were found to be <1%, the provisional target rate. The CFA results ranged from < 2% to meet the provisional target rate but prevalence in some testing sites were as high as 3.4%, over the target rate. In school-aged children tested, the antibody prevalence was >2% target rate and some of those who had high antibody response also tested positive for CFA. The 3 children of the 137 children tested who were positive for CFA were also found to be positive for mf. Compelling evidence of the ability to find hot-spots and a more accurate determination of ongoing transmission have been demonstrated.

This comprehensive study highlights the value of including antibody testing to define correctly the transmission status of EUs. It also demonstrates how the TAS method of assessment is not sensitive for post-PCT studies. All EUs tested meet the criteria of TAS to stop PCT and if antibody testing were not done in these areas, it could potentially cause the elimination efforts to be wasted because transmission can be re-

established. The investigators recommend the use of the Bm14 Antibody CELISA as part of a comprehensive assessment study.

7.5 DISCUSSION

Feedback from early multicentre trials by Dr Gary Weil et al. from 2004-2008 and the Gates/Global Alliance to Eliminate Lymphatic Filariasis (GAELF) multi-country trials have been instrumental in the final design of the Bm14 ELISA. The standardisation process took approximately eight years because of the need to address specific programme requirements of the GAELF. Optimisation of the kit was completed following the reports from the prototype evaluations but some of the published investigations mentioned above have utilised the prototype kit version. The optimisation process ensured that the kit performance was consistent by using reference samples for validation.

The early multi-centre trials by Dr Weil's group raised the issue of kit stability seen with the prototype kit when a cool room used to store the Bm14 ELISA kits at WU failed during a weekend. This incident exposed the kits to >30^{o}C temperature for an unknown amount of time. The prototype kit was expected to withstand variable temperature (2 – 40^{o}C) outside the recommended 2-8^{o}C storage temperature for a maximum of 7 days. The reported incident resulted in a more detailed optimisation of assay design which revealed that the plate coating buffers that contained neonatal calf serum (NCS) was affected by high temperatures that caused the unstable plates (see Chapter 2). After modifying the plate-coating buffers to address stability, it was also found that removing NCS, which was in the original prototype plate version, not only improved stability but has also increased sensitivity. It is possible that NCS, being a biological material, is sensitive to higher temperatures and, therefore, causes the breakdown of antigens it is coating. Using the classical carbonate-bicarbonate plate coating buffer improved the stability of the plates. It is also noted that the NCS blocks the antigen binding sites by competition, therefore removing this from the coating buffer frees up the binding sites. This resulted in increased assay sensitivity seen in the final kit version released at the beginning of the year 2009, after the Gates/GAELF multi-country trials.

One of the important lessons learnt from the evaluations was the failure to highlight the need to follow instructions as stated in the IFU. A diagnostic test must always be used as intended by the manufacturer. The Bm14 ELISA was designed for use with serum samples from whole blood although the majority of the Gates/GAELF trials had used filter paper blood spots as the source of serum. ELISA design and development can be exacting because the kit contains some components, buffers and reagents that work together specifically. This means that introducing any factor such as the use of blood spot serum eluates, which is not the intended sample for use, renders the assay invalid. To address this shortfall, each laboratory needs to optimise and adopt the Bm14 Antibody CELISA for use with the preferred sample other than the intended serum from whole blood. This was done successfully at the WHO Support Centre collaborating laboratory for LF at JCU by Joseph and Melrose for the purpose of epidemiological work in the PICT. However, other evaluation laboratories failed to follow the kit instructions and used filter paper blood spots without prior validation of methods.

It is clear that communication lines between the user and manufacturer must be open to ensure that the end users are guided. All stakeholders (end-users, elimination project managers, developers etc.) need to be clear of their project requirements or objectives and the expected outcomes.

This chapter has summarised the general applications of the standardised Bm14 Antibody CELISA kit and how the release of a prototype kit and final version kits were used. It has also included the problems reported at the manufacturing laboratory that helped in the design and re-design of the assay to address the requirements of the intended purpose of the kit. The lessons learnt were discussed above and can be used as a guide for future development of similar diagnostic kits.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 DISCUSSION AND CONCLUSIONS

The global LF elimination programme is moving swiftly towards the set objectives by the target year of 2020. Halfway through the programme, the remaining 71 countries have implemented preventive chemotherapy (PCT) and are progressing towards the final stages of elimination (WHO, 1010). Reliable diagnostic tools are imperative in determining the end-points of PCT so a programmatic decision can be made on whether to stop PCT or continue with additional rounds. Suitable diagnostic tools are also important for the surveillance stages after PCT has been stopped, to verify that no resurgence of infection has occurred. After fifteen years, the strategy of the elimination programme has been successful in achieving an estimated 46% reduction of the *at-risk* population (Hooper et al., 2014). This remarkable progress is due to a concerted effort. It encompasses the integration of activities with the neglected tropical diseases (NTDs), vector control, morbidity management and the improvement of screening methods and diagnostic tools that should see the global effort towards its goal. Although there are challenges that are currently being debated, one of these is the lack of a unified method for transmission assessment.

In the WHO (2013b) LF training guide in monitoring and transmission assessment survey (TAS), the recommended diagnostic tests for use are the detection of mf from blood films and the antigen rapid test (RDT) detection using ICT BinaxNow card tests for *W.bancrofti*. For areas of *Brugia* spp., the Brugia Rapid test for antibody is used. At the commencement of PCT programmes, the mapping stages have used the methods of mf and antigen RDT detection. However, as the PCT progresses through towards the final stages of the programme, these may not be adequate diagnostic tests for use in monitoring and transmission assessment. The levels of antigen and mf at the final stages of PCT are very low, and in effect, the sensitivity of these diagnostic tests are no longer adequate. What is needed for the global elimination programme is to retract to the proposed antibody detection strategy for the final stages of PCT.

The inclusion of antibody testing for a comprehensive transmission assessment study was highlighted by a number of investigators in the LF research community (Lammie et al., 2004; Melrose et al., 2004; Grady et al., 2007; Weil et al., 2011; Mladonicky et al., 2009; Joseph et. al., 2010b; Rao et al., 2014; Chu et al., 2013). The rationale for the use of antibody tests in sentinel groups is because antibody response to LF parasite exposure is quick and can easily be detected by reliable diagnostic tools. The Bm14 Antibody CELISA was developed and standardised in this research work for this specific purpose. It was optimised to detect very low immunological changes to LF IgG4 in serum samples from a sentinel group. In essence, antibody testing is the most practical method of determining whether the transmission is present in an assessment unit (AU). As discussed by Berguist, Johansen & Utzinger (2009), the study of helminths follows a pattern when it comes to the diagnostic approach to elimination methods. They point out the need for a diagnostic approach that takes into account the changing situation when prevalence, distribution and severity of disease change. For the WHO TAS method, it seems, the same diagnostic tools are being used for both, mapping and monitoring stages. There needs to be a comprehensive method of assessment of transmission at these final stages of the programme that lends itself to an approach that is tailored specifically for a particular AU. Every AU is in varying degrees of prevalence and the one-fit-all method of transmission assessment is not suitable (Rao et al., 2014).

Diagnostic assays need to be reliable for it to be of any practical use for clinical diagnosis of samples or epidemiological research use. To ensure the reliability of a diagnostic kit, standardisation and optimisation to the intended purpose is paramount. This is a process that cannot be replicated in a research laboratory because of the lack of industry-based knowledge and infrastructure. Commercial methods of standardisation and manufacture, including documentation and quality control procedures have been covered. Since every diagnostic kit that is produced and sold in Australia and overseas needs to be registered with the Australian regulatory body, the TGA, anyone who wants to develop a test needs to be aware of certain regulatory rules that needs to be inbuilt in the design of a diagnostic test. The purpose of regulation is to ensure that the test being developed is of high quality, it performs as intended, and there is no risk of harming the health of an individual or the public. The important process of regulatory affairs applied for the Bm14 Antibody ELISA registration with the TGA is covered in this study. It will provide information that can be used by other diagnostic researchers when traversing the confusing regulatory affairs

framework. It will also provide information on the important requirements for producing a reliable diagnostic test that can be applied for any similar test.

After the development of the Bm14 Antibody CELISA, the test was used to investigate a research question on the role of IgG1 in the early detection of infection. Results of the experiments in Chapter 6 shows that IgG1 is equally present in the presence of IgG4 but in lower levels. Both IgG1 and IgG4 are seen in all age groups who do not have circulating filarial antigen (CFA) detected. These reactions are LF specific, and IgG4 as we know, can be used as a surrogate for an active infection, but what do the persistent levels of IgG1 represent? A switch from IgG1 to IgG4 was not evident and it is assumed that this subclass switching may appear in infants as they are exposed in a high-transmission area. The response to early infection may vary in low-transmission area and this would be an area for further investigation. To investigate the mechanisms of subclass switching and what they represent in an age-profile study, additional work is needed using appropriate samples for study. Perhaps samples from a younger age group or infants in a sentinel site or low transmission area.

The Bm14 Antibody CELISA was successfully used in the transmission assessment of elimination programmes across the globe. Chapter 7 summarises these investigations to support the application of the Bm14 Antibody CELISA. One of the “lessons learnt” in this translational research is the sharing of information among stakeholders that are considered integral in diagnostic test design and development. The aims, requirements and objectives of a project need to be clear for all concerned. For example, the Bm14 Antibody CELISA was originally designed and developed for use with serum samples. All the experimental work was based on serum samples, the assay optimisation is specific for serum samples and very exact. The introduction of filter paper blood spots for use with the Bm14 Antibody CELISA was found to give high background results as reported by some investigators, although the work of Joseph et al., (2010) using filter paper blood spots resulted in its favourable application for use with the Bm14 Antibody CELISA. The use of filter paper blood spots for the global elimination programmes only came after the Bm14 Antibody ELISA was released for external evaluations for the Global Alliance to Eliminate Lymphatic Filariasis (GAELF)/Gates Foundation project (GAELF, 2007). Additional work is needed to adopt the current assay for use with filter paper blood spots.

The development of the RDT version of the ELISA was intended to be used as an alternative test for field work. There is a growing need for reliable RDTs for field-work because of its ease of use and convenience. Field-work for malaria eradication programmes has used the RDT test successfully for many years now. Similarly, the Bm14 RDT dipstick can potentially be a field-based test where the laboratory-based ELISA is not practical. The initial functionality tests resulted in performance comparable to the ELISA format and has the potential to be used for transmission assessment of elimination programmes. The lack of further evaluations to verify the performance characteristics needs to be addressed to investigate the usability of the dipstick. It will require independent evaluations similar to the multi-centre trials for the Bm14 ELISA.

8.2 FUTURE DIRECTIONS

The completion of this research has raised some questions that need to be investigated by further research. These are:

(a) Why is IgG1 persistently present in exposed children who also have IgG4?

(b) The development of an antibody quantification method can assist in determining further research into the antibody response to LF. More work is needed to develop an LF antibody IgG1 and IgG4 quantification method.

(c) Upon exposure, when does the subclass switching from IgG1 to IgG4 occur? Studies in low transmission area or sentinel are antibody response may yield some useful results.

(2) The Bm14 Antibody CELISA can be optimised to work well with filter paper blood spots. Further research is recommended to adopt the assay for filter paper blood spot for use without affecting the performance characteristics.

(a) What is causing the reported background problems when using filter paper blood spots?

(b) What is the correct ratio of filter paper blood spot required that is equivalent to serum? Comparative studies are required to determine the correct ratio that all laboratories for the elimination programme can use.

(3) What are the performance characteristics of the Bm14 RDT dipstick test? Multi-trial evaluations are required to determine the performance characteristics of the dipstick test. The assay needs to be evaluated for field use to determine the practicality and usability for the elimination programmes.

REFERENCES

Achidi, E. A., Anchang, J. K., Minang, J. T., Ahmadou, M. J., & Troye-Blomberg, M. (2005). Studies on Plasmodium falciparum isotypic antibodies and numbers of IL-4 and IFN-γ secreting cells in paired maternal cord blood from South West Cameroon. *International journal of infectious diseases*, *9*(3), 159-169.

Addis, D., Bettinger, J., & Dreyer, G. (2000). The role of albent and prevention of problems associated with lymphatic filariasis: part 1. *Learner's Guide*, 1-51.

Addiss, D. G., Louis-Charles, J., Roberts, J., LeConte, F., Wendt, J. M., Milord, M. D., Lammie, P.J. & Dreyer, G. (2010). Feasibility and effectiveness of basic lymphedema management in Leogane, Haiti, an area endemic for bancroftian filariasis. *PLoS neglected tropical diseases*, *4*(4), e668. Doi: 10.1371/journal.pntd.0000668.

Adjobimey, T., & Hoerauf, A. (2010). Induction of immunoglobulin G4 in human filariasis: an indicator of immunoregulation. *Annals of tropical medicine and parasitology*, *104*(6), 455-464.

A history of pregnancy testing. (2015), p.6. Retrieved from http://history.nih.gov/exhibits/thinblueline/timeline.html

Akobeng, A. K. (2007). Understanding diagnostic tests 3: receiver operating characteristic curves. *Acta paediatrica*, *96*(5), 644-647. doi:10.1111/j.1651-2227.2006.00180.x.

Alexander, N. D. (2015). Are we nearly there yet? Coverage and compliance of mass drug administration for lymphatic filariasis elimination. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, tru204.

Allen, J. E., & Maizels, R. M. (2011). Diversity and dialogue in immunity to helminths. *Nature Reviews Immunology*, *11*(6), 375-388.

Anderson, W. (1997). Geography, Race and Nation: Remapping 'Tropical' Australia, 1890-1930. *Historical Records of Australian Science*, *11*(4), 457-468.

Awogun, I. A. (1978). A New Staining Technique For Microfilariae. *Biotechnic & Histochemistry*, *53*(4), 187-189.

Babayan, S. A., Allen, J. E., & Taylor, D. W. (2012). Future prospects and challenges of vaccines against filariasis. *Parasite immunology*, *34*(5), 243-253. Doi:10.111/j.1365-3024.2011.01350.x.

Babayan, S. A., Attout, T., Harris, A., Taylor, M. D., Le Goff, L., Vuong, P. N., ... & Bain, O. (2006). Vaccination against filarial nematodes with irradiated larvae provides long-term protection against the third larval stage but not against subsequent life cycle stages. *International journal for parasitology*, *36*(8), 903-914.

Babu, S., Blauvelt, C., Kumaraswami, V. & Nutman, T.B. (2005). Diminished expression and function of TLR in lymphatic filariasis: A novel mechanism of immune dysregulation. Journal of Immunology, 175:1170-1176. doi:10.4049/jimmunol.176.7.3885

Babu B., Nayak A., Dhal, K., Acharya, A., Jangid, P., & Mallick, G. (2002). The economic loss due to treatment costs and work loss to individuals with chronic lymphatic filariasis in rural communities of Orissa, India. Acta tropica, 82(1), 31-38.

Babu, S. & Nutman, T.B. (2012). Immunopathogenesis of lymphatic filarial disease. *Seminars in Immunopathology*, 34 (6): 847-861. doi:10.1007/s00281-012-0346-4.

Babu, S. & Nutman, T.B. (2014). Immunology of lymphatic filariasis. Parasite Immunology, 36: 338-346. Doi:10.1111/pim.12081.

Bal, M. S., Beuria, M. K., Mandal, N. N., & Das, M. K. (2003). Parasite antigenaemia and IgG4 antibodies to a filarial protease in an endemic human population in India. Journal of helminthology, 77(04), 287-290.

Bartholomay, L. C., & Christensen, B. M. (2002). Vector-parasite interactions in mosquito-borne filariasis. In *The filaria* (pp. 9-19). Springer US.

Beaver, P. C. (1970). Filariasis without Microfilaremia Am J Trop Med Hyg March 1970 19: 181-189. *Am J Trop Med Hyg*, *19*, 181-189.

Bell, D. (1967). Membrane filters and microfilariae: a new diagnostic technique. *Annals of tropical Medicine and Parasitology*, *61*(2), 220.

Bergquist, R., Johansen, M. V., & Utzinger, J. (2009). Diagnostic dilemmas in helminthology: what tools to use and when?. *Trends in parasitology*, *25*(4), 151-156.

Boakye, D. A., Baidoo, H. A., Glah, E., Brown, C., Appawu, M., & Wilson, M. D. (2007). Monitoring lymphatic filariasis interventions: Adult mosquito sampling, and improved PCR–based pool screening method for Wuchereria bancrofti infection in Anopheles mosquitoes. *Filaria journal*, *6*(1), 13.

Bockarie, M. J., & Deb, R. M. (2010). Elimination of lymphatic filariasis: do we have the drugs to complete the job?. *Current opinion in infectious diseases*, *23*(6), 617-620. Doi: 10.1097/QCO.ob013e32833fdee5.

Bockarie, M. J., Fischer, P. E. T. E. R., Williams, S. A., Zimmerman, P. A., Griffin, L. Y. S. A. G. H. T., Alpers, M. P., & Kazura, J. W. (2000). Application of a polymerase chain reaction-ELISA to detect Wuchereria bancrofti in pools of wild-caught Anopheles punctulatus in a filariasis control area in Papua New Guinea. *The American journal of tropical medicine and hygiene*, *62*(3), 363-367.

Bockarie, M. J., Pedersen, E. M., White, G. B., & Michael, E. (2009). Role of vector control in the global program to eliminate lymphatic filariasis. *Annual review of entomology*, *54*, 469-487.

Boggild, A. K., Keystone, J. S., & Kain, K. C. (2004). Tropical pulmonary eosinophilia: a case series in a setting of nonendemicity. *Clinical infectious diseases*, *39*(8), 1123-1128.

Boreham, P.F.L. and Marks, E.N., 1986. Human filariasis in Australia: introduction, investigation and elimination. In *Proc R Soc Qld* (Vol. 97, pp. 23-52).

Brady, M. (2014). Seventh meeting of the Global Alliance to Eliminate Lymphatic Filariasis: reaching the vision by scaling up, scaling down, and reaching out. *Parasites & vectors*, *7*(1), 1-19.

Brantus, P. (2009). Ten years of managing the clinical manifestations and disabilities of lymphatic filariasis. *Annals of Tropical Medicine & Parasitology*, *103*(Supplement-1), 5-10. Doi:10.1179/000349809X12502035776432.

Bundy, D.A.P., Grenfell, B.T. & Rajagopalan, P.K. (1991). Immunoepidemiology of lymphatic filariasis: the relationship between infection and disease. Immunology Today, 12(3): A71-5

Burgess, G. W. (Ed.). (1988). *ELISA Technology in Diagnosis and Research: Short Course: Papers*. Graduate School of Tropical Veterinary Science, James Cook University of North Queensland.

Burkot, T. R., Durrheim, D. N., Melrose, W. D., Speare, R., & Ichimori, K. (2006). The argument for integrating vector control with multiple drug administration campaigns to ensure elimination of lymphatic filariasis. *Filaria journal*, *5*(1), 10.

Caballero, Z. C., Sousa, O. E., Marques, W. P., Saez-Alquezar, A., & Umezawa, E. S. (2007). Evaluation of serological tests to identify *Trypanosoma cruzi* infection in humans and determine cross-reactivity with *Trypanosoma rangeli* and *Leishmania* spp. *Clinical and vaccine immunology*, *14*(8), 1045-1049.

CDC, (1993). Recommendations of the International Task Force for Disease Eradication. Retrieved from http://www.cdc.gov/mmwr/PDF/rr/rr4216.pdf

Chandra, G. (2008). Nature limits filarial transmission. *Parasit Vectors*, *1*, 13.

Chandrashekar, R., Curtis, K. C., Li, B. W., & Weil, G. J. (1995). Molecular characterization of a *Brugia malayi* intermediate filament protein which is an excretory-secretory product of adult worms. Molecular and biochemical parasitology, 73(1), 231-239.

Chandrashekar, R., Curtis, K. C., Ramzy, R. M., Liftis, F., Li, B. W., & Weil, G. J. (1994). Molecular cloning of *Brugia malayi* antigens for diagnosis of lymphatic filariasis. *Molecular and biochemical parasitology*, *64*(2), 261-271.

Chandy, A, Thakur, A.S., Singh M.P. & Manigauha A. (2011). A review of neglected tropical disease: filariasis. Asian Pacific Journal of Tropical Medicine, 4 (7), 581-586. doi:10.1016/S1995-7645(11)60150-8.

Cheng, T. C. (1964). The phasmid nematodes. The Biology of Animal Parasites. (pp. 447-471). W.B. Saunders Company, London,

Chernin, E. (1983). Sir Patrick Manson's studies on the transmission of biology of filariasis. Reviews of Infectious Diseases, 5 (1): 148-166.

Chu, B. K., Deming, M., Biritwum, N. K., Bougma, W. R., Dorkenoo, A. M., El-Setouhy, M., ... & Won, K. Y. (2013). Transmission assessment surveys (TAS) to define endpoints for lymphatic filariasis mass drug administration: a multicenter evaluation. PLoS neglected tropical diseases, 7(12), e2584.

Chularerk, P., & Desowitz, R. S. (1970). A simplified membrane filtration technique for the diagnosis of microfilaremia. Journal of Parasitology, 56(3), 623-624.

Cobbold, T.S. (1877). Discovery of the adult representative of microscopic filariae. Lan Cox, F.E.G. (2002). History of Human Parasitology. Clinical Microbiology Reviews, 15 (4), 595-612, doi:10.1128/CMR.15.4.595-612.2002cet, ii: 70-71.

Cross, J.H. (1996). Filarial Nematodes. In S. Baron (Ed.), Medical Microbiology (4th ed), University of Texas Medical Branch at Galveston, TX.

Dakshinamoorthy, G., Samykutty, A. K., Munirathinam, G., Reddy, M. V., & Kalyanasundaram, R. (2013). Multivalent fusion protein vaccine for lymphatic filariasis. *Vaccine*, *31*(12), 1616-1622.

Das, P.K. & Shenoy, R.K. (2008). Helminthic diseases: Filariasis. In J. Griffiths, J. Maguire, H.K. Heggenhougen & S.R. Quah (Eds.), *Public Health and Infectious Diseases,* Academic Press, CA.

David, A., & Tapp, E. (1992). *The mummy's tale: the scientific and medical investigation of Natsef-Amun, priest in the temple at Karnak*. Michael O'Mara Books

Day, K. P., Gregory, W. F., & Maizels, R. M. (1991). Age-specific acquisition of immunity to infective larvae in a bancroftian filariasis endemic area of Papua New Guinea. *Parasite immunology*, *13*(3), 277-290.

Demedts, P., Vermeulen-Van Overmeir, C., & Wery, M. (1987). Simultaneous use of *Plasmodium falciparum* crude antigen and red blood cell control antigen in the enzyme-linked immunosorbent assay for malaria. *The American journal of tropical medicine and hygiene*, *36*(2), 257-263.

Denham, D. A. (1975). The diagnosis of filariasis. *Ann Soc Belg Med Trop*, *55*, 517-24.

Denham, D. A., Dennis, D. T., Ponnudurai, T., Nelson, G. S., & Guy, F. (1971). Comparison of a counting chamber and thick smear methods of counting microfilariae. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *65*(4), 521-526.

Dennis, D. T., Dreyer, G., Ismail, M. M., Kumaraswami, V., Mak, J. W., Mataika, J. U., ... & Huijin, Z. (1992). Lymphatic filariasis, the disease and its control. *WHO technical report series*, (821), 1-71.

De Savigny, D.H. (1980). Studies on nematode secretory antigen in immunodiagnosis with special reference to toxocariasis. (Doctoral dissertation, University of London, UK).

de Souza, D. K., Koudou, B., Kelly-Hope, L. A., Wilson, M. D., Bockarie, M. J., & Boakye, D. A. (2012). Diversity and transmission competence in lymphatic filariasis vectors in West Africa, and the implications for accelerated elimination of Anopheles-transmitted filariasis. *Parasit Vectors*, *5*, 259.

Dissanayake, S., Xu, M., & Piessens, W. F. (1992). A cloned antigen for serological diagnosis of *Wuchereria bancrofti* microfilaremia with daytime blood samples. *Molecular and biochemical parasitology*, *56*(2), 269-277.

Drakeley C, Cook J (2009) Chapter 5. Potential contribution of sero-epidemiological analysis for monitoring malaria control and elimination: historical and current perspectives. *Adv Parasitol* 69: 299–352 doi:10.1016/S0065-308X(09)69005-9.

Dreyer, G., Addiss, D., Dreyer, P., & Noroes, J. (2002). Basic lymphoedema management. *HollisNH: Hollis Publishing*.

Dreyer, G., Santos, A., Norões, J., Rocha, A., & Addiss, D. (1996). Amicrofilaraemic carriers of adult Wuchereria bancrofti. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *90*(3), 288-289.

EDMA (2005) EDMA Classification. Retrieved from http://www.edma-ivd.be/index.php?page=Global-IVD-Classification

Eldridge, B.F. (1992). Patrick Manson and the discovery age of vector biology. *Journal of American Mosquito Control Association*, 8 (3): 215-220.

Engvall, E., & Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry*, *8*(9), 871-874.

Engvall, E., & Perlmann, P. (1972). Enzyme-linked immunosorbent assay, ELISA III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *The Journal of Immunology*, *109*(1), 129-135.

Evans, D. B., Gelband, H., & Vlassoff, C. (1993). Social and economic factors and the control of lymphatic filariasis: a review. *Acta tropica*, *53*(1), 1-26.

Favaloro, E. J., Plebani, M., & Lippi, G. (2011a). Regulation of in vitro diagnostics (IVDs) for use in clinical diagnostic laboratories: towards the light or dark in clinical laboratory testing?. Clinical Chemistry and Laboratory Medicine, 49(12), 1965-1973.

Favaloro, E. J., Lippi, G., & Koutts, J. (2011b). Laboratory testing of anticoagulants: the present and the future. Pathology-Journal of the RCPA, 43(7), 682-692.

Fischer, P., Supali, T., Wibowo, H., Bonow, I., & Williams, S. A. (2000). Detection of DNA of nocturnally periodic *Brugia malayi* in night and day blood samples by a polymerase chain reaction-ELISA-based method using an internal control DNA. *The American journal of tropical medicine and hygiene*, *62*(2), 291-296.

Fischer, P., Wibowo, H., Pischke, S., CKERT, P., Liebau, E., Ismid, I. S., & Supali, T. (2002). PCR-based detection and identification of the filarial parasite *Brugia timori* from Alor Island, Indonesia. *Annals of Tropical Medicine & Parasitology*, *96*(8), 809-821.

Frances, S. P., Baade, L. M., Kubofcik, J., Nutman, T. B., Melrose, W. D., McCarthy, J. S., & Nissen, M. D. (2008). Seroconversion to filarial antigens in Australian defence force personnel in Timor-Leste. *The American journal of tropical medicine and hygiene*, *78*(4), 560-563.

Freedman, D. O., de Almeida Filho, P. J., Besh, S., e Silva, M. C. M., Braga, C., & Maciel, A. (1994). Lymphoscintigraphic analysis of lymphatic abnormalities in symptomatic and asymptomatic human filariasis. *Journal of Infectious Diseases*, *170*(4), 927-933.

Frost, L. J., & Reich, M. (2009). *Access: how do good health technologies get to poor people in poor countries?*. Bibliomotion, Inc. Retrieved from www.accessbook.org/downloads/

GAELF. (2007). Resolving the critical challenges facing LF Elimination: Activities Update, An overview of the grant to the Global Alliance by the Bill & Melinda Gates Foundation, Issue 1, Retrived from www.filariasis.org/pdfs/.../LFgrant_Newsletter2.pdf

Garraud, O., Nkenfou, C., Bradley, J. E., Perler, F. B., & Nutman, T. B. (1995). Identification of recombinant filarial proteins capable of inducing polyclonal and antigen-specific IgE and IgG4 antibodies. *The Journal of Immunology*, *155*(3), 1316-1325.

Gass, K., de Rochars, M. V. B., Boakye, D., Bradley, M., Fischer, P. U., Gyapong, J., ... & Lammie, P. (2012). A multicenter evaluation of diagnostic tools to define endpoints for programs to eliminate bancroftian filariasis. *PLoS neglected tropical diseases*, *6*(1), e1479.

Goff, L. L., Maréchal, P., Petit, G., Taylor, D. W., Hoffmann, W., & Bain, O. (1997). Early reduction of the challenge recovery rate following immunization with irradiated infective larvae in a filaria mouse system. *Tropical Medicine & International Health*, *2*(12), 1170-1174.

Goldsmid, J. M., Rogers, S., Parsons, G. S., & Chambers, P. G. (1976). The intestinal protozoa and helminths infecting Africans in the Gatooma region of Rhodesia. *The Central African journal of medicine*, *22*(5), 91.

Grady, C. A., De Rochars, M. B., Direny, A. N., Orelus, J. N., Wendt, J., Radday, J., Mathieu, E., Roberts, M.J., Streit, T.G., Addiss, D.G. & Lammie, P. J. (2007). Endpoints for lymphatic filariasis programs. *Emerging infectious diseases*, *13*(4), 608.

Graves, P. M., Makita, L., Susapu, M., Brady, M. A., Melrose, W., Capuano, C., ... & Kelly-Hope, L. A. (2013). Lymphatic filariasis in Papua New Guinea: distribution at district level and impact of mass drug administration, 1980 to 2011. Parasit Vectors, 6(7).

Gyapong, M., Gyapong, J. O., Adjei, S., Vlassoff, C., & Weiss, M. (1996). Filariasis in northern Ghana: some cultural beliefs and practices and their implications for disease control. *Social science & medicine*, *43*(2), 235-242.

Gyapong, M., Gyapong, J., Weiss, M., & Tanner, M. (2000). The burden of hydrocele on men in Northern Ghana. *Acta tropica*, *77*(3), 287-294.

Haarbrink, M., Terhell, A. J., Abadi, K., Asri, M., De Medeiros, F., & Yazdanbakhsh, M. (1999). Anti-filarial IgG4 in men and women living in *Brugia malayi*-endemic areas. *Tropical Medicine & International Health*, *4*(2), 93-97.

Haarbrink, M., Terhell, A., Abadi, K., van Beers, S., Asri, M., & Yazdanbakhsh, M. (1995). IgG4 antibody assay in the detection of filariasis. *The Lancet*, *346*(8978), 853-854.

Harb, M., Faris, R., Gad, A.M., Hafez, O.N., Ramzy, R. & Buck, A.A. (1993). The resurgence of lymphatic filariasis in the Nile delta. *Bulletin of the World Health Organisation*, 71 (1): 49-54.

Heydon, G. M. (1927). Report of Investigation into Malaria and Filariasis in Cairns and elsewhere. *Health*, *5*(5), 133-140.

Heydon, G. M. (1931). Some common Queensland mosquitoes as intermediate hosts of Wuchereria bancrofti (Filaria bancrofti). *Parasitology*, *23*(04), 415-427.

Hira, P. R. (1976). *Wuchereria bancrofti*: the staining of the microfilarial sheath in giemsa and haematoxylin for diagnosis. *Medical journal of Zambia*, *11*(4), 93-96.

Hoerauf, A. (2008). Filariasis: new drugs and new opportunities for lymphatic filariasis and onchocerciasis. Current Opinion in Infectious Diseases, 21: 673-681. Doi:10.1097/QCO.0b013e328315cde7.

Hooper, P. J., Chu, B. K., Mikhailov, A., Ottesen, E. A., & Bradley, M. (2014). Assessing Progress in Reducing the At-Risk Population after 13 Years of the Global Programme to Eliminate Lymphatic Filariasis.

Hopkins, D. R., Eigege, A., Miri, E. S., Gontor, I., Ogah, G., Umaru, J., ... & Richards, F. O. (2002). Lymphatic filariasis elimination and schistosomiasis control in combination with onchocerciasis control in Nigeria. *The American journal of tropical medicine and hygiene*, *67*(3), 266-272.

Horton, J., Witt, C., Ottesen, E. A., Lazdins, J. K., Addiss, D. G., Awadzi, K., ... & Weerasooriya, M. V. (2000). An analysis of the safety of the single dose, two drug regimens used in programmes to eliminate lymphatic filariasis. *Parasitology*, *121*(S1), S147-S160.

Hotez, P. J. (2008). *Forgotten people, forgotten diseases: the neglected tropical diseases and their impact on global health and development*. ASM Press.

Hotez, P. J., Fenwick, A., Savioli, L., & Molyneux, D. H. (2009). Rescuing the bottom billion through control of neglected tropical diseases. *The Lancet*, *373*(9674), 1570-1575.

Hudson, L., Hay, F. C., & Hudson, L. (1976). *Practical immunology* (pp. 1-3). Oxford:: Blackwell Scientific.

Hussain et al., (1992). Control of allergic reactivity in human filariasis. Predominant localization of blocking antibody to the IgG4 subclass.Journal of immunology, May 1, 1992 vol. 148 no. 9 2731-2737

Hussain, R. A. B. I. A., Grögl, M., & Ottesen, E. A. (1987). IgG antibody subclasses in human filariasis. Differential subclass recognition of parasite antigens correlates with different clinical manifestations of infection. *The Journal of Immunology*, *139*(8), 2794-2798.

Ichimori, K., King, J. D., Engels, D., Yajima, A., Mikhailov, A., Lammie, P., & Ottesen, E. A. (2014). Global programme to eliminate lymphatic filariasis: the processes underlying programme success. *PLoS neglected tropical diseases*, *8*(12), e3328.

ISO (2012). ISO15225 Medical devices - Quality management - Medical device nomenclature data structure. British Standards, UK. Retrieved from http://www.infostore.saiglobal.com.

ISO (2012). ISO13485 Medical devices – quality management systems – requirements for regulatory purposes. British Standards, UK. Retrieved from Retrieved from http://www.infostore.saiglobal.com.

Itoh, M., Weerasooriya, M. V., Qiu, G., Gunawardena, N. K., Anantaphruti, M. T., Tesana, S., ... & Kimura, E. (2001). Sensitive and specific enzyme-linked immunosorbent assay for the diagnosis of Wuchereria bancrofti infection in urine samples. *The American journal of tropical medicine and hygiene*, *65*(4), 362-365.

Jacobsen, R.H. (1996). Principles of validation of diagnostic assays for infectious diseases. Manual of standards for diagnostic tests and vaccines, p. 8—15, OIE.

Jacobson, R. H. (1998). Principles of validation of diagnostic assays for infectioius diseases. *Diagnosis and epidemiology of animal diseases in Latin America*, 15.

Jin, Y., Shen, C., Huh, S., Sohn, W. M., Choi, M. H., & Hong, S. T. (2013). Serodiagnosis of toxocariasis by ELISA using crude antigen of *Toxocara canis* larvae. The Korean journal of parasitology, 51(4), 433-439.

Joseph, H., (2010b). Lymphatic filariasis elimination: residual endemnicity, spatial clustering and future surveillance using the new filariasis CELISA diagnostic assay. PhD thesis, JCU. Retrieved from http://eprints.jcu.edu.au/12000.

Joseph, H., Maiava, F., Naseri, T., Taleo, F., Ake, M., Capuano, C., & Melrose, W. (2011b). Application of the Filariasis CELISA Antifilarial IgG4 Antibody Assay in Surveillance in Lymphatic Filariasis Elimination Programmes in the South Pacific. *Journal of tropical medicine*, *2011*.

Joseph, H., Maiava, F., Naseri, T., Silva, U., Lammie, P., & Melrose, W. (2011). Epidemiological assessment of continuing transmission of lymphatic

filariasis in Samoa. *Annals of Tropical Medicine & Parasitology*, *105*(8), 567-578.

Joseph, H., Moloney, J., Maiava, F., McClintock, S., Lammie, P., & Melrose, W. (2011a). First evidence of spatial clustering of lymphatic filariasis in an Aedes polynesiensis endemic area. Acta tropica, 120, S39-S47.

Joseph, H. M., & Melrose, W. (2010). Applicability of the filter paper technique for detection of antifilarial IgG4 antibodies using the Bm14 Filariasis CELISA. *Journal of parasitology research*, *2010*.

Jullien, P., Somé, J. D. A., Brantus, P., Bougma, R. W., Bamba, I., & Kyelem, D. (2011). Efficacy of home-based lymphoedema management in reducing acute attacks in subjects with lymphatic filariasis in Burkina Faso. *Acta tropica*, *120*, S55-S61. doi:10.1016/j.actatropica.2011.03.007.

Kalyanasundaram, R., & Balumuri, P. (2011). Multivalent vaccine formulation with BmVAL-1 and BmALT-2 confer significant protection against challenge infections with *Brugia malayi* in mice and jirds. *Research and reports in tropical medicine*, *2011*(2), 45.

Kazura, J. W. (2002). Lymphatic filarial infections: an introduction to the filariae. In *The Filaria* (pp. 1-8). Springer US.

Kazura, J.W. (2002). Lymphatic filarial infections: An introduction to the filariae. In T.R. Klei & T.V. Rajan. (Eds.), *World Class Parasites: Volume 5 The Filaria*. Kluwer Academic Publishers, MA

Keiser & Nutman, (2002), Vaccines for Filarial Infections, The Filaria: World Class

Keiser, P.B. & Nutman , T.B. (2002). Update on lymphatic filarial infections. *Current Infectious Disease Reports*, 4: 65-69.

Kelly-Hope, L. A., Molyneux, D. H., & Bockarie, M. J. (2013). Can malaria vector control accelerate the interruption of lymphatic filariasis transmission in Africa; capturing a window of opportunity. *Parasit Vectors*, *6*, 39.

Kemeny, D. M., & Challacombe, S. J. (Eds.). (1988). *ELISA and other solid phase immunoassays: theoretical and practical aspects*. John Wiley & Sons. Parasites Volume 5, 2002, pp 167-178

King, C. L., & Nutman, T. B. (1993). IgE and IgG subclass regulation by IL-4 and IFN-gamma in human helminth infections. Assessment by B cell precursor frequencies. *The Journal of Immunology*, *151*(1), 458-465.

Kiple, K.F. (1993). *The Cambridge world history of human disease* (p. 1008) (A. Browne Ed.) Cambridge: Cambridge University Press.

Klüber, S., Supali, T., Williams, S. A., Liebau, E., & Fischer, P. (2001). Rapid PCR-based detection of Brugia malayi DNA from blood spots by DNA Detection Test Strips™. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *95*(2), 169-170.

Knott, J. (1935). The periodicity of the microfilaria of *Wuchereria bancrofti*. Preliminary report of some injection experiments. *Transactions of the Royal Society of tropical Medicine and Hygiene*, *29*(1), 59-64.

Kubofcik, J., Fink, D. L., & Nutman, T. B. (2012). Identification of Wb123 as an Early and Specific Marker of *Wuchereria bancrofti* Infection.

Kumaraswami, V. (2000). The clinical manifestations of lymphatic filariasis. *Lymphatic filariasis*, 103-26.

Kurniawan-Atmadja, A., Sartono, E., Partono, F., Yazdanbakhsh, M., & Maizels, R. M. (1998). Antibody responses to filarial infective larvae are not dominated by the IgG4 isotype. *Parasite immunology*, *20*(1), 9-17.

Kurniawan, A., Yazdanbakhsh, M., Van Ree, R., Aalberse, R., Selkirk, M. E., Partono, F., & Maizels, R. M. (1993). Differential expression of IgE and IgG4 specific antibody responses in asymptomatic and chronic human filariasis. *The Journal of Immunology*, *150*(9), 3941-3950.

Kurstak, E. (1985). Progress in enzyme immunoassays: production of reagents, experimental design, and interpretation. Bulletin of the World Health Organization, 63(4), 793.

Lakhal, S., Mekki, S., Ben-Abda, I., Mousli, M., Amri, F., Aoun, K., & Bouratbine, A. (2012). Evaluation of an enzyme-linked immunosorbent assay based on crude Leishmania histone proteins for serodiagnosis of human infantile visceral leishmaniasis. *Clinical and Vaccine Immunology*, *19*(9), 1487-1491.

Lalitha, P., Ravichandran, M., Suba, S., Kaliraj, P., Narayanan, R. B., & Jayaraman, K. (1998). Quantitative assessment of circulating antigens in human lymphatic filariasis: a field evaluation of monoclonal antibody-based ELISA using blood collected on filter strips. *Tropical Medicine & International Health*, *3*(1), 41-45.

Lal, R. B., & Ottesen, E. A. (1988). Enhanced diagnostic specificity in human filariasis by IgG4 antibody assessment. *Journal of Infectious Diseases*, *158*(5), 1034-1037.

Lammie, P. J., Weil, G., Noordin, R., Kaliraj, P., Steel, C., Goodman, D., Lakshmikanthan, V.B. & Ottesen, E. (2004). Recombinant antigen-based antibody assays for the diagnosis and surveillance of lymphatic filariasis–a multicenter trial. *Filaria Journal*, *3*(1), 9.

Laurence, B.R. (1970). The curse of Saint Thomas. *Medical History*, 14 (4): 352-363.

Levine, R. A., Wardlaw, S. C., & Patton, C. L. (1989). Detection of haematoparasites using quantitative buffy coat analysis tubes. *Parasitol Today*, *5*(4), 132-4.

Lizotte, M. R., Supali, T., Partono, F., & Williams, S. A. (1994). A polymerase chain reaction assay for the detection of *Brugia malayi* in blood. *The American journal of tropical medicine and hygiene*, *51*(3), 314-321.

London, C. A., Abbas, A. K., & Kelso, A. (1998). Helper T cell subsets: heterogeneity, functions and development. *Veterinary immunology and immunopathology*, *63*(1), 37-44.

Lu, A. G., Valencia, L. B., Llagas, L., Aballa, L., & Postrado, L. (1988). *Filariasis: a study of knowledge, attitudes and practices of the people of Sorsogon.*

Social and Economic Research Project Reports No. 1. TDR/SER/PRS/1 WHO, Geneva.

MacDonald, A. S., Araujo, M. I., & Pearce, E. J. (2002). Immunology of parasitic helminth infections. *Infection and Immunity*, *70*(2), 427-433. Doi: 10.1128/IAI.70.2.427-733.2002.

Mackerras, M. J. (1958). The Decline of Filariasis in Queensland. *Medical Journal of Australia*, *1*(21), 702-4.

Mahanty, S. & Nutman, T.B. (1995). Immunoregulation in human lymphatic filariasis: the role of interleukin 10. Parasite Immunology, 17 (8): 385-392. DOI: 10.1111/j.1365-3024.1995.tb00906.x

Maizels, M. M., (2000). Lymphatic Filariasis: A historical perspective (T.B. Nutman, Ed.). Imperial College Press, MD.

Maizels, M.M., Bundy D.A.P., Selkirk, M.E., Smith, D.F. & Anderson, R.M. (1993). Immunological modulation and evasion by helminth parasites in human populations. *Nature,* 365: 797-805.

Maizels, R. M., Allen, J. E., & Yazdanbakhsh, M. (2000). Immunology of lymphatic filariasis: current controversies. Lymphatic filariasis, 1, 217-244.

Maizels, R. M., Blaxter, M. L., & Scott, A. L. (2001). Immunological genomics of *Brugia malayi*: filarial genes implicated in immune evasion and protective immunity. *Parasite immunology, 23*(7), 327-344.

Mand, S., Debrah, A. Y., Klarmann, U., Mante, S., Kwarteng, A., Batsa, L., ... & Hoerauf, A. (2011). The role of ultrasonography in the differentiation of the various types of filaricele due to bancroftian filariasis. *Acta tropica*, *120*, S23-S32.

Mand, S., Marfo-Debrekyei, Y., Dittrich, M., Fischer, K., Adjei, O., & Hoerauf, A. (2003). Animated documentation of the filaria dance sign (FDS) in bancroftian filariasis. *Filaria journal*, *2*(1), 3.

Mannonen, S., & Riikonen S. (2006). In vitro-diagnostics (IVD) compliance and what it means in practice. *American Biotechnology Laboratory 24*(8), 18.

Mathews, H. M., Fisher, G. U., & Kagan, I. G. (1969). The persistence of malaria antibody in Tobago, West Indies, following eradication as measured by the indirect haemagglutination test. Retrieved from http://whqlibdoc.who.int/malaria/WHO_Mal_70.722.pdf

McCarthy, J. (2000). Diagnosis of lymphatic filarial infection. *Nutman TB, Imperial College Press, London*, 127-141.

McGraw, E. A., & O'Neill, S. L. (2013). Beyond insecticides: new thinking on an ancient problem. *Nature Reviews Microbiology*, *11*(3), 181-193.

McNulty, S. N., Mitreva, M., Weil, G. J., & Fischer, P. U. (2013). Inter and intra-specific diversity of parasites that cause lymphatic filariasis. *Infection, Genetics and Evolution*, *14*, 137-146.

McSorley, J.H., & Maizels, R.M. (2012). Helminth Infections and host immune regulation. Clinical Microbiology Reviews, 25(4): 585-608.

Melrose, W. D. (2002). Lymphatic filariasis: new insights into an old disease. *International journal for parasitology*, *32*(8), 947-960.

Melrose, W. D. (2004). *Lymphatic filariasis: A review 1862-2002*. Warwick Educational Publishing Inc.

Melrose, W. D., Durrheim, D. D., & Burgess, G. W. (2004). Update on immunological tests for lymphatic filariasis. *Trends in parasitology*, *20*(6), 255-257.

Melrose, W. D., Turner, P. F., Pisters, P., & Turner, B. (2000). An improved Knott's concentration test for the detection of microfilariae. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *94*(2), 176.

Miller, T. S., & Nesbitt, J. W. (2014). *Walking Corpses: Leprosy in Byzantium and the Medieval West*. Cornell University Press.

Mladonicky, J.M., King, J.D., Liang, J.L., Chambers, E., Pa'au, M., Schmaedick, M.A., Burkot, T.R., Bradley, M. and Lammie, P.J., 2009. Assessing transmission of lymphatic filariasis using parasitologic, serologic, and entomologic tools after mass drug administration in American Samoa. *The American journal of tropical medicine and hygiene*, *80*(5), 769-773.

Mohanty, M. C., Satapathy, A. K., Sahoo, P. K., & Ravindran, B. (2001). Human bancroftian filariasis–a role for antibodies to parasite carbohydrates. *Clinical & Experimental Immunology*, *124*(1), 54-61.

More, S. J., & Copeman, D. B. (1990). A highly specific and sensitive monoclonal antibody-based ELISA for the detection of circulating antigen in bancroftian filariasis. *Tropical medicine and parasitology: official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur Technische Zusammenarbeit (GTZ)*, *41*(4), 403-406.

Muller, R. and Buttner, P. (2009) HS2402: An introduction to practical biostatistics in medicine and public health. James Cook University, Townsville.

Mylonakis, M. E., Papadopoulos, E., Koutinas, A. F., Paitaki, C., & Leontides, L. (2004). Comparative methodology for the detection and differentiation of circulating microfilariae of *Dirofilaria immitis* in the dog. *Journal of helminthology*, *78*(02), 137-140.

Nakane, P. K., & Kawaoi, A. (1974). Peroxidase-labeled antibody a new method of conjugation. *Journal of Histochemistry & Cytochemistry*, *22*(12), 1084-1091.

Nelson, G. (1996). Lymphatic filariasis. In F.E.G. Cox (Ed.), *The Wellcome Trust Illustrated History of Tropical Disease* (pp.294-303). The Wellcome Trust, London, United Kingdom.

Nguyen, N. L., Plichart, C., & Esterre, P. (1999). Assessment of immunochromatographic test for rapid lymphatic filariasis diagnosis. *Parasite*, *6*(4), 355-358.

Nicolas, L. (1997). New tools for diagnosis and monitoring of bancroftian filariasis parasitism: the Polynesian experience. *Parasitology Today*, *13*(10), 370-375.

Nielsen, N. O., Bloch, P., & Simonsen, P. E. (2002). Lymphatic filariasis-specific immune responses in relation to lymphoedema grade and infection status. II. Humoral responses. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *96*(4), 453-458.

Njenga, S. M., Wamae, C. N., Mwandawiro, C. S., & Molyneux, D. H. (2007). Immuno–parasitological assessment of bancroftian filariasis in a highly endemic area along the River Sabaki, in Malindi district, Kenya. *Annals of tropical medicine and parasitology*, *101*(2), 161-172.

Noordin, R., Aziz, R. A., & Ravindran, B. (2004). Homologs of the *Brugia malayi* diagnostic antigen BmR1 are present in other filarial parasites but induce different humoral immune responses. *Filaria journal*, *3*(1), 10.

Nuchprayoon, S. (2009). DNA-based diagnosis of lymphatic filariasis. *Southeast Asian J Trop Med Public Health*, *40*(5), 904-913.

Nutman, T. B. (1991). Experimental infection of humans with filariae. *Review of Infectious Diseases*, *13*(5), 1018-1022.

Nutman, T.B. & Kumaraswami, V. (2001). Regulation of the immune response in lymphatic filariasis: perspectives on acute and chronic infection with Wuchereria bancrofti in South India. Parasite Immunology, 23(7): 389-399.

Oliver, C. (1999). Conjugation of colloidal gold to proteins. In L.C. Javois (Ed.), *Immunocytochemical Methods and Protocols* (5th ed.) (pp. 331-334). Humana Press Inc., Totowa, New Jersey.

Ottesen, E.A. (1980). Immunopathology of lymphatic filariasis in man. Springer Seminars in Immunopathology, 2(4): 373-385. Doi:10.1007/BF01857174.

Ottesen, E.A. (1989). Filariasis now. American Journal of Tropical Medicine and Hygiene, 41(3) 9-17.

Ottesen, E. A. (2006). Lymphatic filariasis: treatment, control and elimination. *Advances in parasitology*, *61*, 395-441.

Ottesen, E. A., Hooper, P. J., Bradley, M., & Biswas, G. (2008). The global programme to eliminate lymphatic filariasis: health impact after 8 years. *PLoS Negl Trop Dis*, *2*(10), e317.

Ottesen, E. A., Duke, B. O., Karam, M., & Behbehani, K. (1997). Strategies and tools for the control/elimination of lymphatic filariasis. *Bulletin of the World Health Organization*, *75*(6), 491.

Ottesen, E. A., Kumaraswami, V., Paranjape, R., Poindexter, R. W., & Tripathy, S. P. (1981). Naturally occurring blocking antibodies modulate immediate hypersensitivity responses in human filariasis. *J Immunol*, *127*(5), 2014-2020.

Ottesen, E.A. & Nutman, T.B. (1992). Tropical pulmonary eosinophilia. Annual Review of Medicine, 43: 417-424. Doi:10.1146/annurev.me.43.020192.

Ottesen, E. A., Skvaril, F., Tripathy, S. P., Poindexter, R. Ý., & Hussain, R. (1985). Prominence of IgG4 in the IgG antibody response to human filariasis. *The Journal of Immunology*, *134*(4), 2707-2712.

Paily, K. P., Hoti, S. L., & Das, P. K. (2009). A review of the complexity of biology of lymphatic filarial parasites. *Journal of Parasitic Diseases*, *33*(1-2), 3-12.

Palmieri, J. R., Purnomo, Dennis, D. T., & Marwoto, H. A. (1980). Filarid parasites of South Kalimantan (Borneo) Indonesia. *Wuchereria kalimantani* sp. n.(Nematoda: Filarioidea) from the silvered leaf monkey, Presbytis cristatus Eschscholtz 1921. *The Journal of parasitology*, 645-651.

Panicker, K. N., Krishnamoorthy, K., Sabesan, S., & Prathiba, J. (1991). Comparison of effects of mass annual and biannual single dose therapy with diethylcarbamazine for the control of Malayan filariasis. *The Southeast Asian journal of tropical medicine and public health*, *22*(3), 402-411.

Person, B., Addiss, D. G., Bartholomew, L. K., Meijer, C., Pou, V., & Van den Borne, B. (2006). Health-seeking behaviors and self-care practices of Dominican women with lymphoedema of the leg: implications for lymphoedema management programs. *Filaria journal*, *5*(1), 13. Doi:10.1186/1475-2883-5-13.

Person, B., Bartholomew, L. K., Gyapong, M., Addiss, D. G., & van den Borne, B. (2009). Health-related stigma among women with lymphatic filariasis from the Dominican Republic and Ghana. *Social Science & Medicine*, *68*(1), 30-38.

Pharoah Mentuhotep II image http://parasitology.kasralainy.edu.eg/draft-plan-statushttp://parasitology.kasralainy.edu.eg/draft-plan-status

Pichon, G. (2002). Limitation and facilitation in the vectors and other aspects of the dynamics of filarial transmission: the need for vector control against Anopheles-transmitted filariasis. *Annals of Tropical Medicine & Parasitology*, *96*(2), S143-S152.

Piessens, W. F. (1981). Lymphatic filariasis in humans: an immunologic maze. *Annals of internal medicine*, *95*(6), 778-779.

Piessens, W. F., Wadee, A. A., & Kurniawan, L. (1987). Regulation of immune responses in lymphatic filariasis. In *Filariasis* (pp. 164-173). Wiley New York.

Pinkston, P., Vijayan, V. K., Nutman, T. B., Rom, W. N., O'Donnell, K. M., Cornelius, M. J., ... & Yenokida, G. (1987). Acute tropical pulmonary eosinophilia. Characterization of the lower respiratory tract inflammation and its response to therapy. *Journal of Clinical Investigation*, *80*(1), 216.

Plichart, C., & Lemoine, A. (2013). Monitoring and evaluation of lymphatic filariasis interventions: an improved PCR-based pool screening method for high throughput *Wuchereria bancrofti* detection using dried blood spots. *Parasites & vectors*, *6*(1), 110.

Plichart, C., Sechan, Y., Davies, N., & Legrand, A. M. (2006). PCR and dissection as tools to monitor filarial infection of Aedes polynesiensis mosquitoes in French Polynesia. *Filaria journal*, *5*(1), 2. Doi: 10.1186/1475-2883-5-2.

Portano, F. (1987). Filariasis. CIBA Foundation Symposium 127 (pg.25), John Wiley & Sons, Chichester, UK

Portano, F. (1978). Clinical features of timorian filariasis among immigrants to an endemic area in West Flores, Indonesia. *The Southeast Asian journal of tropical medicine and public health*, *9*(3), 338-343.

Rahmah, N., Lim, B. H., Anuar, A. K., Shenoy, R. K., Kumaraswami, V., Hakim, S. L., ... & Ramachandran, C. P. (2001a). A recombinant antigen-based IgG4 ELISA for the specific and sensitive detection of *Brugia malayi* infection. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *95*(3), 280-284.

Rahmah, N., Taniawati, S., Shenoy, R. K., Lim, B. H., Kumaraswami, V., Anuar, A. K., ... & Ramachandran, C. P. (2001b). Specificity and sensitivity of a rapid dipstick test (Brugia Rapid) in the detection of *Brugia malayi* infection. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *95*(6), 601-604.

Rahman, R. A., Hwen-Yee, C., & Noordin, R. (2007). Pan LF-ELISA using BmR1 and BmSXP recombinant antigens for detection of lymphatic filariasis. *Filaria Journal*, *6*(1), 10.

Rajan, T.V. (2000). Lymphatic Filariasis: A historical perspective (T.B. Nutman, Ed.). Imperial College Press, MD.

Rajan, T.V. (2005). Natural course of lymphatic filariasis: Insights from epidemiology, experimental human infections, and clinical observations. American Journal of Tropical Medicine and Hygiene, 73 (6): 995-998.

Rajan T. V & Gundlapalli. (1997). Lymphatic filariasis. In D. O. Freedman (Ed.), *Immunopathogenetic Aspects of Disease Induced by Helminth Parasites. Chemical Immunology*, vol 66 (pp. 125–158). Karger Medical and Scientific Publishers, Basel.

Rajasekariah, G. H. R., Kay, G. E., Russell, N. V., & Smithyman, A. M. (2003). Assessment of assay sensitivity and precision in a malaria antibody ELISA. *Journal of Immunoassay and Immunochemistry*, *24*(1), 89-112.

Rajasekariah, G. H. R., Ryan, J. R., Hillier, S. R., Lisa, P. Y., Stiteler, J. M., Cui, L., ... & Martin, S. K. (2001). Optimisation of an ELISA for the serodiagnosis of visceral leishmaniasis using in vitro derived promastigote antigens. *Journal of immunological methods*, *252*(1), 105-119.

Rajasekariah, G. R., Parab, P. B., & Subrahmanyam, D. (1991). Detection of Wuchereria bancrofti specific antigens in the serum of endemic residents. *Tropical medicine and parasitology: official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur Technische Zusammenarbeit (GTZ)*, *42*(2), 103-105.

Ramaiah, K. D., & Kumar, K. V. (2000). Effect of lymphatic filariasis on school children. *Acta Tropica*, *76*(2), 197-199.

Ramaiah, K. D., & Ottesen, E. A. (2014). Progress and impact of 13 years of the Global programme to eliminate Lymphatic Filariasis on reducing the Burden of filarial disease.

Ramirez, B. L., Hernandez, L., Alberto, F. F., Collins, M., Nfonsam, V., Punsalan, T., & Kron, M. A. (2004). Contrasting *Wuchereria bancrofti* microfilaria rates in two Mangyan-populated Philippine villages. *The American Journal of Tropical Medicine and Hygiene*, *71*(1), 17-23.

Rao, K. V. N., Eswaran, M., Ravi, V., Gnanasekhar, B., Narayanan, R. B., Kaliraj, P., ... & Scott, A. L. (2000). The *Wuchereria bancrofti* orthologue of *Brugia malayi* SXP1 and the diagnosis of bancroftian filariasis. *Molecular and biochemical parasitology*, *107*(1), 71-80.

Rao, R. U., Atkinson, L. J., Ramzy, R. M., Helmy, H., Farid, H. A., Bockarie, M. J., ... & Weil, G. J. (2006). A real-time PCR-based assay for detection of *Wuchereria bancrofti* DNA in blood and mosquitoes. *The American journal of tropical medicine and hygiene*, *74*(5), 826-832.

Rao, R. U., Nagodavithana, K. C., Samarasekera, S. D., Wijegunawardana, A. D., Premakumara, W. D., Perera, S. N., ... & Weil, G. J. (2014). A comprehensive assessment of lymphatic filariasis in Sri Lanka six years after cessation of mass drug administration. *Plos Neglected Tropical Diseases*, 8(11), e3281

Ravindran, B., Satapathy, A. K., Sahoo, P. K., & Mohanty, M. (2003). Protective immunity in human lymphatic filariasis: problems and prospects. *Medical microbiology and immunology*, *192*(1), 41-46.

Rebollo, M. P., & Bockarie, M. J. (2013). Toward the elimination of lymphatic filariasis by 2020: treatment update and impact assessment for the endgame.

Reeve, D. M., (2010). Comparative accuracy and 'field friendly' effectiveness of diagnostic tools for lymphatic filariasis and neurocyticercosis in Papua New Guinea and Timor-Leste with consideration on the impact of parasitic reduction programs. Prefessional Doctorate (Research) thesis, James Cook University. Retrieved from http://eprints.jcu.edu.au/19042

Rocha, A., Addiss, D., Ribeiro, M. E., NoroTes, J., Baliza, M., Medeiros, Z., & Dreyer, G. (1996). Evaluation of the Og4C3 ELISA in *Wuchereria bancrofti* infection: infected persons with undetectable or ultra-low microfilarial densities. *Tropical Medicine & International Health*, *1*(6), 859-864.

Roll Back Malaria and World Health Organization. (2000). *New perspectives: malaria diagnosis: report of a joint WH. Retrieved from http://apps.who.int/iris/bitstream/10665/66321/1/WHO_CDS_RBM_2000.14.pdf*

Romagnani, S. (2000). T-cell subsets (Th1 versus Th2). Annals of Allergy, Asthma, and Immunology, 85(1): 9-18.

Romagnani, S., Parronchi, P., D'Elios, M.M., Romagnani, P., Annuziato, F.,Piccinni, M.P., Samppgnaro, S., Mavillia, C., De Carli, M., Maggi, E. &

Del Prete, G.F.(1997). An update on human Th1 and Th2 cells. International Archives of Allergy Immunology, 113(1-3): 153-156.

Sahu, B. R., Mohanty, M. C., Sahoo, P. K., Satapathy, A. K., & Ravindran, B. (2008). Protective immunity in human filariasis: a role for parasite-specific IgA responses. *Journal of Infectious Diseases*, *198*(3), 434-443.

Samykutty, A., Dakshinamoorthy, G., & Kalyanasundaram, R. (2010). Multivalent vaccine for lymphatic filariasis. *Procedia in Vaccinology*, *3*, 12-18. Doi:10.1016/j.provac.2010.11.003.

Schellekens, S. M., Ananthakrishnan, S., Stolk, W. A., Habbema, J. D. F., & Ravi, R. (2005). Physicians' management of filarial lymphoedema and hydrocele in Pondicherry, India. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *99*(1), 75-77. Doi:10.1016/j.trstmh.2004.05.001.

Schuetz, A., Addiss, D. G., Eberhard, M. L., & Lammie, P. J. (2000). Evaluation of the whole blood filariasis ICT test for short-term monitoring after antifilarial treatment. *The American journal of tropical medicine and hygiene*, *62*(4), 502-503.

Scott, A. L. (2000). Lymphatic Filariasis: A historical perspective (T.B. Nutman, Ed.). Imperial College Press, MD.

Simonsen, P. E., & Dunyo, S. K. (1999). Comparative evaluation of three new tools for diagnosis of bancroftian filarial based on detection of specific circulating antigens. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *93*(3), 278-282.

Simonsen, P. E., & Magesa, S. M. (2004). Short Communication: Observations on false positive reactions in the rapid NOW® Filariasis card test. *Tropical Medicine & International Health*, *9*(11), 1200-1202.

Simonsen, P. E., Meyrowitsch, D. W., Jaoko, W. G., Malecela, M. N., Mukoko, D., Pedersen, E. M., ... & Michael, E. (2002). Bancroftian filariasis infection, disease, and specific antibody response patterns in a high and a low

endemicity community in East Africa. *The American journal of tropical medicine and hygiene*, *66*(5), 550-559.

Southgate, B. A. (1974). A quantitative approach to parasitological techniques in Bancroftian filariasis and its effect on epidemiological understanding. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *68*(3), 177-185.

Southgate, B. A., & Hamilton, P. J. S. (1974). Problems of clinical and biological measurements in the epidemiology and control of filarial infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *68*(3), 177-185.

Srividya, A., Pani, S. P., Rajagopalan, P. K., Bundy, D. A. P., & Grenfell, B. T. (1991). The dynamics of infection and disease in Bancroftian filariasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *85*(2), 255-259.

Steel, C., Kubofcik, J., Ottesen E.A. and Nutman, T.B. (2012a) Antibody to the filarial antigen Wb123 reflects reduced transmission and decreased exposure in children born following single mass drug administration (MDA), Plos Neglected Tropical Diseases 6(12): e1940. doi:10.1371/journal.pntd.0001940.

Steel, C., Kubofcik, J., Ottesen, E. A., & Nutman, T. B. (2012b). Antibody to the filarial antigen Wb123 reflects reduced transmission and decreased exposure in children born following single mass drug administration (MDA).

Supali, T., Rhamah, N., Djuardi, Y., Sartono, E., Ruckert, P. & Fischer, P. (2004). Detection of filarial-specific IgG4 antibodies using Brugia Rapid test in individuals from an area highly endemic for *Brugia timori*. Acta Tropica, 90: 255-261. doi:10.1186/1756-3305-6-299.

Terhell, A. J., Haarbrink, M., Abadi, K., Bronneberg, D. C., Tieleman, M. C., Asri, M., & Yazdanbakhsh, M. (1996). A filter paper technique for the detection of anti-filarial IgG4 in lymphatic filariasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *90*(2), 196-198.

Terhell, A. J., Price, R., Koot, J., Abadi, K., & Yazdanbakhsh, M. (2000). The development of specific IgG4 and IgE in a paediatric population is influenced by filarial endemicity and gender. *Parasitology, 121*(05), 535-543

Terhell, A., Wahyuni, S., Pryce, A., Koot, J., Abadi, K., & Yazdanbakhsh, M. (2002). Anti-filarial and total lgG4 and IgE antibody levels are correlated in mothers and their offspring. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 96*(3), 334-339.

TGA. (2010). IVD medical devices: Definitions and links. Retrieved from https://www.tga.gov.au/ivd-medical-devices-definitions-links.

TGA. (2011a). *Overview of the regulatory framework for in-vitro diagnostic medical devices*. Retrieved from https://www.tga.gov.au/overview-regulatory-framework-vitro-diagnostic-medical-devices.

TGA. (2011b). Australian regulatory guidelines for medical devices (ARGMD). Retrieved from https://www.tga.gov.au/publication/australian-regulatory-guidelines-medical-devices-argmd.

TGA. (2012). Therapeutics Goods (Medical Devices) Regulations 2002 (amended from Therapeutic Goods Act 1989). Retrieved from http://www.comlaw.gov.au/Details/F2012C00424.

TGA. (2013). IVD guidance documents. Retrieved from https://www.tga.gov.au/ivd-guidance-documents

TGA. (2014a). *Medical devices notices & standard orders*. Retrieved from https://www.tga.gov.au/medical-devices-notices-standards-orders.

TGA. (2014b). *Essential principles checklist (medical devices).* Retrieved from https://www.tga.gov.au/form/essential-principles-checklist-medical-devices

Uzicanin, A., Lubega, I., Nanuynja, M., Mercader, S., Rota, P., Bellini, W., & Helfand, R. (2011). Dried blood spots on filter paper as an alternative specimen for measles diagnostics: detection of measles immunoglobulin

M antibody by a commercial enzyme immunoassay. *Journal of Infectious Diseases*, *204*(suppl 1), S564-S569.

Van den Berghe, L., Peel, E., & Chardome, M. (1963). Two Sheathed Microfilariae in Animals from the Congo Basin Forest. *The Journal of Parasitology*, 471-473.

Van der Neut Kolfschoten, M., Schuurman, J., Losen, M., Bleeker, W. K., Martínez-Martínez, P., Vermeulen, E., ... & Parren, P. W. (2007). Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science*, *317*(5844), 1554-1557.

Vanamail, P., Subramanian, S., Das, P. K., Pani, S. P., & Rajagopalan, P. K. (1990). Estimation of fecundic life span of *Wuchereria bancrofti* from longitudinal study of human infection in an endemic area of Pondicherry (south India). *The Indian journal of medical research*, *91*, 293-297.

Voller, A., Bartlett, A., & Bidwell, D. E. (1978). Enzyme immunoassays with special reference to ELISA techniques. *Journal of Clinical Pathology*, *31*(6), 507-520.

Voller, A., Bidwell, D. E., & Bartlett, A. (1976). Enzyme immunoassays in diagnostic medicine: Theory and practice. *Bulletin of the World Health Organization*, *53*(1), 55–65.

Voller, A., & Bidwell, D. E. (1986). The enzyme-linked immunosorbent assay (ELISA) test for the identification of blood-meals of haematophagous insects. *Bulletin of entomological research*, *76*(02), 321-330.

Voller, A., Bidwell, D., Huldt, G., & Engvall, E. (1974). A microplate method of enzyme-linked immunosorbent assay and its application to malaria. *Bulletin of the world health organization*, *51*(2), 209.

Wartman, W.B. (1947). Filariasis in American armed forces in World War II. *Medicine*, *26*(4), 333-394.

Washington University of St. Louis. (2014). ELISA protocol (human IgG4 antibody), ELISA for human IgG4 Ab to filarial antigen Bm14-HIS.

Retrieved from http://www.filariasiscenter.org/molecular-resources/research-materials/elisa-protocol-human-igg4-antibody.

Weil, G. J. (1990). Parasite antigenemia in lymphatic filariasis. *Experimental parasitology*, *71*(3), 353-356.

Weil, G. J. (2005). Diagnostic tools for filariasis elimination programmes. WHO. Retrieved from http://www/who.int/tdr/publications/publications/swg_lymph_fil.htm.

Weil, G. J., Curtis, K. C., Fakoli, L., Fischer, K., Gankpala, L., Lammie, P. J., ... & Fischer, P. U. (2013). Laboratory and field evaluation of a new rapid test for detecting *Wuchereria bancrofti* antigen in human blood. *The American journal of tropical medicine and hygiene*, *89*(1), 11-15.

Weil, G. J., Curtis, K. C., Fischer, P. U., Won, K. Y., Lammie, P. J., Joseph, H., Melrose, W.D. & Brattig, N. W. (2011). A multicenter evaluation of a new antibody test kit for lymphatic filariasis employing recombinant *Brugia malayi* antigen Bm-14. *Acta tropica*, *120*, S19-S22.

Weil, G. J., Hussain, R., Kumaraswami, V., Tripathy, S. P., Phillips, K. S., & Ottesen, E. A. (1983). Prenatal allergic sensitization to helminth antigens in offspring of parasite-infected mothers. *Journal of Clinical Investigation*, *71*(5), 1124.

Weil, G. J., Lammie, P. J., & Weiss, N. (1997). The ICT filariasis test: a rapid-format antigen test for diagnosis of bancroftian filariasis. *Parasitology today*, *13*(10), 401-404.

Weil, G. J., & Liftis, F. A. N. Y. A. (1987). Identification and partial characterization of a parasite antigen in sera from humans infected with *Wuchereria bancrofti*. *The Journal of Immunology*, *138*(9), 3035-3041.

Weil, G. J., & Ramzy, R. M. (2007). Diagnostic tools for filariasis elimination programs. *Trends in parasitology*, *23*(2), 78-82.

WHO, (1997). WHA 50.29 Retrieved from http://www.who.int/lymphatic_filariasis/policy/en/

WHO, 2007 Report of the Ninth Workshop for Pacific Lymphatic Filariasis Programme Mangers, WHO, Fiji, pp. 1-38. Remove as not yet used <<1% CFA acceptance in PacELFs>

WHO , 2010, Progress report 2000-2009 and strategic plan 2010-2020 of the global programme to eliminate lymphatic filariasis: halfway towards eliminating lymphatic filariasis

WHO (2011). Monitoring and epidemiological assessment of mass drug administration.

WHO. (2012). Accelerating Work to Overcome the Global Impact of Neglected Tropical Diseases; A Roadmap for Implementation. Retrieved from http://www.who.int/neglected_diseases/NTD_RoadMap_2012_Fullversion.pdf

WHO, (2013a) Lymphatic Filariasis: GPELF, Practical entomology, A handbook for national elimination programmes. Retrieved from http://apps.who.int/iris/bitstream/10665/87989/1/9789241505642_eng.pdf

WHO, (2013b). Lymphatic Filariasis: GPELF, Training in monitoring and epidemiological assessment of mass drug administration for eliminating lymphatic filariasis. Retrieved from *whqlibdoc.who.int/publications/2011/9789241501484_eng.pdf*

WHO. (2013). *Global Health Observatory (GHO) data: Lymphatic filariasis, situations and trends.* Retrieved from http://www.who.int/gho/neglected_diseases/lymphatic_filariasis/en/.

WHO. (2014a). Lymphatic filariasis: Programmes. Retrieved from http://www.who.int/lymphatic_filariasis/disease/en/

WHO (2015). Global Health Observatory (GHO) data. Lymphatic filariasis; Situations and trends. Retrived from http://www.who.int/gho/neglected_diseases/lymphatic_filariasis/en/

Williams, S. A., Laney, S. J., Bierwert, L. A., Saunders, L. J., Boakye, D. A., Fischer, P., ... & Ottesen, E. A. (2002). Development and standardization of a rapid, PCR-based method for the detection of *Wuchereria bancrofti* in mosquitoes, for xenomonitoring the human prevalence of bancroftian filariasis. *Annals of tropical medicine and parasitology*, *96*(2), 41-46.

Witt, C., & Ottesen, E. A. (2001). Lymphatic filariasis: an infection of childhood. *Tropical Medicine & International Health*, *6*(8), 582-606.

Wright, P. F., Nilsson, E., Van Rooij, E. M., Lelenta, M., & Jeggo, M. H. (1993). Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Revue scientifique et technique (International Office of Epizootics), 12(2), 435-450.

Wynd, S., Durrheim, D. N., Carron, J., Selve, B., Chaine, J. P., Leggat, P. A., & Melrose, W. (2007a). Socio-cultural insights and lymphatic filariasis control–lessons from the Pacific. *Filaria journal*, *6*(1), 3.

Wynd, S., Melrose, W. D., Durrheim, D. N., Carron, J., & Gyapong, M. (2007b). Understanding the community impact of lymphatic filariasis: a review of the sociocultural literature. *Bulletin of the World Health Organization*, *85*(6), 493-498.

Xie, H., Bain, O., & Williams, S. A. (1994). Molecular phylogenetic studies on filarial parasites based on 5S ribosomal spacer sequences. Parasite, 1(2), 141-151.

Yahathugoda, T. C., Wickramasinghe, D., Weerasooriya, M. V., & Samarawickrema, W. A. (2005). Lymphoedema and its management in cases of lymphatic filariasis: the current situation in three suburbs of Matara, Sri Lanka, before the introduction of a morbidity-control programme. *Annals of tropical medicine and parasitology*, *99*(5), 501-510. Doi:10.1179/136485905X46450.

Zhong, M., McCarthy, J., Bierwert, L., Lizotte-Waniewski, M., Chanteau, S., Nutman, T. B., ... & Williams, S. A. (1996). A polymerase chain reaction

assay for detection of the parasite *Wuchereria bancrofti* in human blood samples. *The American journal of tropical medicine and hygiene*, *54*(4), 357-363.

Ziperstein J, Dorkenoo M, Datagni M, Drexler N, Murphy M, Sodahlon Y, Mathieu E. (2014). Final program evaluation methods and results of a National Lymphedema Management Program in Togo, West Africa. Journal of Epidemiology and Global Health, 4(2):125-33. doi: 10.1016/j.jegh.2013.11.001.

Zouré, H. G., Wanji, S., Noma, M., Amazigo, U. V., Diggle, P. J., Tekle, A. H., & Remme, J. H. (2011). The geographic distribution of Loa loa in Africa: results of large-scale implementation of the Rapid Assessment Procedure for Loiasis (RAPLOA). *PLoS Negl Trop Dis*, *5*(6), e1210.

APPENDIX 1

Set of Serum samples used for ELISA Evaluations

	Prefix used	Description	Comments
	NEI	Australian Non-endemic serum sample set 1	
	NEC	Australian non-endemic sample set 2	Cancer patients
	NEW	Non-endemic from USA	
	SLM	Defined set of samples from Sri Lanka	Wayne Melrose
	EG	Endemic from Egypt	
	EWP	Endemic from GW Naru (PNG) set 1	
	EWV	Endemic samples from GW	VJCsamples
	EM	Endemic from PNG Set 2	Wayne melrose
	NI	New Ireland samples PNG	
	Ref–ve	Non-endemic sample from USA	
	RefE-ve	Endemic negative	
	Kit –ve	Commercial negative pool sourced from non-endemic population	
	Kit +ve	Highly positive sample from Sri Lanka	

APPENDIX 2

Filariasis Bm14 Antibody CELISA Product Insert

Lymphatic Filariasis Bm14 Antibody CELISA

BACKGROUND

Lymphatic filariasis (LF), caused by the filarial worm, *Wuchereria bancrofti,* and transmitted through a mosquito vector, affects more than 100 million people in more than 83 countries worldwide[1]. A further billion people are at risk from the infection, which in its chronic stage (elephantiasis) has profound physical, social, and economic consequences, and represents the world's fourth leading cause of permanent disability. The disease has been targeted by the WHO for eradication by the year 2020 through an international programme of mass drug administration (MDA)[2] operated by affected countries in collaboration with the Global Alliance to Eliminate Lymphatic Filariasis (GAELF).

To monitor the effectiveness of the LF eradication programme a number of diagnostic tools have been employed, including microfilaremia quantitation and parasite antigen immunoassays, both of the rapid and ELISA format. Specific IgG4 anti – filarial antibodies have also been proposed as an effective marker for interruption of LF transmission, particularly in young children[3].

The Bm14 Antibody CELISA Kit is an indirect ELISA for the detection of human IgG4 antibodies to the filarial recombinant antigen, Bm14[4,5,6]. Added sensitivity and specificity result from the use of a monoclonal anti – IgG4 indicator. The test is designed to be used with serum samples as well as blood spot eluates collected on filter paper in the field.

INTENDED USE AND PRINCIPLE OF THE TEST

This test is an indirect ELISA for the detection of Bm14 specific IgG4 antibody. Antibody is bound by the recombinant antigen Bm14 coated on the inner surface of the test strip. Peroxidase-conjugated antibody to human IgG4 is added and reacts with bound antibody. A chromogenic substrate for peroxidase is added. If antibody to LF is present there is a reaction which results in the development of a blue colour, which is in proportion to the serum level of the antibody. Addition of stopping solution changes the blue colour to yellow.

CONTENTS OF THE KIT

FAMW	Celisa Plate – 1x96 wells - (single use only)	5 plates
ab	Positive Control	0.1mL
ac	Negative Control	0.1mL
FASD	Sample Diluent (10x)	60mL
FAPO	Enzyme Conjugate (100x)	0.6mL
FACD	Conjugate Diluent	60 mL
FAPT	PBS/Tween (20x)	250mL
FASC	Substrate Chromogen (TMB) (20x)	3.0mL
FASB	Substrate Buffer	60mL
FASS	Stopping Solution	30mL

English

Store all components at 2-8^{O}C.Expiry dates are clearly marked on each kit component and on the box. Expiry dates do not change once opened.

MATERIALS REQUIRED BUT NOT PROVIDED

Micropipettes and tips; clean glassware or plastic containers for solutions; humid chamber; ELISA washer; and Spectrophotometer to read absorbances at a single wavelength of 450nm.

PRECAUTIONS

For in vitro diagnostic use only. Reagents should not be used after the expiry date shown on the label. If protective packaging is damaged, contact your local distributor and ask for a replacement. Do not mix reagents from different kits. Thimerosal preservative added to some components is a poison. Exercise caution when handling these components. The stopping solution is corrosive. Avoid contact with skin, eyes and mucous membranes. Dispense all reagents with care to avoid cross contamination of wells. Avoid exposure of the substrate to light. Treat all clinical and control material as though potentially infectious and dispose of in accordance with local operating regulations. For further information, please refer to the Material Safety Data Sheet.

INSTRUCTIONS FOR USE

Preparation of Wash Buffer

If crystals are present, warm the concentrate to dissolve. For each microplate, add 50mL PBS-Tween concentrate FAPT to 950mL of distilled water. Label the bottle WASH BUFFER. Store at 2-8^{o}C.

Preparation of Sample Diluent

Prepare the working Sample Diluent FASD (1x) by diluting the 10x Concentrate in distilled water. Mix the buffer thoroughly before using to dilute test samples.

Preparation of Samples

Serum or Plasma

Collect patient blood samples by standard venipuncture procedure. The test may be used with serum or plasma. The serum or plasma should be stored below -10°C if the analysis is delayed. Prepare a 1/100 dilution of the positive control ab, the negative control ac and the patient specimens using the prepared Sample Diluent FASD (1x) above, ensuring proper mixing. Record the position of each diluted SAMPLE on a work sheet.

Blood Spot Eluates

The recommended filter paper for use is the TropBio filter paper (WHO format). Each filter paper circle absorbs ~10uL of blood. Upon drying at room temperature, the spotted blood circles should be stored at 2-8 °C for short term storage and transportation or -20°C in a freezer for long term storage until ready for testing. Prepare to elute the sample by carefully removing one circle and completely immersing in a tube of 500uL of prepared working Sample Diluent FASD (1x). Leave the samples refrigerated at 2-8 °C overnight. The following day, vortex the contents of the tube to ensure complete elution of sample. The eluate is equivalent to a 1/100 serum dilution and can be used in the Bm14 Antibody CELISA assay without further dilution.

Assay Procedure

1. Bring all reagents to room temperature (18-25 °C) before use.
2. Prepare WASH BUFFER (see Preparation of Wash Buffer).
3. Prepare the working Sample Diluent FASD (1x) (See Preparation of Sample Diluent).
4. Remove required number of FAMW strips. Reseal the foil bag containing unused microwell strips immediately with tape.
5. Pipette 100µL of the SAMPLE, ab and ac, into individual microwells. Include two positive and two negatives in each assay run. Cover and incubate for one (1) hour at 37 °C in a humid chamber. (see Preparation of Samples using serum, plasma or blood spot from filter paper).
6. In the last 10 minutes of the incubation period, prepare the working strength CONJUGATE. Add 10µL of Enzyme Conjugate FAPO to 990µL of FACD and mix thoroughly (allow 1mL per strip of 8 wells).
7. Wash the wells preferably using an automatic plate/strip washer or manually as follows:
   - Empty contents from the wells. Refill with the WASH BUFFER.
   - Repeat this process a further three (3) times. After the fourth wash, bang inverted wells dry on absorbent tissue.
   - NB: take care when flicking out plates, hold side of frame firmly to hold strips in place.
8. Add 100µL of CONJUGATE to each well. Incubate for 45 minutes at 37 °C in a humid chamber.
9. In the last 10 minutes of the incubation period, prepare the working strength SUBSTRATE. Add 50µL of Substrate Chromogen FASC to 950µL of Substrate Buffer FASB and mix thoroughly (allow 1mL per strip of 8 wells). The stability of the solution is 30 minutes.
10. Repeat washing as in step 7.
11. Add 100µL of fresh SUBSTRATE and incubate in the dark (covered) at room temperature for 15 minutes.
12. Add 100µL of Stopping Solution FASS. Tap the plate to mix.
13. Read the results visually or in a spectrophotometer at 450nm or 450/620 nm.

READING AND INTERPRETATION OF RESULTS AND DIAGNOSIS

Photometrically

It is optimal to use a dual beam ELISA reader. Blank machine against the well designated as the blank. Within 30 minutes of stopping the reaction read absorbance of all wells including all the controls at 450nm on an ELISA reader.

For the test results to be accepted the controls must read as follows:

Control	O.D Value (450/620nm)
Negative Control	< 0.20
Positive Control	> 0.40

Note: A cut-off value can be determined by testing a panel of known negative samples. Cut-off value = mean + 3 Standard Deviations (+3SD). A pilot study conducted in- house with 50 blood spot eluates from Australian residents without prior exposure to LF gave a cut-off of 0.25 OD units (mean +3SD)

Visual Reading

Results can also be read visually. Note colour of the specimen wells and compare with the colour of the negative control. If the colour, read visually, of a specimen well is above that of the negative control, then the specimen contains antibody.

WASTE DISPOSAL

Dispose of any unused components as biohazardous waste. For more information, please refer to the MSDS.

SENSITIVITY, SPECIFICITY, & OTHER DATA ON THE BM14 ANTIBODY CELISA KIT

Data on the Bm14 Antibody CELISA test performance can be obtained from your local distributor or by contacting Cellabs.

INDEMNITY NOTICE

Modifications or changes made in the recommended procedure may affect the stated or implied claims. A positive or negative result does not preclude the presence of other underlying causative agents. Cellabs and its agents and distributors shall not be liable for damages under these circumstances

Lymphatic Filariasis Bm14 Antibody CELISA

PREAMBULE

L'agent de la filariose lymphatique (FL) est la filaire *Wuchereria bancrofti,*qui est transmise par un moustique et qui infecte plus de 100 millions de personnes dans plus de 83 pays du monde[1]. Plus d'un milliard de personnes risquent cette infection qui dans son stade chronique (éléphantiasis) a de profondes conséquences physiques, sociales et économiques et représente la quatrième cause d'handicap permanent au monde. L'éradication de cette maladie par l'OMS est prévue en l'année 2020 grâce à un programme de chimiothérapie de masse (CTM)[2] opéré par les pays affectés en collaboration avec l'Alliance Globale pour l'Elimination de la Filariose Lymphatique (AGEFL).

Afin de suivre l'efficacité du programme d'éradication FL, un nombre d'outils diagnostics ont été employés, y compris la mesure de la microfilarémie et d'antigènes parasitaires par dosages immunologiques, en formats rapides et ELISA. Les anticorps spécifiques IgG4 anti-filariose ont également été proposés comme marqueurs efficaces de l'interruption de la transmission FL, en particulier chez les jeunes enfants[3].

La trousse Bm14 Antibody CELISA est un ELISA indirecte pour détecter les anticorps IgG4 humains de l'antigène recombinant Bm14[4,5,6]. L'usage d'un anticorps monoclonal de détection anti-IgG4 augmente la sensibilité et la spécificité du dosage. Le test est conçu pour usage sur échantillons de sérum ainsi que sur échantillons d'élution de gouttes de sang sur papier filtre collectés sur le terrain.

PRINCIPE DU TEST ET INDICATIONS D'EMPLOI

Ce test est un ELISA indirecte pour détecter les anticorps IgG4specifiques Bm14. L'anticorps est lié par l'antigène recombinant Bm14 fixé sur la surface interne des puits de dosage. Un anticorps anti-IgG4 humain conjugué à la peroxydase est ajouté et réagit avec l'anticorps immobilisé. Un substrat chromogène pour peroxydase est ajouté. Si l'anticorps LF est présent, une réaction se produit conduisant au développement d'une couleur bleue, d'intensité proportionnelle au niveau d'anticorps dans le sérum. L'addition d'un réactif d'arrêt change la couleur au jaune.

COMPOSITION DU COFFRET

FAMW	Plaque CELISA – 1x96 puits (usage unique)	5 plaques
ab	Contrôle positif	0.1mL
ac	Contrôle négatif	0.1mL
FASD	Réactif de dilution des échantillons (10x)	60mL
FAPO	Conjugué enzymatique (100x)	0.6mL
FACD	Diluant de conjugué enzymatique	60 mL
FAPT	PBS/Tween (20x)	250mL
FASC	Substrat chromogène (TMB) (20x)	3.0mL
FASB	Tampon du substrat	60mL
FASS	Solution d'arrêt	30mL

Conserver tous les composants à 2-8°C. Les dates de péremption sont clairement marquées sur chaque composant et sur le coffret de la trousse. L'ouverture n'altère pas les dates de péremption.

MATERIELS REQUIS NON FOURNIS

Micropipettes et embouts; verrerie propre ou récipients plastiques pour solutions; eau distillée; chambre humide; laveur ELISA; spectrophotomètre capable de lire à 450 nm.

PRECAUTIONS

Produit à usage uniquement *in vitro*. Ne pas utiliser après la date de péremption indiquée sur l'étiquette. Si l'emballage est abîmé, contactez votre fournisseur local pour un remplacement. Ne pas mélanger les réactifs de coffrets différents. Certains réactifs contiennent du Thimerosal comme préservatif, qui est un poison. Manipulez ces réactifs avec soin. La solution d'arrêt est corrosive. Evitez tout contact avec la peau, les yeux ou les membranes muqueuses. Ajouter les réactifs en évitant tout risque de contamination croisée des puits. Eviter d'exposer le substrat à la lumière. Les échantillons cliniques et les contrôles doivent être considérés comme potentiellement infectieux et jetés selon les procédures en vigueur. Consultez la fiche de sécurité du produit (notice MSDS) pour plus amples informations.

INSTRUCTIONS D'EMPLOI

Préparation du tampon de lavage

Si des cristaux apparaissent dans le concentré, réchauffer le réactif pour les dissoudre. Pour chaque plaque de micropuits, ajouter 50mL de concentré PBS/Tween FAPT à 950mL d'eau distillée. Libeller la bouteille WASH BUFFER. Conserver à 2-8°C.

Préparation du réactif de dilution des échantillons

Préparer la dilution de travail du réactif de dilution des échantillons FASD (1x) en diluant le concentré x10 avec de l'eau distillée. Mélanger le tampon vigoureusement avant usage sur échantillons à tester.

Préparation des échantillons

Sérum ou plasma

Collecter les spécimens sanguins par ponction veineuse courante. Le dosage peut se faire sur plasma ou sérum. Conserver les échantillons de plasma ou sérum à -10°C si l'analyse est retardée. Préparer une dilution au 1/100 du contrôle positif ab,du contrôle négatif ac et des échantillons patients à l'aide du réactif WASH BUFFER et bien mélanger. Repérer la position des échantillons dilués SAMPLE sur la feuille de travail.

Elution de gouttes de sang sur papier filtre

L'emploi du papier filtre TropBio (format OMS) est recommandé. Chaque cercle de papier filtre absorbe ~10uL de sang. Apres séchage à température ambiante, les papiers filtres utilisés doivent être conservés à 2-8°C à court terme ou au congélateur à -20°C pour un stockage de plus longue durée jusqu'à leur emploi. Eluer les échantillons en retirant délicatement un cercle et en l'immergent dans un tube de 500uL de solution de travail de réactif de dilution des échantillons FASD (1x). Laisser les échantillons au réfrigérateur à 2-8°C durant la nuit. Le lendemain, agiter au vortex le contenu du tube d'élution de l'échantillon. Cette élution est équivalente à une dilution du sérum au 1/100 et peut être utilisée dans la trousse Bm14 Antibody CELISA sans dilution supplémentaire.

Mode D'emploi

1. Ramener tous les réactifs à température ambiante (18-25°C) avant l'emploi.
2. Préparer le WASH BUFFER (voir Préparation du Tampon de Lavage).
3. Préparer la solution de travail FASD (1x) (voir Préparation du Reactif de Dilution des Echantillons).
4. Retirer le nombre requis de micropuits FAMW. Immédiatement refermer le sac contenant les micropuits restants avec du ruban adhésif.
5. Ajouter 100µL de SAMPLE, de ab et de ac dans leurs micropuits individuels. Inclure deux contrôles positifs et deux contrôles négatifs dans chaque série de dosage. Couvrir et incuber une (1) heure à 37°C en chambre humide. (voir Préparation des Echantillons – Elution de gouttes de sang sur papier filtre)
6. Dans les 10 dernières minutes de l'incubation, préparer la solution de travail de CONJUGATE. Ajouter 10µL de Conjugué enzymatique FAPO à 990µL de FACD et mélanger vigoureusement (prévoir 1mL de solution de travail par barrette de 8 micropuits).
7. Laver les micropuits au laveur automatique ou manuellement comme suit :
   - Vider le contenu des micropuits. Remplir les micropuits de WASH BUFFER.
   - Répéter l'opération trois (3) fois. Vider le contenu des puits après le quatrième lavage.
   - NB : opérez cette étape avec précaution, en serrant le cadre de la plaque à micropuits pour éviter qu'ils ne s'en délogent.
8. Ajouter 100µL de CONJUGATE dans chaque puits. Incuber 45 minutes à 37°C en chambre humide.
9. Dans les 10 dernières minutes de l'incubation, préparer la solution de travail de SUBSTRATE. Ajouter 50µL de Substrat chromogène FASC à 950µL de Tampon de substrat FASB et mélanger vigoureusement (prévoir 1 mL de solution de travail par barrette de 8 micropuits). Cette solution de travail est stable pendant 30 minutes.
10. Répéter le lavage comme à l'étape 7.
11. Ajouter 100µL de SUBSTRATE frais et incuber (couvert) à l'obscurité pendant 15 minutes à température ambiante.
12. Ajouter 100µL de solution d'arrêt FASS. Taper la plaque pour mélanger.
13. Lire les résultats visuellement ou au spectrophotomètre à 450nm ou à 450/620nm.

LECTURE, INTERPRETATION DES RESULTATS ET DIAGNOSTIC

Lecture spectrophotométrique

Il est préférable d'utiliser un spectrophotomètre à double longueur d'onde. Calibrer la machine contre un blanc désigné. Lire l'absorbance de chaque puit y compris contrôles dans un lecteur à microplaque ELISA dans les 30 minutes suivant l'addition de la solution d'arrêt. Pour être valides, les valeurs des contrôles doivent être comme suit :

Contrôle	Valeur D.O. (450/620 nm)
Contrôle négatif	< 0.20
Contrôle positif	> 0.40

Note: Un seuil discriminant peut être déterminé à l'aide d'un groupe d'échantillons négatifs confirmés. Seuil discriminant = moyenne + 3 Déviations Standard (+ 3 DS). Une étude pilote effectuée par Cellabs sur 50 échantillons sanguins d'élution sur papier filtre provenant de résidents Australiens sans exposition préalable à FL a produit un seuil discriminant de 0.25 unités DO (moyenne + 3 DS).

Lecture visuelle

Les résultats peuvent être lus visuellement. Observer l'intensité couleur des échantillons en comparaison à celle du contrôle négatif. Si un échantillon présente une couleur visuellement clairement supérieure à celle du contrôle négatif, cet échantillon contient des anticorps.

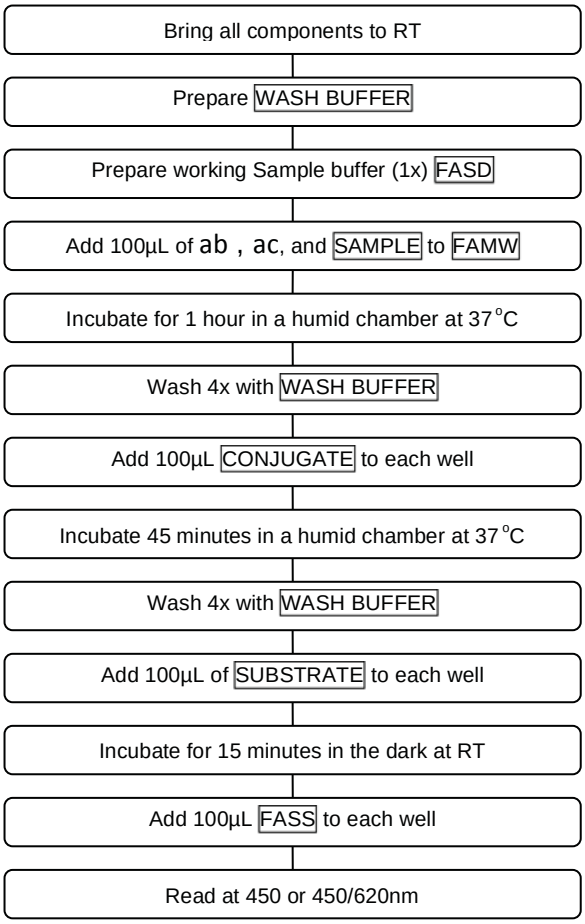
DECHETS

Jetez tout composant inutilisé dans la poubelle aux déchets biologiques. Consultez la fiche de sécurité du produit (notice MSDS) pour plus amples informations.

SENSIBILITE, SPECIFICITE ET AUTRES DONNEES DU TEST
Des données sur la performance du test Bm14 Antibody CELISA sont disponible auprès de votre distributeur local ou en contactant Cellabs.

NOTICE D'INDEMNITE
Toute modification ou variation du protocole d'emploi recommandé peut affecter les performances annoncées du produit. Un résultat positif ou négatif n'exclue pas la présence d'autres agents causatifs sous-jacents. Cellabs et ses agents et distributeurs ne sont légalement responsables d'aucun dommage dans de telles circonstances.

FIGURE 1 Lymphatic Filariasis Bm14 Antibody CELISA DIAGRAM FOR USE



APPENDIX 3

Therapeutics Goods Order No. 34 (TGO34)

COMMONWEALTH OF AUSTRALIA
Therapeutic Goods Act 1966
THERAPEUTIC GOODS ORDER NO. 34
STANDARD FOR DIAGNOSTIC GOODS OF HUMAN ORIGIN

I, PETER STAPLES, Minister of State for Aged, Family and Health Services, pursuant to Section 17 of the Therapeutic Goods Act 1966, by this Order REVOKE Therapeutic Goods Order No. 19 made on the 23 September 1984, and pursuant to sections 13, 15 and 23F of that Act by this Order -

(a) DETERMINE that with respect to quality and method of preparation, the standard for goods which contain materials of human origin and which are intended as laboratory reagents for diagnostic tests, shall be the standard specified in this Order;
(b) DIRECT that for the purposes of Sections 19, 20 and 22 of the said Act, such goods shall be labelled in the manner specified in this Order; and
(c) DETERMINE that the procedures to be carried out in the production of such goods shall include the procedures specified in this Order.

Application

1.(1) Subject to sub-clauses (2) and (3), the goods to which this Order applies are goods which contain materials of human origin and which are intended as laboratory reagents in diagnostic tests.
(2) Clauses 7 to 9 inclusive do not apply to goods manufactured by a process such that human immunodeficiency virus (HIV) would be inactivated.
(3) This Order does not apply to -
(a) goods which, due to either the nature or scarcity of the final product, cannot be prepared from material that is non-reactive when tested for hepatitis B virus surface antigen (HBsAg) or antibody to HIV; or
(b) goods which have been treated at 60°C for 10 hours.

Interpretation

2. In this Order -
"donor" means a person from whom body fluids or tissues are obtained for the purpose of preparing diagnostic goods;
"donor serum" means serum or plasma obtained from a donor and which is intended for the preparation of diagnostic goods of human origin;
"final bulk" means the finished homogeneous material present in the container from which the final containers are filled;
"final lot" means a collection of sealed final containers that have been filled during one working session from a single final bulk and further processed under conditions which ensure its physical, chemical and microbial homogeneity;
"goods" means goods for therapeutic use as defined in the Therapeutic Goods Act 1966;
"HBsAg" means hepatitis B virus surface antigen;
"HIV" means any human immunodeficiency virus known to cause acquired immune deficiency syndrome;
"manufacture" includes the process of collection and testing of starting material as well as subsequent processing of that material into diagnostic goods;
"pooled sera" means a pool of all donor serum or plasma which is processed into a final bulk or final lot; and
"reference panel of sera" means the panel of human sera which is designated by the Commonwealth Department of Community Services and Health as the Australian reference panel of human sera for tests for hepatitis B virus surface antigen.

Source material

3. Goods shall consist of or be derived from source material obtained from donors who are free from -
(a) signs of infectious diseases transmissible by blood products; and
(b) signs of injection with narcotics,
unless, by its nature, such source material must be obtained from donors suffering from specific illness.

Test for hepatitis B virus surface antigen in donor serum

4.
(1) A serological test for HBsAg complying with the specifications of clause 6 shall be carried out on individual donor sera during manufacture.
(2) Donor serum giving a positive reaction for HBsAg shall not be used to prepare goods to which this Order applies.

Test for hepatitis B virus surface antigen in final bulk or final lot

5.
(1) A serological test for HBsAg complying with the specifications of clause 6 shall be carried out by the manufacturer on the final bulk or the final lot.
(2) A final bulk which gives a positive reaction for HBsAg that can be neutralised with antibody to HBsAg shall not be used to prepare goods to which this Order applies.
(3) The final lot, when tested for the presence of HBsAg in accordance with clause 6, shall give a negative reaction.

Sensitivity of hepatitis B virus surface antigen test

6.

(1) The sensitivity of the serological test for HBsAg shall be sufficient to give a positive reaction in all twenty sera labelled FH001 -FHO17 and FHO23 - FHO25 in the reference panel of sera.

(2) A positive control serum known to be weakly reactive shall be included in each test and a positive reaction shall be obtained with this positive control serum for the test to be valid.

Test for antibody to human immunodeficiency virus in donor serum

7.

(1) A serological test for antibody to HIV that is a test in accordance with clause 9 shall be carried out on individual donor sera during manufacture.

(2) Donor serum which gives a positive reaction to a test referred to in clause 9 shall not be used to prepare goods to which this Order applies.

Test for antibody to human immunodeficiency virus in final bulk or final lot

8.

(1) Unless the sera to be used in the manufacture of diagnostic goods of human origin has been collected in premises licensed for such a purpose, being subject to inspection, and using tests approved by the competent Health Authority of a particular country, whose standards are recognised by the Secretary of the Commonwealth Department of Community Services and Health, and supplied with specific certification from the primary manufacturer that the units of blood used in the manufacture of identified production lots were tested for antibody to HIV and found non-reactive, then a serological test for antibody to HIV complying with the specifications of clause 9 shall be carried out by the manufacturer on the pooled sera prior to further consolidation, or on the final bulk, or the final lot.

(2) A pooled sera or final bulk which gives a positive reaction to a test referred to in clause 9, shall not be used to prepare goods to which this Order applies.

(3) The final lot, when tested for the presence of antibody to HIV in accordance with a test referred to in clause 9, shall give a negative reaction.

Sensitivity and specificity of human immunodeficiency virus antibody test

9.

(1) The test for the presence of antibody to HIV shall, if performed in Australia, be either:-

(a) a test performed using a test kit specified in the Schedule to this Order; or

(b) a test performed using a method of greater sensitivity and specificity than that used in a test kit which has been so specified.

(2) The results of a test referred to in paragraph 9 (1) (b) will take precedence over the results of a test referred to in paragraph 9 (1) (a).

(3) The test for the presence of antibody to HIV shall, if performed outside Australia, be either:-

(a) a test performed using a test kit of equal sensitivity and specificity to one specified in paragraph 9 (1) (a); or

(b) a test performed using a method of greater sensitivity and specificity than that used in a test kit which has been so specified.

(4) The results of a test referred to in paragraph 9 (3) (b) will take precedence over the results of a test referred to in sub-clause 9 (3) (a).

Labelling requirements

10. Unless the goods are goods manufactured by a process such that human immunodeficiency virus would be inactivated -

(a) the goods shall include an information leaflet containing -

(i) certification that tests for HBsAg and antibody to HIV have been performed on the goods and have been found to be negative; and

(ii) a warning that the product may be infectious; and

(b) the label attached or affixed to goods shall contain an expiry date or recommended shelf life.

Dated this third day of August 1990

PETER STAPLES
Minister of State for
Aged, Family and Health
Services

APPENDIX 4

Cellabs Pty Ltd
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Brookvale 2100
www.cellabs.com.au

Phone +(61) 2 9905 0133
Fax +(61) 2 9905 0420
Email: cellabs@cellabs.com.au

Product Code:

LF1 (1 Plate kit)
LF2 (5 Plate kit)

LF2.01 (2 September 2014)

TropBio

ELISA KIT FOR DETECTING AND QUANTIFYING *Wuchereria bancrofti* ANTIGEN

MANUFACTURED BY CELLABS PTY LTD.

Cellabs

CELLABS PTY LTD

WWW.CELLABS.COM.AU

LF2.01

Lymphatic filariasis

An antigen detection assay has been developed by TropBio and James Cook University of North Queensland for the detection of Wuchereria bancrofti (Bancroftian filariasis) infection in man. This assay has been marketed to fulfil a perceived need in the testing of human patients suspected of being infected with this parasite.

Lymphatic filariasis in man is caused by infection with the filarial parasites W. bancrofti and Brugia spp. These parasites inhabit the lymphatics and cause disease by obstruction and secondary inflammatory changes to lymph vessels. The disease is transmitted by mosquitoes which ingest microfilariae during feeding on an infected host and transmit the infective larvae to other individuals at a subsequent feeding.

Throughout the world, more than 90 million people are affected by lymphatic filariasis. Most live in the humid tropics in areas such as Africa (south of the Sahara), Egypt, the Indian subcontinent, South-East Asia, China, Madagascar, Papua New Guinea, the Pacific Islands, the Philippines and Central and South America (World Health Organisation, 1984).

Acute symptoms of lymphatic filariasis primarily involve lymphadenitis and lymphangitis. Recurring fever and pain of affected lymph nodes are the normal sequelae. In some patients, symptoms may be less specific, with fever and malaise being the only symptoms. Some infected individuals are asymptomatic.

Lymphoedema becomes apparent after repeated episodes of lymphadenitis, and swelling of the limbs or scrotum may occur. Lymphoedema and elephantiasis may affect the leg, arm and scrotum and occasionally the vulva and breasts, but they are usually restricted to the leg below the knee.

With brugian filariasis, more severe inflammatory changes are noted in the lymphatics, whereas bancroftian filariasis has a more extensive swelling of the entire limb.

Filariasis in previously unexposed migrants to an endemic area has a similar clinical course, but can be manifested earlier (6 - 8 weeks) than the normal 7 - 8 month clinical incubation period. Microfilaraemia is an uncommon finding in these migrant individuals, reflecting an intense immunological reaction to the parasite (Partono et al., 1977).

References

More, S.J., and Copeman, D.B. (1990) A highly specific and sensitive monoclonal antibody-based ELISA for the detection of circulating antigen in bancroftian filariasis. Tropical Medicine and Parasitology 41: 403- 406

Partono, F., Oemijati, S. and Hudojo, I. (1977) Malayan filariasis in Central Sulawesi (Celebes), Indonesia. Southeast Asian Journal of Tropical Medicine and Public Health 8: 452-458

Turner P., Copeman B., Gerisl, D. and Speare R (1993) A comparison of the OG4C3 antigen capture ELISA, the Knott test, an IgG_4 assay and clinical signs, in the diagnosis of Bancroftian filariasis. Tropical Medicine and Parasitology 44: 45-48

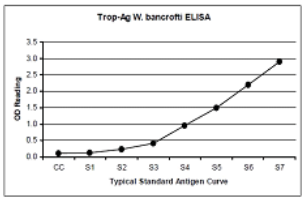
World Health Organisation (1984) Lymphatic Filariasis. Fourth Report of the WHO Expert Committee on Filariasis, Geneva. WHO Technical Services 702

READING AND INTERPRETATION OF RESULTS

Read the microwell plate at duale wavelength in a compatible ELISA plate reader, blanked against air. For the test results to be accepted, standard 1 (Negative control) and Standard 7 (Positive Control) must read as follows:

Standard 1 OD <0.2
Standard 7 OD >2.0

A typical standard curve for the seven standard antigens is shown below. These values are used to allocate the test samples into one of eight titre groups according to the table shown below



Using the seven standard samples it is possible to allocate the test samples into eight titre groups according to the following table. The titre groups are very useful for population studies. Moore and Copeman (1990) allocated antigen units to the seven controls.

Allocation of samples to titre groups

Titre group	Absorbance	Standard No	Antigen units
1	< Control sample No 1	1	<1.0
2	# Control sample No 2	2	32
3	# Control sample No 3	3	128
4	# Control sample No 4	4	512
5	# Control sample No 5	5	2,048
6	# Control sample No 6	6	8,192
7	# Control sample No 7	7	32,000
8	> Control sample No 7		

Studies of serum samples of uninfected Australian residents

Serum:	Samples	Mean	Standard Deviation
Study 1	303	OD 0.149	0.019
Study 2	66	OD 0.063	0.129

Blood filter paper samples:

Study 1	100	OD 0.149	0.045

Standard 1 control sample does not contain any antigen (<OD 0.2). Test samples allocated in titre group 1 can be considered non-reactors (negative). Samples allocated in titre group 2 are equivocal reactors (grey area) and the test may be repeated. Test samples allocated in titre groups 3-7 are positive reactors containing circulating filarial antigen (CFA).

Note: If using the antigen unit method of interpretation, ensure that standards 1-7 results for each plate follow the typical OD values for each standard as shown in the standard curve in Figure 1.

In a previous study in New Guinea, Ghana, the Philippines and India this group represented up to 10% of the test samples. None of the Australian samples from an uninfected population were allocated to group 3. It is very likely that samples allocated to group 3 are reacting in the assay. Further data will be collected on this group to determine their status.

Further Reading:

Burgess, G.W. and Smith, J.R. (1997) The development and marketing of an ELISA to detect Wuchereria bancrofti antigenaemia. 2 International Conference on the Control of Lymphatic Filariasis. Townsville, Australia.

Laltha, P., Ravichandran, M., Suba, S., Kalilaj, P., Narayanan, R.B. and Jayaraman, K. (1998) Quantitative assessment of circulating antigens in human lymphatic filariasis: a field evaluation of monoclonal antibody-based ELISA using blood collected on filter strips. Tropical Medicine and International Health 3:41-45

Lammie, P.J., Reiss, M.D., Dimock, K.A., Streit., T.G., Roberts, J.M. and Eberhard, M.L. (1998) Longitudinal analysis of the development of filarial infection and antifilarial immunity in a cohort of Haitian children. American Journal of Tropical Medicine and Hygiene 59:217-221

Rocha, A., Addiss, D., Ribeiro, M.E., Noroes, J., Medeiros, Z. and Dreyer, G. (1996) Evaluation of the Og4C3 ELISA in Wuchereria bancrofti infection: infected persons with undetectable or ultra-low microfilarial densities. Tropical Medicine and International Health 1:859-864

Simonsen, P.E. and Dunyo, S.K. (1999) Comparative evaluation of three new tools for diagnosis of Bancroftian filariasis based on detection of specific circulating antigens. Transactions of the Royal Society Tropical Medicine and Hygiene 93: 278-282

CONTENTS

	Code	KF1	KF2
Microwell plates (Og4C3)	FGMW	1	5
Sample Diluent at working strength	FGSD	30mL	120mL
Antibody and Conjugate Diluent (10x)	FGCD	5mL	100mL
Standard Antigens (1-7)	FGPC	-	0.8mL
Standard Antigen No. 7 (Positive Control)	FGPC	0.6mL	-
Standard Antigen No. 1 (Negative Control)	FGNC	0.6mL	-
Rabbit anti-onchocerca antibody (120x)	FGAB	0.07mL	0.35mL
Anti-rabbit HRP-conjugate (120x)	FGPO	0.07mL	0.35mL
ABTS Chromogen (ready to use)	FGSC	12mL	60mL
Wash Buffer (20x).	FGPT	100mL	250mL

METHOD FOR SERUM OR PLASMA SAMPLES

All steps carried out at room temperature. Ensure that all reagents and the microtitre plates are at room temperature before use.. Quantities indicated below refer to the use of ONE plate.
To prepare wash buffer, dilute 25 ml of wash buffer (20x) from the dispensing bottle to 500mL of distilled water.

Sample Preparation

To prepare samples (serum or plasma), add 100uL of test sample to 300uL of sample diluent in a suitable tube for boiling eg. Eppendorf microcentrifuge tubes (pierce the lid with a fine needle to allow air to escape during boiling). Do not boil or dilute standards 1-7.

Place the tubes into a 100°C boiling water bath for five minutes. After boiling, centrifuge the samples at 2,000g for 15 minutes (racked tubes) or 10,000g for five minutes (Eppendorf tubes). The clear supernatant fluid contains the heat stable antigen.

1. Add 50uL aliquots of boiled sample supernatant fluid to a test well. Up to 80 samples can be tested per plate.

   Add 50uL of Standard Antigens (1-7) and conjugate control (use sample diluent) in duplicates using two strips of a plate. Refer to the plate layout diagram on Page 3.

2. Incubate by placing the plate in a humid container for 1.5 hours at room temperature. *(Note: using an overnight incubation will result in high OD readings that will not produce a typical standard curve).*

3. Wash the plate three times with wash buffer, invert and tap gently to remove residual buffer.

4. Dilute 50uL of the anti-Onchocerca antibody (Yellow cap) to 6 mL of antibody diluent (Blue solution). Mix thoroughly. Add 50 uL of diluted rabbit-anti onchocerca antibody to all wells. Place the plate in a humidity chamber and incubate for one hour at room temperature.

6. Wash the plate three times as before.

7. Dilute 50 uL of Anti-rabbit HRPO conjugate (Purple cap) to 6 mL of antibody diluent. Mix thoroughly. Add 50 uL of diluted conjugate to all wells. Place in a humidity chamber and incubate for one hour at room temperature.

8. Wash the plate three times as before.

9. Add 100uL of ABTS (do not dilute) to each well and incubate for one hour in the dark at room temperaure.

10. Read the plate using a spectrophotometer at 414 nm, or dual wavelengths of 414 / 492 nm.

METHOD FOR FILTER PAPER SAMPLES

Note: This kit does not contain 1% hydrogen peroxide (H_2O_2). You are required to prepare the solution in Step 5 of the method. Filter paper discs can be ordered separately. See ordering information at the back of this kit insert.

1. **Preparation of filter paper samples:** Cut three protrusions from the filter paper disk and add them to a suitable tubes eg. 2 mL Eppendorf microtubes. Protrusions can be cut in half to allow the disks to reach the bottom of the tubes.

Add 200 uL of sample diluent to each tube and eave the blood samples to elute overnight at 2-8°C.

Next morning, place the tubes into a 100°C water bath for five minutes

After heating in the water bath, centrifuge the samples at 2,000 *g* for 15 minutes. The supernatant contains the heat stable antigen.

2. Add 50uL aliquots of boiled sample supernatant fluid to a test well. Up to 80 samples can be tested per plate.

Add 50uL of Standard Antigens 1-7 (do not boil) and conjugate control (use sample diluent) in duplicates using two strips of a plate. Refer to the plate layout diagram on Page 3.

3. Incubate by placing the plate in a humid container for 1.5 hours at room temperature. *(Note: using an overnight incubation will result in high OD readings that will not produce a typical standard curve)*

4. Wash the plate three times with wash buffer, invert and tap gently to remove residual buffer.

5. Prepare a 1% hydrogen peroxide solution for ONE plate by adding 400uL of hydrogen peroxide (~30%) to 12mL of 1x wash buffer.

6. Add 50uL of the 1% hydrogen peroxide solution to all test wells and incubate for 10 minutes at room temperature. Do not add 1% hydrogen peroxide to standards and controls.

7. Wash the plate three times with wash buffer, invert and tap gently to remove residual buffer.

8. Dilute 50uL of the anti-Onchocerca antibody (Yellow cap) to 6 mL of antibody diluent (Blue solution). Mix thoroughly. Add 50 uL of diluted rabbit anti-onchocerca antibody to all wells. Place the plate in a humidity chamber and incubate for one hour at room temperature.

9. Wash the plate three times as before.

10. Dilute 50 uL of Anti-rabbit HRPO conjugate (Purple cap) to 6 mL of antibody diluent. Add 50 uL of diluted conjugate to all wells. Place in the plate in a humidity chamber and incubate for one hour at room tempeature.

11. Wash the plate three times as before.

12. Add 100uL of ABTS (do not dilute) to each well and incubate for one hour in the dark.

13. Read the plate using a spectrophotometer at 414 nm, or dual wavelengths of 414 / 492 nm.

Standard ELISA plate layout

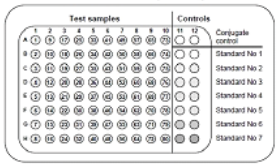


Figure 1: ELISA plate layout

NOTES ON THE ASSAY

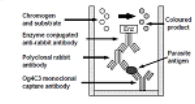
This kit has been designed to be used in conjunction with serum or plasma samples. Samples are boiled in an EDTA solution and centrifuged. This treatment has been shown to dissociate antigen/antibody complexes. The target antigen is heat stable and is retained in the supernatant. Following treatment there can be up to a fourfold rise in antigen titre.

For filter paper blood samples, a 1% hydrogen peroxide (H_2O_2) solution step is required to reduce the interference of endogenous peroxidase released by the haemolysis of red blood cells on the filter paper.

Test samples can be handled in a 96 well microtitre format using racked tubes. The racks are modified to allow them to be placed into a boiling water bath. Subsequently the racks can be centrifuged in a rotor with microtitre buckets at approximately 2,000 g for 15 minutes.
The 96 well microtitre plates supplied are coated with a monoclonal antibody (Og4C3) which has been shown to specifically recognise only Wuchereria bancrofti antigen in human sera. Og4C3 will not cross react with human sera infected with Onchocerca volvulus, Brugia malayi, Brugia timori, Loa loa, Mansonella perstans, Strongyloides stercoralis, Dracunculus medinensis or Ascaris lumbricoides. The cattle parasite Onchocerca gibsoni is recognised by this monoclonal antibody and is used to standardise the ELISA.

The indicator antibody is produced by vaccinating rabbits with purified Onchocerca gibsoni antigen. Finally the rabbit globulin is detected by goat anti-rabbit globulin conjugated to horseradish peroxidase. The substrates solution which contains the chromogen ABTS produces a green colour which has maximum absorption at 414 nm.

ELISA configuration (antigen detection) for *W. bancrofti*



PRECAUTIONS

For research use only. Reagents should not be used after the expiry date shown on the label. Do not mix reagents from different kits. Exercise caution when handling the kit components. Dispense all reagents with care to avoid cross-contamination of wells. Treat all clinical and control material as though potentially infectious and dispose of in accordance with local operating regulations. For further information, please refer to the Material Safety Data Sheet.

APPENDIX 5

FILARIA Ab CELISA

1. Scope: This procedure outlines the specifications of the Filaria Ab Celisa Kit.

2. Final Kit Performance Specifications:

Product Name:	Filaria Ab Celisa
Product Code:	KF3
Kit Format:	480 Tests (5 Plate)
Specificity:	Human IgG4

QC Procedure:	Doc. No. 2105		
QC Records:	Final Kit should be QC tested against a previously released reference kit and recorded on Doc No. 2203. It is only once this document is QMC signed-off and the ELISA Kit – Documentation Compliance Check List for Release (Doc. No. 2216) is stamped "Approved" that the kit Batch Release Form can be completed.		
Batch Release Form:	Doc. No. 2254		
Specifications:		**450nm**	**450/620nm**
	Blank:	<0.15	< 0.10 OD units
	QC Negative Control:	<0.25	< 0.20 OD units
	Kit Negative Control:	<0.25	< 0.20 OD units
	QC Positive Control (Low):	>0.4	> 0.4 OD units
	QC Positive Control (High):	>2.0	> 2.0 OD units
	Kit Positive Control:	>2.5	> 2.5 OD units
	Cut-Off:		

3. Final Kit Components and Expiry

Kit Components	Code	Quantity	Vial Type	RMS	Expiry @ 2 – 8 °C	Prod'n Doc. No.	QA Doc. No.
Celisa Plate	FAMW	5 x 96 wells	Large Foil Bag	8.1.313	12 mths	1127	1127
Positive Control	FAPC	1 x 0.05mL	0.5 Nalgene Tube (Red Lid)	8.1.285	18 mths	2031	2105
Negative Control	FANC	1 x 0.05mL	0.5 Nalgene Tube (Clear Lid)	8.1.285	18 mths	2035	2105
Sample Diluent (10x)	FASD	1 x 60mL	60 mL Nalgene	8.1.261	24 mths	4113	2105
Enzyme Conjugate [100x]	FAPO	1 x 0.6mL	2.0 mL vial (Blue Lid)	8.1.298	12 mths	41121	2105
Conjugate Diluent	FAPO	1 x 60mL	60 mL Nalgene	8.1.261	24 mths	4160	2105
PBS/Tween [20x]	FAPT	1 x 250mL	250mL Nalgene Bottle	8.1.309	24 mths	4152	2105
Substrate Chromogen	FASC	1 x 3.0mL	8mL Amber Vial	8.1.301	24 mths	4105	2105
Substrate Buffer	FASB	1 x 60mL	60mL Nalgene Bottle	8.1.261	24 mths	4154	2105
Stopping Solution	FASS	2 x 30mL	30mL Nalgene	8.1.3	24	4158	2105

			Bottle	06	mths		
Product Insert	LF3	1	N/a	N/a	N/a	N/a	N/a

4. Expiry of Final Kit

The expiry of the Final Kit will be equivalent to the expiry of the component with the earliest expiry date, with a maximum shelf life of 12 months at 2 – 8 ^{o}C.

5. Documentation

Refer to documents referenced throughout this document.

APPENDIX 6

RDT List of Current Patents

RDT Patents affecting the manufacture of needed rapid tests for LF

Patent Number	Description	How it affects LF RDT	Patent Issue Date	Patent Expiry Date	Comments
US 5602040	Assays – analytical test device eg. Pregnancy testing using a) hollow casing; b) porous carrier; c) bibulous sample receiver; d) reaction zones (test line and control lines); e) movement of reagent through porous carrier	Cassette format assay in general however, not patented in Australia	12 May1994	Expected date 2009 - 2011	No Australian Patent
US 5622871 US 6187598 US 6228660	Capillary immunoassay and device therefor comprising mobilizable particulate labelled reagents	Immobilising rAg on the test line and anti-mouse Ig on the control line. Liquid phase movement over these immobilised particles. Lateral flow rapid test in cassette format	22 Apr 1997 13 Feb 2001 8 May 2001	2017 2021 2021	Australia Patent 6007486 7/1985
US 5656503	Test device for detecting analytes in biological samples	Lateral flow rapid test in cassette format	12 Aug 1997	2017	Australia Patent 6007486 7/1985
US 6352862	Analytical test device for immunoassays and methods of using the same	Lateral flow rapid test in cassette format and use of 10un porous material. Flow of immunoreagents through membrane for reaction at test lines	5 Mar 2002	2022	Australia Patent 6007486/7/1985
US 5714389 US 5989921 US 645982	Test device and method for colored particle immunoassay	N/A – lateral flow rapid test in an elongated casing, slightly different method than that of the cassette format.	3 Feb 1998 23 Nov 1999 26 Nov 2002	2018 2019 2022	A63502/86 10/1986
US 5120643	Process for immunochromatography with colloidal particles	Lateral flow and the use of specific binding reagents, labelled colloidal particles and test and control lines on membrane	9 June 1992	2012	No Australian Patent
US 5578577	Method for storing labile proteins	Specific binding reagents in a lateral flow. Impregnation of colloidal particles on solid substrate	26 Nov 1996	2016	No Australian Patent
US 6534320 US 6534320	Process for immunochromatography with colloidal particles	Specific binding reagents in a lateral flow. Use of conjugates and immobilised labile proteins on solid substrate including its resolubilisation.	18 Mar 2003 18 Mar 2003	2023 2023	No Australian Patent
US 4943522	Lateral flow, non-bibulous membrane assay protocols	Lateral flow with test and control zones and use of blood samples	24 Jul 1990	2010	No Australian Patent

Note: This information was compiled on the 7th of August 2014 using US Patent Database (www.uspto.gov). Patents can be adjusted and extended under current law therefore patents need to be checked for expiry date correctness before referencing. Patents with revision containing additional claims have also been re-issued, therefore extending patent expiry dates. US patents issued after 1995 is valid for a 20 year term.

APPENDIX 7

Panel of reference samples used for evaluating the Bm14 dipsticks.

Sample	Blood	Serum	Bm14 ELISA OD	Neg/ Pos
NegDD	Yes			Neg
NegRH	Yes			Neg
NegNO	Yes			Neg
NegAS	Yes			Neg
NegAW	Yes			Neg
NegRef	No	Yes	0.061	Neg
OnchoST	No	Yes		Neg
OnchoWK	No	Yes		Neg
W.bST	No	Yes	3.5+	Neg
W.bWK	No	Yes		Neg
Ref +veSB	Yes		ND	Pos
Ref +ve Wb-09	No	Yes	3.2	Pos

Sample	Blood	Serum	Bm14 ELISA		Neg/ Pos
			Serum OD	Blood OD	
DSS1	Yes	No	-	0.021	Neg
DSS2	Yes	Yes	3.167	3.116	Pos
DSS3	Yes	Yes	2.999	3.300	Pos
DSS4	Yes	Yes	2.909	3.281	Pos
DSS5	Yes	Yes	3.190	3.291	Pos
DSS6	Yes	Yes	0.203	0.198	Neg
DSS7	Yes	Yes	1.830	2.645	Pos
DSS8	Yes	Yes	1.570	2.849	Pos
DSS9	Yes	Yes	2.275	2.831	Pos
DSS10	Yes	Yes	1.776	2.780	Pos
DSS11	No	Yes	-	1.595	Pos
MP1	Yes	Yes	0.988	1.145	Pos
MP2	Yes	Yes	2.946	3.011	Pos
MP3	Yes	Yes	3.357	3.468	Pos

APPENDIX 8

TGA FRAMEWORK 2014 TECHNICAL FILE
INDEX FOR CONFORMITY ASSESSMENT

Section	Description	CA Form
3a	EC Certificate	3.4.2
3b	Re-certification audit report 2013	3.4.2
3c	TGA ISO Certificate	3.4.4
3d	TGA Audit Report 2010	3.4.4
4	Critical Supplier Details	4
6	IVD Details	6
A1-a	Internal Audits Doc. No. 144,145,146 Management Review Doc. No. 102	Attch 1
A1-b	Overview of Manufacturing stages	
A1-c	Requirements Related to Product	
A1-d	Quality Manual	
A1-e	Purchasing SOPs Doc.No. 8105, 8107	
A1-f	Post-market monitoring system	
A1-g	Advisory Notice procedures	
A1-h	NC/CAPA Procedures	
A1-i	Records Control Procedures	
A1-j	Essential Principles Checklist	
A1-k	Risk Management Report	
A1-l	Clinical Evidence - N/A for IVDs	
A1-m	Labelling (a) IFU (b)	
A1-n	Advertising Material	
B	Letter of abridgement of fees	
C	Regulatory History/ Recalls or FSCA etc.	

APPENDIX 9

CELLABS OPERATING PROCEDURE

Section: 0
Doc.No.:202.6
Page 1of 5
IssueDate: 26/02/15

DESIGN CONTROL

Prepared By:
AuthorisedBy:

PRODUCT DESIGN & DEVELOPMENT: PLANNING AND CHECKLIST

Product Name: Bm14 Filariasis Ab CELISA **Description:** ELISA for the detection of exposure to LF

Project Manager (PM): Dr Rajasakeriah
Managing Director (MD) *approvals*: Dr Smithyman
Quality and Regulatory *stage approvals*: Diane Dogcio
Production: Neil Marshall (NM), Amber Blair (AB)

Project Title: Development of a new ELISA product for the detection of exposure to LF. Specific for brugian and bancroftian filariasis using serum samples.

Project Plan and Schedule

	Project Stage	Start Date	Expected End Date	Actual End	PM	MD	Comments
Stage 1	Market needs and requirements Project start-up meetings and documentation (1a-1e)	June 2003	Expected to finalise by end of 2003	June 2004	X	X	
Stage 2	**Design & Development Planning**	June 2004	Oct 2004	Oct 2004	x	x	
	Design and Development Input				x	x	
	Product prototype construction	2007	2008	2008			
	Input Requirements (refer to checklist 3a-3g)	2007	2008	2010			
	Review of Inputs and time adjustment	2008	2008	2011			
	Design and Development Output				x	x	
	Functional and Performance test	2003		2011			
	Safety Review/ Risk Assessment	2010		ongoing			
	Regulatory requirements plan	2009		2010			
	Regulatory documentation	2009		2010			
	Staff training	2009		2010			
	Marketing plan	2009		2010			
	Review of Outputs (refer to checklist 4a – 4g)	2010		ongoing			
Stage 3	**Design and Development Review on prototype kit.**				x	x	
	Review of results vs requirements	2008		2010			
	Discuss kit performance and determine if ready for GMP	2009		2010			
Stage 4	**Design and Development Verification**				x	x	
	Review results of verification tests. Decide if kits are ready for GMP production.	2009		2010			
Stage 5	**Design and Development Validation**				x	x	
	Arrange external validation	2008		2010			
	Follow-up of validation results	2008		2010			
	Review of validation results	2008		2010			
Stage 6	**Marketing notes**				x	x	See list of paper journals written for the assay.
	Customer Feedback						
	Other Marketing notes						

APPENDIX 10

CONTENTS

APPENDIX 11

Contents

Page

APPENDIX 12

[redacted]

Section 0
Doc. No.: 139.07
Page 1 of 7
Issue date: 30/06/2014
Prepared by:
Authorised by:

Risk Management

1. **Scope:** This document describes the Risk Management process for all Cellabs product range. This document is applicable to the life-cycle of IFA and ELISA kits that will serve to identify hazards that may be associated with each product, its evaluation of identified risks, control measures and the monitoring of the implemented control measures

2. **Definitions:**

End-user : Trained laboratory personnel and/or healthcare provider or physician
Refer to ISO14971 Section 2 for other terms and definitions.

3. **The risk management process:**

- The risk management process is the responsibility of the Quality Assurance and Regulatory Manager (QARM) or designate who will authorise personnel who have the knowledge, experience to perform risk assessments. Risk analysis, risk evaluation, risk control and production and post-production information shall be performed using Figure B1 of ISO 14971 as a guide. Risk Assessment sheets at to be used as for recording each assessment (Appendix 1) and filed under Risk Management File located above the QA desk. A copy is to be filed under the Design, Development and Commercialisation Records for each product.

- The risk assessment process is applicable to the life-cycle of each product, determined by the shelf life at an average of **18 months** for IFA and ELISA products. The general risk management plan includes a risk assessment to be performed **within three months, before and after the last risk assessment date** for each product and reviewed as part of the periodical document review.

- ISO13641 Elimination of reduction of risk of infection related to in vitro diagnostic reagents is used as a reference throughout the design of IFA and ELISA kits as part of the management process. ISO14971 is used as guidance to assess the risk of the current products sold as IVD.

4. **Risk Analysis, Evaluation and Control**

A Risk Assessment can be performed when:
- a new product is under development;
- a major change is proposed to a product;
- and a reportable adverse event/incident occurs.

5. **Risk Management Report**

A Risk Management report shall be prepared to state the implementation of the risk management plan, the outcomes of identified and evaluated risks, and the residual risks (if applicable) and the methods implemented to gather production and post-production information.

6. **Production and Post-production information.**

Production and post-production information can be gathered and documented in Appendix 1. The information shall be evaluated to determine if risk assessment is necessary. Information to consider may be the following:
- Review of current batch files available for each product for any changes including Customer Complaints (CC), Non Conformities (NC) and CAPA implemented for each product.
- Information such as CC or NC associated with each product.
- New standards applicable to methods used in production.
- Safety and risk information of similar products already in the market.
- Other relevant information.

7. **Other considerations when performing risk evaluations for products:**

To reduce risk consider:
- Rationale for using Potentially Infectious / Infectious material. Must comply with TGO34
- Warnings in information supplied by manufacturer (dependent on level of risk)

Appendices: Appendix 1: Risk Assessment Report Form
Appendix 2: Failure Mode Effects Analysis

ORIGINAL

APPENDIX 13

A multicenter evaluation of a new antibody test kit for lymphatic filariasis employing recombinant *Brugia malayi* antigen Bm-14

Kurt C. Curtis[a], Peter U. Fischer[a], Kimberly Y. Won[b], Patrick J. Lammie[b], Hayley Joseph[c], Wayne D. Melrose[c], Norbert W. Brattig[d], Gary J. Weil[a]. [a]Infectious Diseases Division, Washington University School of Medicine, St. Louis, MO, USA, [b]Centers for Disease Control and Prevention, Atlanta, GA, USA, [c]School of Public Health and Tropical Medicine, James Cook University, Townsville, Australia, [d]Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

Abstract

Antibody tests are useful for mapping the distribution of lymphatic filariasis (LF) in countries and regions and for monitoring progress in elimination programs based on mass drug administration. Previous antibody tests have suffered from poor sensitivity and/or specificity or from a lack of standardization. We conducted a multicenter evaluation of a new commercial ELISA that detects IgG4 antibodies to the recombinant filarial antigen Bm14. Four laboratories tested a shared panel of coded sera that included 56 samples from people with microfilaremic *Wuchereria bancrofti* or *Brugia* infections and 25 control samples. Qualitative results were identical in all four test sites. In addition, each laboratory tested samples from their own serum banks. The assay detected antibodies in 32 of 36 sera (91%) from people with Brugian filariasis and in 96 of 98 sera (98%) from people with Bancroftian filariasis. Specificity testing showed that many sera from patients with other filarial infections such as onchocerciasis had positive antibody tests. Specificity was otherwise excellent, although 3 of 30 sera from patients with ascariasis and 4 of 51 with strongyloidiasis had positive antibody tests; it is possible that some of these people had previously lived in filariasis-endemic areas. Antibody test results obtained with eluates from blood dried on filter paper were similar to those obtained with serum tested at the same dilution. This test may be helpful for diagnosing LF, especially for patients with clinical signs of filariasis. The test also has attractive features for use in public health programs in LF endemic countries, namely robustness, high throughput, and the ability to test filter paper blood samples. These features should help to make antibody testing a practical tool for monitoring the progress of filariasis elimination programs and for post-MDA surveillance.

Introduction

The Global Programme to Eliminate Lymphatic Filariasis (GPELF) uses mass drug administration (MDA) to reduce filarial infection rates below those required for sustained transmission with the goal of permanently eliminating LF by the year 2020 (Ottesen et al.,2008; World Health Organization, 2008). GPELF relies on diagnostic tests to identify and map LF-endemic areas and to monitor the impact of interventions. A recent review discussed the value of different diagnostic tests (serum antigen and antibody assays and detection of parasite DNA in vector mosquitoes) for different phases of LF elimination programs (Weil and Ramzy, 2007). In addition to their value as tools for diagnosing individual patients, **filarial antibody tests** are useful for identifying endemic areas and for following antibody rates in young children over time as a means of assessing changes in transmission rates following MDA (Ramzy et al.,2006; Weil et al.,2008). Several studies have shown that an ELISA that detects IgG4 antibodies to the recombinant filarial antigen Bm14 (GenBank Acc. No. M95546.1, also known as BmM14, similar to BmSXP-1, GenBank M98813) is a sensitive marker for infections with or exposure to *B. malayi* or *W. bancrofti* (Chandrashekar et al.,1994; Ramzy et al.,1995; Lammie et al.,2004). Since antibodies to Bm14 clear only slowly after treatment (Helmy et al.,2006), the Bm14 ELISA is especially useful for use in serial surveys of young children to assess changes in LF transmission following MDA (Ramzy et al.,2006; Weil and Ramzy 2007). Known limitations of the test are its cross-reactivity with sera from patients infected with other filarial parasites (eg., *Onchocerca volvulus* and *Loa Loa*) and the fact that it was a non-commercial, research grade test (Lammie et al.,2004). A company has recently marketed an ELISA test kit based on Bm14 that employs standardized reagents and an optimized protocol.

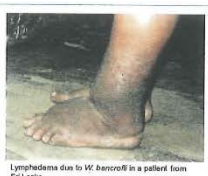
The purpose of this study was to conduct an independent, multicenter trial to evaluate the performance of the commercial Bm14 antibody test kit.

Materials and methods

The Filariasis CELISA Test (Cellabs, Brookvale, NSW, Australia) is an indirect ELISA that detects IgG_4 antibodies to the recombinant *B. malayi* antigen Bm14. The Washington University laboratory (WU) also tested serum samples with a non-commercial ELISA for IgG4 antibodies to Bm14 (the research laboratory Bm14 test), as previously described (Ramzy et al.,1996).

Elution of antibodies from dried blood samples: Venous blood was collected in EDTA from donors with no history of exposure to LF. Thirty µl aliquots of blood were centrifuged, and 15 µl of serum was removed. We then added 15 µl of test sera and resuspended the blood. Ten µl aliquots of antibody-spiked blood samples were pipetted onto round filter paper circles (TropBio, Townsville, Australia), and these were dried and stored at 5°C until use (1-3 wks). Elution was performed in 500 µl of the kit elution buffer overnight at 5°C. Samples were centrifuged, and eluates were tested by ELISA in parallel with the same serum samples used to spike the blood diluted 1:100 in sample diluent.

Serum sets and test laboratories: WU assembled a shared panel of 81 de-identified and coded serum or plasma samples that was tested blindly by the following four laboratories: WU, Bernhard Nocht Institute for Tropical Medicine, (BNI), Centers for Disease Control and Prevention (CDC), and James Cook University (JCU). The shared panel included serum samples from persons with *Wuchereria bancrofti* (n=29), *B. malayi* (n=11), or *B. timori* (n=15) microfilaremia from different countries (India, Indonesia, Sri Lanka, and Egypt), non-endemic sera from residents of St. Louis, USA (n=20), and sera from patients with strongyloidiasis with no history of exposure to filariasis (n = 6). These sera had been previously tested with the research version of the Bm14 antibody test. Positive sera were selected to represent a range of antibody reactivity. Laboratories were also encouraged to test their own panels of sera from persons with filariasis (Mf+) and negative control sera. Dr. Thomas Nutman kindly provided sera obtained from patients with ascariasis collected in an area of Ecuador that is non-endemic for filariasis or onchocerciasis.

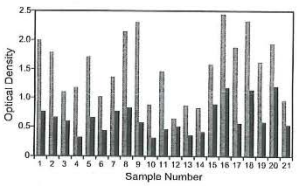


Lymphedema due to *W. bancrofti* in a patient from Sri Lanka.

Immunolocalization (red) of Bm14 antigen in a female *B. malayi*

Results

Figure 1



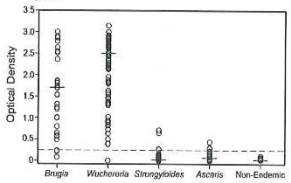
Sensitivity of the Bm14 antibody test. Figure 1 compares OD values obtained for 21 sera from patients with LF with the Filariasis CELISA test and the research laboratory Bm14 test.

These results show that the CELISA test kit produced higher OD values than the research laboratory test.

Antibody results obtained with the Filariasis CELISA test for different patient groups are shown in **Figure 2.** Sensitivity was evaluated with Mf-positive filariasis sera from two study laboratories (WU and CDC). Sera tested included samples in the shared panel and other samples from serum banks at these laboratories. Thirty-two of 35 sera (91%) from people with *B. malayi* or *B. timori* infections had positive antibody tests. These sera were from India and Indonesia. Ninety-six of 98 sera (98%) from people with *W. bancrofti* microfilaremia had positive tests. These sera were from India, Egypt, Papua New Guinea, Sri Lanka, Haiti, and Kenya. The difference in % positivity with *Brugia* and *Wuchereria* sera was not statistically significant ($P = 0.22$).

These results show that the test is highly sensitive for LF infection.

Figure 2



Specificity results are also shown in Figure 2. All 20 nonendemic normal sera in the shared panel were negative at all study sites. All six sera in the shared panel from patients with strongyloidiasis were negative at all study sites. Overall, 4 of 51 sera from patients with strongyloidiasis had positive Bm14 antibody tests (specificity 92.2%). It is possible that some or all of the patients with positive tests had been exposed to filarial infections in the past.

Five of 5 sera from patients with ascariasis tested at CDC were negative with OD values < 0.1. Three of 15 sera from Turkish patients with ascariasis tested at BNI were positive with OD values of 0.289-0.440. Travel histories were not available for these patients. None of 10 Ecuadoran sera from patients with ascariasis tested at WU were positive. None of 6 nonendemic (USA) sera with rheumatoid factor titers ≥ 1:64 were positive.

The BNI laboratory reported that 19 of 25 sera (76%) from onchocerciasis patients living in an area of Ghana without bancroftian filariasis had positive antibody tests. Twenty-two of 24 sera (92%) from an area in Uganda that is highly endemic for *Mansonella perstans* and *M. streptocerca* had positive antibody tests. There is some onchocerciasis in this area but no *W. bancrofti*. In contrast, only 3 of 12 *M. perstans* sera from a different area in Uganda with low-level onchocerciasis endemicity had positive antibody tests.

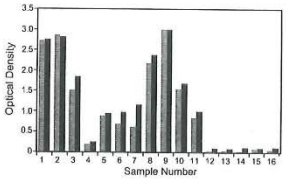
These results suggest that the CELISA test is specific for filarial infections, but it does not provide a species-specific diagnosis.

Interlaboratory reproducibility was excellent. Qualitative results for the 81 sera in the shared serum panel were identical in all four laboratories. OD values for filariasis samples varied between laboratories with a mean coefficient of variation (CV) of 0.16 (SD 0.15). OD values for the 20 control serum samples in the shared panel were low in all of the test laboratories with mean values ≤ 0.053; the range of OD values obtained in different laboratories for individual control sera in the shared panel was -0.014 to 0.135.

Antibody tests of blood dried on filter paper. OD values obtained with eluates from dried blood were similar to those obtained with serum tested at the same dilution (Figure 3).

These results suggest that dried blood samples are as good as serum for antibody testing. However, additional studies are needed to validate the test with paired blood and serum samples from LF patients.

Figure 3



Summary and discussion

The CELISA test has several advantages over the research laboratory Bm14 ELISA:

- Higher sensitivity
- GMP manufacturing with standardized reagents should improve reproducibility.
- Formally validated shelf life of 12 months at 5°C.

The test appears to have excellent sensitivity and specificity for *W. bancrofti* and *Brugia* infections. Cross-reactivity with other filarial infections such as onchocerciasis will limit the value of this test for diagnosing or monitoring LF in sub-Saharan Africa.

Interlaboratory reproducibility was excellent.

Results obtained with filter paper blood samples are promising. However, further studies with paired serum and dried blood samples are needed to validate blood spot testing.

Physicians may find this test useful to support a diagnosis of lymphatic filariasis in patients with a history of exposure to the parasite(s) and clinical signs of the disease.

Antibody testing of sentinel populations may be useful for monitoring late stages of filariasis elimination programs and for post-MDA surveillance.

APPENDIX 14

TGA ARTG Licence for Bm14 Antibody CELISAm

Australian Government

Department of Health

Therapeutic Goods Administration

Australian Register of Therapeutic Goods Certificate

Issued to

Cellabs Pty Ltd

for approval to supply

Parasitic infectious diseases IVDs

ARTG Identifier	231975
ARTG Start date	18/12/2014
Product Category	Medical Device Included - IVD Class 2
GMDN	CT356
GMDN Term	Parasitic infectious diseases IVDs
Intended Purpose	For the detection of parasitic infectious diseases IVD using immunofluorescence assay (IFA), Enzyme-linked Immunoassay (ELISA) and rapid diagnostic test (RDT).

Manufacturer Details	Address	Certificate number(s)
Cellabs Pty Ltd	Unit 7 / 27 Dale Street BROOKVALE, NSW, 2100 Australia	DV-2014-MC-16198-1

ARTG Standard Conditions

The above Medical Device Included - IVD Class 2 has been entered on the Register subject to the following conditions:

- The automatic conditions applicable to the inclusion of all kinds of medical devices in the Register are as specified in section 41FN of the Therapeutic Goods Act 1989.,
- The standard conditions that are imposed under section 41FO of the Therapeutic Goods Act 1989 when kinds of medical devices are included in the Register are as set out in the following paragraphs.,
- For a medical device included in the Register under Chapter 4 and imported into Australia, the Sponsor must ensure that information about the Sponsor is provided in such a way as to allow the sponsor to be identified.,
- Each sponsor shall retain records of the distribution of all of the sponsor's medical devices included in the Register under Chapter 4. In the case of records relating to a Class AIMD medical device, Class III medical device, or Class IIb medical device that is an implantable medical device, the distribution records shall be retained for a minimum period of 10 years. In the case of records relating to any other device, the distribution records shall be retained for a minimum period of 5 years.,
- The sponsor of a medical device included in the Register under Chapter 4 shall keep an up to date log of information of the kind specified in Regulation 5.8.,
- Where a medical device included in the Register, contains a substance which is included in the Fourth Schedule to the Customs (Prohibited Imports) Regulations or the Eighth Schedule to the Customs (Prohibited Exports) Regulations the Sponsor shall, at the time of importation or exportation of the medical device, be in possession of a licence and a permission for importation or exportation of each consignment of the goods as required by those regulations.,
- A sponsor shall ensure that a medical device within their control is stored and transported in accordance with the instructions and information provided by the manufacturer.,
- It is a condition of inclusion in the ARTG that the sponsor of a medical device that is a Class 4 IVD provides three consecutive annual reports to the Head of the Office of Product Review, Therapeutic Goods Administration following inclusion of the device in the ARTG (as specified in 5.8 of the regulations). Annual reports are due on 1 October each year. Reports should be for the period 1 July to 30 June. The first report following the date of inclusion in the ARTG must be for a period of at least six months but no longer than 18 months. Subsequent reports are to be provided on 1 October for a further 2 years. The annual report must include all complaints and adverse events received by the manufacturer relating to problems with the use of the device that have been received by them over the year.,
- Goods which would require an application audit under Regulation 5.3 if subject to a separate application for entry in the Register cannot be included under this ARTG entry until a request to vary the