PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST INTERFERON GAMMA OF ASIAN ELEPHANT *Elephas maximus* FOR TUBERCULOSIS DIAGNOSIS IN ELEPHANTS BY INTERFERON GAMMA RELEASE ASSAY

Mr. Songkiat Songthammanuphap

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การผลิตมอนอคลอนอลแอนติบอดีต่ออินเตอร์เฟียรอนแกมมาของช้างเอเชีย *Elephas maximus* เพื่อการตรวจวินิจฉัยวัณโรคในช้างโดยทดสอบการหลั่งอินเตอร์เฟียรอนแกมมา

นายทรงเกียรติ ทรงธรรมานุภาพ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Mr. Songkiat Songthammanuphap
Field of Study	Microbiology and Microbial Technology
Thesis Advisor	Associate Professor TANAPAT PALAGA, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the Faculty of Science
(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Associate Professor NARAPORN SOMBOONNA, Ph.D.)

..... Thesis Advisor
(Associate Professor TANAPAT PALAGA, Ph.D.)

..... Examiner
(Associate Professor CHULEE YOMPAKDEE, Ph.D.)

..... External Examiner
(Wandee Yindeeyoungyeon, Ph.D.)

ทรงเกียรติ ทรงธรรมานุภาพ : การผลิตมอนอคลอนอลแอนติบอดีต่ออินเตอร์เฟียรอนแกมมาของช้างเอเชีย *Elephas maximus* เพื่อการตรวจวินิจฉัยวัณโรคในช้างโดยทดสอบ การหลั่งอินเตอร์เฟียรอนแกมมา. (PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST INTERFERON GAMMA OF ASIAN ELEPHANT *Elephas maximus* FOR TUBERCULOSIS DIAGNOSIS IN ELEPHANTS BY INTERFERON GAMMA RELEASE ASSAY) อ.ที่ปรึกษาหลัก : รศ. ดร.ธนาภัทร ปาลกะ

วัณโรคเป็นโรคติดต่อจากสัตว์สู่คนที่ส่งผ่านจากสัตว์ที่ติดเชื้อไปยังมนุษย์ มีการรายงานว่าสัตว์ป่า เช่น ช้าง และ สัตว์ตระกูลลิง สามารถติดเชื้อในกลุ่ม *Mycobacterium tuberculosis* (MTB) complex ซึ่งเป็นสาเหตุของวัณโรคในสัตว์ได้เช่นกัน วิธีวินิจฉัยวัณโรคโดยทั่วไปที่ใช้ในมนุษย์ เช่น การตรวจเอกซเรย์ปอด และการตรวจวัณโรคโดยทดสอบทางผิวหนังไม่สามารถทำได้ในช้าง นอกจากนี้วิธีมาตรฐานโดยการเพาะเลี้ยงเชื้อจากน้ำล้างงวงช้างเป็นวิธีที่มีความไวต่ำ ใช้เวลานาน และสามารถตรวจพบวัณโรคได้ในระยะที่มีการแสดงอาการของโรคเท่านั้น ดังนั้น การตรวจวินิจฉัยที่แม่นยำ ครอบคลุมทุกระยะของการติดเชื้อ MTB จึงเป็นสิ่งจำเป็น Interferon gamma release assay (IGRA) เป็นวิธีทางเลือกสำหรับการตรวจวินิจฉัยวัณโรค ซึ่งเป็นวิธีตรวจหาอินเตอร์เฟียรอนแกมมา (IFNγ) ที่มีการหลั่งจากเซลล์เม็ดเลือดขาวที่ถูกกระตุ้นด้วยแอนติเจนจำเพาะของ MTB จุดประสงค์ของการศึกษานี้คือ การพัฒนา IGRA เพื่อใช้สำหรับการวินิจฉัยวัณโรคในช้างและสัตว์ป่าชนิดอื่นๆ ใช้เปปไทด์ซึ่งประกอบไปด้วยบริเวณอนุรักษ์ของกรดอะมิโนของ IFNγ จากสัตว์เลี้ยงลูกด้วยน้ำนม 10 สปีชีส์เป็นอิมมิวโนเจนสำหรับการกระตุ้นการผลิตแอนติบอดีจำเพาะในหนูเมาส์ในหมู่แอนติบอดี 12 โคลน ที่ทำปฏิกิริยาได้สูงต่อโปรตีนรีคอมบิแนนท์อินเตอร์เฟียรอนแกมมาของช้าง (reIFNγ) มอนอคลอนอลแอนติบอดีจากไฮบริโดมาหมายเลข nF1C3#15 ซึ่งเป็นชนิด IgM ถูกเลือกเพื่อใช้เป็นแอนติบอดีตรึงในวิธี IgM sandwich ELISA เพื่อเปรียบเทียบกับ IgG sandwich ELISA ที่พัฒนาก่อนหน้านี้ โดยใช้มอนอคลอนอลแอนติบอดีซึ่งเป็น IgG ที่ได้จากการใช้ reIFNγ ในการกระตุ้นภูมิคุ้มกัน ความไวของ IgM sandwich ELISA และ IgG sandwich ELISA มีขีดจำกัดต่ำสุด (LOD) ของการตรวจวัดที่ 190 นาโนกรัมต่อมิลลิลิตร และ 0.257 นาโนกรัมต่อมิลลิลิตร ตามลำดับ ดังนั้น IgG sandwich ELISA เหมาะสำหรับการพัฒนาต่อไป จากการใช้ IGRA ที่พัฒนาขึ้นในการวินิจฉัยวัณโรคในเลือดตัวอย่างจากช้างหกสิบเอ็ดตัวอย่างโดยมี PPD จาก *M. bovis* เป็นแอนติเจน พบว่า 37.7% ของจำนวนตัวอย่างทั้งหมดมีการวินิจฉัยให้ผลเป็นลบ 4.9% ไม่สามารถระบุผลได้ และ 57.4% มีผลเป็นบวกต่อการติดเชื้อวัณโรค นอกจากนี้จากการใช้ ESAT-6 ร่วมกับ CFP10 เป็นแอนติเจนในการกระตุ้น PBMC มีการตอบสนองมากกว่าการใช้แอนติเจนจำเพาะของ MTB ESAT-6 และ CFP10 เพียงอย่างเดียว ซึ่งคิดเป็น 31.1%, 21.3% และ 8.2% ตามลำดับ การเปรียบเทียบผลการวินิจฉัยโดย IGRA และ วิธี DPP® VetTB ซึ่งมีจำหน่ายเชิงพาณิชย์ใน 32 ตัวอย่าง แสดงให้เห็นว่า 11 ตัวอย่างให้ผลเหมือนกันทั้งสองวิธี ในขณะที่ 21 ตัวอย่าง ให้ผลบวกต่อการติดเชื้อวัณโรคโดยวิธี IGRA เพียงอย่างเดียว ดังนั้น IGRA ที่ได้พัฒนาขึ้นมีศักยภาพในการใช้เป็นวิธีทางเลือกสำหรับการวินิจฉัยวัณโรคในช้าง โดยมีความแม่นยำเทียบเท่าหรือมากกว่าการใช้ชุดทดสอบเชิงพาณิชย์

สาขาวิชา	จุลชีววิทยาและเทคโนโลยีจุลินทรีย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5971973523 : MAJOR MICROBIOLOGY AND MICROBIAL TECHNOLOGY
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Songkiat Songthammanuphap :
PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST INTERFERON GAMMA OF ASIAN ELEPHANT *Elephas maximus* FOR TUBERCULOSIS DIAGNOSIS IN ELEPHANTS BY INTERFERON GAMMA RELEASE ASSAY. Advisor: Assoc. Prof. TANAPAT PALAGA, Ph.D.

Tuberculosis is a zoonotic disease that can be transmitted from infected animals to humans. Wild animals such as elephants and non-human primate have been reported to be infected by *M. tuberculosis* (MTB) complex, which can cause tuberculosis in animals as well. The common TB diagnostic approaches in human such as chest X-ray and tuberculin skin test are not practical in the elephants. Moreover, the gold standard of the bacteria culture from trunk wash has low sensitivity, is time-consuming and can only detect tuberculosis in the active stage. Therefore, an accurate diagnosis covering all phases of the MTB infection is in need. Interferon gamma release assay (IGRA) is an alternative approach for tuberculosis diagnosis, which detects the interferon gamma (IFNγ) secreted from white blood cells stimulated with MTB antigens. The aim of this study is to develop IGRA for diagnosis of TB in elephants and possibly other wild animals. The peptides spanning the conserved region of IFNγ from ten mammalian species were identified and used as immunogen for stimulating specific antibody production in mice. Among twelve monoclonal antibodies that showed strong reactivity against recombinant elephant IFNγ (reIFNγ), monoclonal antibody from hybridoma No. nF1C3#15, which is an IgM, was chosen as a capture antibody in an IgM sandwich ELISA. This IgM sandwich ELISA was compared to the IgG sandwich ELISA developed previously using IgG from reIFNγ immunization. The sensitivity of IgM sandwich ELISA and IgG sandwich ELISA showed the limit of detection (LOD) at 190 ng/ml and 0.257 ng/ml, respectively. Therefore, the IgG sandwich ELISA was chosen for further development. Using the developed in-house IGRA for elephant TB diagnosis sixty-one elephant peripheral blood mononuclear cell (PBMC) samples were tested using PPD from M. bovis as stimulating antigen. We found that 37.7% of samples were diagnosed as infection negative, 4.9% were indeterminate, and 57.4% showed positive results for potential TB infection. In addition, by using ESAT-6 together with CFP10 as stimulating antigen, the response of the PBMCs was greater than using either ESAT-6 or CFP10 alone, accounting for 31.1%, 21.3%, and 8.2%, respectively. The comparison of the results by in-house IGRA and commercial DPP® VetTB assay using 32 samples, revealed that eleven samples showed positive results in both assays, while twenty-one samples were positive TB infection only with IGRA. In conclusion, the developed in-house IGRA has the potential for using as an alternative approach for elephant TB diagnosis with the same or better accuracy than using a commercial test kit.

Field of Study:	Microbiology and Microbial Technology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

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Songkiat Songthammanuphap

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LIST OF ABBREVIATIONS

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CFP10	Culture filtrate protein 10
CMIR	Cell mediate immune response
Conc	Concentration
ConA	Concanavalin A
CTL	Cytotoxic T lymphocyte
CV	Coefficient of variation
DMSO	Dimethyl sulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
eIFNγ	Elephant interferon gamma
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	Early secretory antigenic target-6
Fab	Fragment antigen-binding
FBS	Fetal bovine serum
Fc	Fragment crystallizable region
g (centrifugation speed)	Gravity

HAT	Hypoxanthine, Aminopterin, and Thymidine
HT	Hypoxanthine, and Thymidine
HRP	Horse-radish peroxidase
I	Indeterminate
IACUC	Institutional Animal Care and Use Committee
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGRA	Interferon gamma release assay
Ig-score	Immunogenicity score
IND	Indeterminate
K	Kilo
kDa	Kilodalton
KLH	Keyhole Limpet Hemocyanin
LOD	Limit of detection
MAPIA	Multi-antigen print immunoassay
mA	Milliampere
mV	Millivolt
mM	Millimolar

M	Molar
min	Minute
ml	Milliliter
mg	Milligram
MP	MTBC infection positive
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MS	MTBC infection suspected
MWCO	Molecular weight cutoff
N	Infection negative
N/A	Not available
ND	Not detected
ng	Nanogram
nm	Nanometer
NO	Nitric oxide
No.	Number
NP	NTM infection positive
NTM	Non-tuberculous mycobacterium
OD	Optical density

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline-Tween
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pg	Picogram
PPD	Purified protein derivative
PPDA	Purified protein derivative from *M. avium*
PPDB	Purified protein derivative from *M. bovis*
PVDF	Polyvinylidene fluoride
PWM	Pokeweed mitogen
RD1	Region of deletion 1
reIFNγ	Recombinant elephant interferon gamma
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean

TB	Tuberculosis
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	Tumor necrosis factor alpha
µl	Microliter
µg	Microgram
ºC	Degree Celsius
v/v	Volume by volume
%	Percentage
:	ratio
x	Times

CHAPTER I
INTRODUCTION

Tuberculosis (TB) is infectious disease caused by *Mycobacterium tuberculosis* complex, especially *Mycobacterium tuberculosis* (MTB). Tuberculosis usually affects the lungs and the pathogens spread from person to person via aerosolized infected droplets when the patients cough and sneeze [1].

MTB is reported to be a zoonotic pathogen that could be transmitted from animals to human. Wild animals such as elephants, non-human primates, psittacine birds, cattle, and tapir have been reported to be infected with MTB by interaction with human [2-5]. In 1998, Michalak *et al.* reported the first case of zoonotic MTB transmission involving elephants [6]. The epidemiological investigation strongly suggests MTB transmission between humans and elephants, as evidenced by DNA fingerprinting. TB is transmitted through close prolonged contact with a person or animal with active TB [6].

TB in elephant can be a chronic infection or latent infection and rarely show clinical symptoms even in the active stage of the infection [6,7]. Infected elephants often show fatigue symptoms, cannot work properly, may have mucus, have labored in breathing and weight loss and eventually death [8]. Because the elephants are large, and have a thick skin, the common diagnosis that are used in humans such as chest X-ray and tuberculin skin test are not practical. Moreover, the bacteria culture from trunk washed which is a gold standard for TB diagnosis has low sensitivity, time-consuming and can detect TB only in the active stage [7]. As for the diagnostic assays detecting the antibody specific for MTB complex antigens in serum have their limitations in yielding false positive result from cross-reactivity with environmental mycobacteria [9]. Hence, an accurate diagnostic approach covering all phase of the infection is required for preventing the epidemic of TB in elephants and transmission between infected animal and human and among the elephants.

As MTB is an intracellular pathogen, the infection triggers a cell-mediated immune response (CMIR) to the infection and the memory response can be induced

by MTB antigens [10]. Interferon gamma (IFNγ) is a cytokine that produced mostly by T helper 1, Cytotoxic T lymphocyte and natural killer cells plays important roles in controlling mycobacterial infection [11]. The main function of this cytokine is to activate macrophages which can kill intracellular mycobacteria, mostly through the production of the nitric oxide (NO) [10]. For this reason, the interferon gamma release assay (IGRA) is an alternative approach for tuberculosis diagnosis. IGRA are *in vitro* blood-based test of the CMIR against MTBC specific antigens. This assay measures IFNγ that is produced from T cells upon stimulation with MTBC specific antigens. Nowadays besides the use in human, IGRA have been developed for tuberculosis diagnosis in many species such as cattle, lion [12] and deer [13].

Previous studies show that IGRA can be used as a tuberculosis diagnosis tool for Asian elephants (*Elephas maximus*), and African elephants (*Loxodonta africana*) [14,15]. However, the IGRA for elephant TB are not commercially available and the diagnostic performance of this approach for elephant TB diagnosis is not well elucidated.

The purpose of this research is to generate monoclonal antibody against the conserved region of IFNγ, especially in Asian elephant. The obtained monoclonal antibodies will be used to develop the IGRA for diagnosis of TB in elephants and other wild animals.

Objectives

1. To generate monoclonal antibodies against interferon gamma of Asian elephant (*Elephas maximus*)
2. To develop elephant IGRA using monoclonal antibodies by sandwich ELISA

CHAPTER II
LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* complex (MTBC) which is a group of bacilli in genus Mycobacterium with positive for acid-fast staining. Bacteria in this group consist of *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canetti*, *M. caprae*, and *M. pinnipedii* [16]. Of these seven species, *M. tuberculosis* (MTB) is the most serious pathogenic mycobacterium follow by *M. bovis* [17].

TB usually affects the lungs, but sometimes it can affect other organs as well. The pathogens spread from person to person mainly via aerosolized infected droplets when the patient is coughing and sneezing [1].

According to the World Health Organization (WHO)'s Global tuberculosis report in 2017 [1], WHO estimated that the global TB incidence rate since 2000 has fallen by 1.4% each year. However, most of the estimated number of cases in 2016 occurred in the South-East Asia Region which accounted for 45% of the world's TB incident cases. Thailand was ranked as one of the thirty countries with high TB burden which has the estimated TB incidence rate at 172 cases per 100,000 population with an uncertainty interval of 102-261 cases [1].

2.2 MTBC and Non-tuberculous mycobacterium (NTM)

Mycobacterium genus can be further dividing into two group; MTBC and NTM. MTBC is a group of Mycobacterium species which is the cause of tuberculosis in humans and animals, while the other members of the genus Mycobacterium except for MTBC and *M. leprae* are categorized as NTM or known as environmental mycobacteria which usually are presence in soil and water. The MTBC and the NTM can be diagnosed and differentiated by using the MTBC specific proteins as an antigen in recall response immunological assays.

2.2.1 Purified protein derivative (PPD)

PPD is prepared from *M. tuberculosis* and currently used in the tuberculin skin test (TST) to diagnose TB infection based on delayed-type hypersensitivity response. These proteins are highly complex and cannot distinguish between MTB infection, *M. bovis* BCG vaccination, or exposure to NTM [18]. However, PPD from *M. bovis* (PPDB) and PPD from *M. avium* (PPDA) has been used as a standard control for MTBC, and NTM, respectively, in immunological assay [14,15,19].

2.2.2 Early secretory antigenic target-6 (ESAT-6) and culture filtrate protein 10 (CFP10)

ESAT-6 and CFP10 are the secreted protein from MTBC encoded by the region of difference 1 (RD1) genes Rv3875 and Rv3874, respectively. These proteins are potent T-cell antigens and were strongly recognized resulting in the interferon gamma (IFNγ) release from peripheral blood mononuclear cells (PBMCs) from human TB patients [20]. Moreover, ESAT-6 was the immunodominant antigen recognized in elephant TB when performed with a multi-antigen print immunoassay (MAPIA) while CFP10 shown less frequently reactive to immunoglobulin G (IgG) [7]. By using these proteins to diagnose TB infection, it is possible to distinguish between BCG vaccination and most NTM from the MTBC infection due to the absence of the RD1 region in BCG vaccine strains [21].

2.3 Immune response to MTB infection

As MTB is an intracellular pathogen, the infection triggers a cell-mediated immune response (CMIR) to control the infection [10]. Interferon gamma (IFNγ) is a cytokine that produced mainly by T helper type 1, cytotoxic T lymphocyte (CTL) and natural killer cells and plays an essential role against mycobacterial infection [11]. The primary function of this cytokine is to synergize with tumor necrosis factor alpha (TNFα) to activate macrophages which can kill intracellular mycobacteria, mostly through the

production of the nitric oxide (NO) and phagolysosome fusion to eliminate the bacteria in the phagosome as shown in Figure 1 [10,22,23]

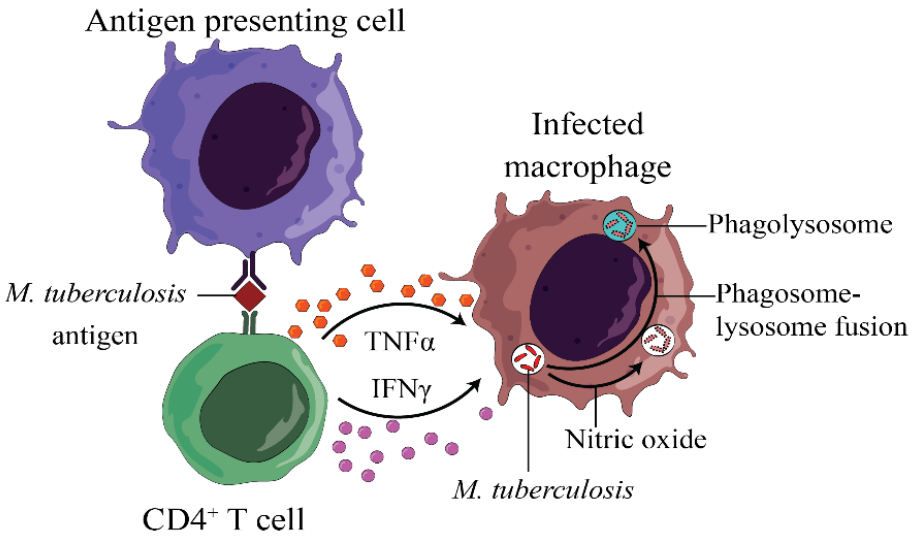


Figure 1 The protective immunity against tuberculosis.
CD4+ T cell plays an important role to control the TB infection. The IFNγ produce from CD4+ T cell coordinate with TNFα to activate the macrophages. The activated macrophages can effectively eliminate the intracellular mycobacteria through the production of NO and phagolysosome fusion.

2.4 Tuberculosis as a zoonotic disease

MTB is reported to be a zoonotic pathogen that could be transmitted from animals to human or *vice versa*. Except for the humans who are known as the natural reservoir hosts for the MTB, many animals such as elephants, non-human primates, psittacine birds, cattle, and tapir have been reported to be infected with MTB by having an interaction with human [2-5]. The infection can cause tuberculosis in these animals. Moreover, the infected animals will become the source of the pathogens and have the potential to transmit this disease back to humans or to other animals [24].

2.4.1 Tuberculosis in elephants

The main cause of TB infection in animals can come from prolonged contact between humans with active TB and animals, especially in captive elephants which show high susceptibility to MTB infection [24]. The susceptibility to MTB infection of the elephants depends on their species. According to the report of Une and Mori in 2007, Asian elephants (*Elephas maximus*) are much more susceptible to the MTB infection than African elephants (*Loxodonta africana*) and therefore, they have a high potential to spread the pathogens to humans [17].

TB in elephants can be a chronic infection and often does not show clinical symptoms until it reaches an active stage of the infection [25]. The infected elephants might show fatigue symptoms, do not work properly, may have mucus, exhibit labored in breathing and weight loss and eventually death [8,26]. Hence, an accurate diagnostic approach covering all phases of MTB infection is required for preventing the epidemic of TB in elephants and the transmission between the infected animal and human and among the elephants.

2.4.1.1 Incidents of MTB infection in elephants

Until now numerous TB in elephant incidents have been reported and summarized in Table 1. In 1998, the first case of zoonotic MTB transmission involving Asian elephants was reported by Michalak *et al.* [6]. The investigation identified evidence of MTB infection in 4 Asian elephants and 11 caretakers. The epidemiological investigation strongly suggests MTB transmission from infected elephants to humans (animal handlers), as evidenced by DNA fingerprinting. TB is possibly transmitted through prolonged close contact with a person or animal with active TB [6].

Between 1996 and November 2010, in the United States, 50 cases of TB in elephants were confirmed by culture test. Of these cases, 46 cases were MTB found in Asian elephant, 3 cases in African

elephant and 1 case was found to be *M. bovis* infection in the African elephant [8].

In 2005, Sweden conducted TB evaluation in captive Asian elephants and found that 6 Asian elephants from Swedish zoo were confirmed by post-mortem using MTB culture. The investigation suggested the outbreak had begun within the herd of lar gibbons and tapirs in the zoo before spreading to one of the Asian elephants, while the other five infected Asian elephants have been diagnosed as TB after post-mortem with an unidentified source of infection [27].

Four captive Asian elephants in Thailand were reported to be positive for TB infection by culture method by Angkawanish, T. *et al*. in 2010. The genotyping analysis showed that MTB was probably transmitted by humans (caretaker) [28].

In 2017, Zachariah, A., *et al*. had confirmed the TB infection in 3 wild Asian elephants by amplifying the targeted bacterial genome and sequencing from the tissue by PCR after post-mortem. From the investigation, can be concluded that cause of the infection may come from the exposure with the infected humans in the area [29].

Taken together, the reported TB infection incidents were mostly found in captive elephants. The primary cause of the MTB infection of the elephants come from close contact with the humans especially with infected humans who have a potential to transmit the causative agent to the elephants. The infected animals subsequently transmit to other elephants or susceptible animals, and cause outbreak between herds or transmit the MTB back to the humans [8,24,28,30]. The incidence of elephant TB infection in Thailand is still not well elucidated due to few incident reports.

Table 1 Summary of the incident reports of MTB infection in Asian elephants.

Reported year	Number of elephants	Number of TB incidents	Country	Diagnostic methods	Ref.
1994 -2010	46	46	USA	Bacterial culture	[8]
2005	6	6	Sweden	Bacterial culture, Post-mortem	[27]
2010	4	4	Thailand	ElephantTB Stat-Pak, Bacterial culture, PCR sequencing	[28]
2014	80	29	Lao PDR	ElephantTB Stat-Pak	[31]
2015	115	15	Nepal	ElephantTB Stat-Pak, MAPIA*	[32]
2016	14	14	Malaysia	ElephantTB Stat-Pak, DPP VetTB	[33]
2017	3	3	India	Post-mortem, PCR amplification and sequencing form tissue samples	[29]

*MAPIA = Multi-antigen print immunoassay, PCR = Polymerase chain reaction

2.4.2 Tuberculosis diagnosis methods in elephants

2.4.2.1 Trunk wash culture

Trunk wash culture is the gold standard method for TB diagnosis in the elephant. This can be done by collecting a sample from an elephant's distal respiratory tract for mycobacterium culture. The Guidelines for the Control of Tuberculosis in Elephants recommends this method as the most practical method for obtaining the sample. However, this procedure can be dangerous to the handles and veterinarians, if the elephant does not cooperate while they are restrained for collecting the samples [34,35]. Also, this method has low sensitivity, time-consuming, and can detect only the active TB stage [7]. Therefore, a more convenient method that can also detect latently infect TB in elephant are needed.

2.4.2.2 Serological test

The serological test is an alternative method for elephant TB diagnosis, along with the trunk wash culture method. This method detects specific serum antibodies against the MTBC antigens [7]. Currently, the serological method was developed into three immune-chromatographic assays. First, the multi-antigen print immunoassay (MAPIA) which is a laboratory test for TB diagnosis based on immunochromatographic principle. This method was performed by using semi-automated micro-spray to immobilize various TB antigens onto the nitrocellulose membranes, followed by chromogenic development to detect the specific antibodies within serum and its reactivity to each antigen [36]. The last two methods are commercialized and called elephant TB-STAT PAK and Dual-path platform (DPP) VetTB assay for elephants which are rapid lateral flow assay [37]. The DPP VetTB assay is the new generation of the rapid lateral flow technology for point of care test for TB in the elephants. The difference between these two tests is the antigens that are used in the assay. The elephant TB-STAT PAK uses the cocktail of several

antigens of MTB, and *M. bovis* printed onto the nitrocellulose membrane in a single band [7]. In contrast, the DPP® VetTB assay uses two antigens (MPB83; *M. szulgai* antigen, and CFP10/ESAT-6; MTBC antigen) which are printed as separating bands [38].

According to the evaluation of the diagnostic potential of these serological tests from Rena Greenwald, *et al.* in 2009, it was showed that all three serological assays could identify all TB infected and non-TB infected correctly [37].

However, Lyashchenko *et al.* reported that by testing 14 MTB infected elephants with these serological tests, the results showed that some elephants were not responded to the immunodominant targets (ESAT-6 and CFP10) which used in the test kits [38]. The elephant TB-STAT PAK has some limitation in yielding false-positive results in non-tuberculous mycobacterium infection by cross-reactivity of the antibody to MBP83 protein from *M. szulgai* [37]. Moreover, the newborn elephant can acquire the MTB reactive antibody from their TB-reactive dams via a placenta. These results indicated that the detectable TB antibody from the calves might not correlate with clinical tuberculosis or the infection [39].

Even though these serological diagnoses show better diagnostic potential, it still needs to perform in parallel with the trunk wash culture method as a confirmatory diagnosis of the TB infection and to improve the accuracy of TB diagnostics [40,41]. In addition, these methods cannot distinguish between active vs. latent TB infection.

2.4.2.3 Interferon gamma release assay (IGRA)

IGRA is an alternative approach for tuberculosis diagnosis. IGRA is *in vitro* blood-based test of the CMIR against specific MTB antigens. This assay measures IFNγ that is produced mainly from T cells upon stimulation with MTB antigens such as ESAT-6, and CFP-10. IFNγ produced by stimulated T cells will be quantified by

enzyme-linked immunosorbent assay (ELISA) as shown in Figure 2. Nowadays besides the commercially used in human (QuantiFERON®-TB-Gold and T-SPOT®.TB), IGRA have been applied for tuberculosis diagnosis in many species such as cattle [42], lion [12] and deer [13] (Table 2).

IGRA has many advantages for diagnosing of TB infection due to the high sensitivity and specificity [43,44]. By using MTB specific antigens for stimulation, this method can reduce the cross-reactivity to most NTM and all BCG vaccine strains [44]. Moreover, IGRA can be used to detect the latent stage of TB infection, but still cannot distinguish between latent and active TB infection alone by itself [45]. Furthermore, blood must be processed within 8-30 hours after collection. After collected, the delay of the blood processing can affect the performance of the test [46,47]. In addition, IGRA required laboratory access and trained personnel to perform.

Previous studies show that IGRA has a potential use as a tuberculosis diagnosis tool for Asian elephants (*Elephas maximus*) and African elephants (*Loxodonta africana*) [14,15]. In 2013, Angkawanish *et al.* developed IGRA for diagnosis in elephant which used the monoclonal antibody as capture and detection antibodies in the assay. Moreover, this study showed the lowest limit of detection of ELISA as one pg/ml. In another study, the polyclonal antibody was used as capture and detection antibodies. The result showed the lowest limit for detecting the recombinant IFNγ in this study is 100 pg/ml [15]. Both studies did not investigate the suitable antigen to be used in IGRA. Furthermore, the IGRA for elephant TB is not currently commercially available.

Table 2 IGRA development for TB diagnosis in animals.

Years	Animals	Reference
2001	Cattle	42
2008	Deer	13
2012	Lion	12
2013	Asian and African elephants	14
2016	Asian elephants	15

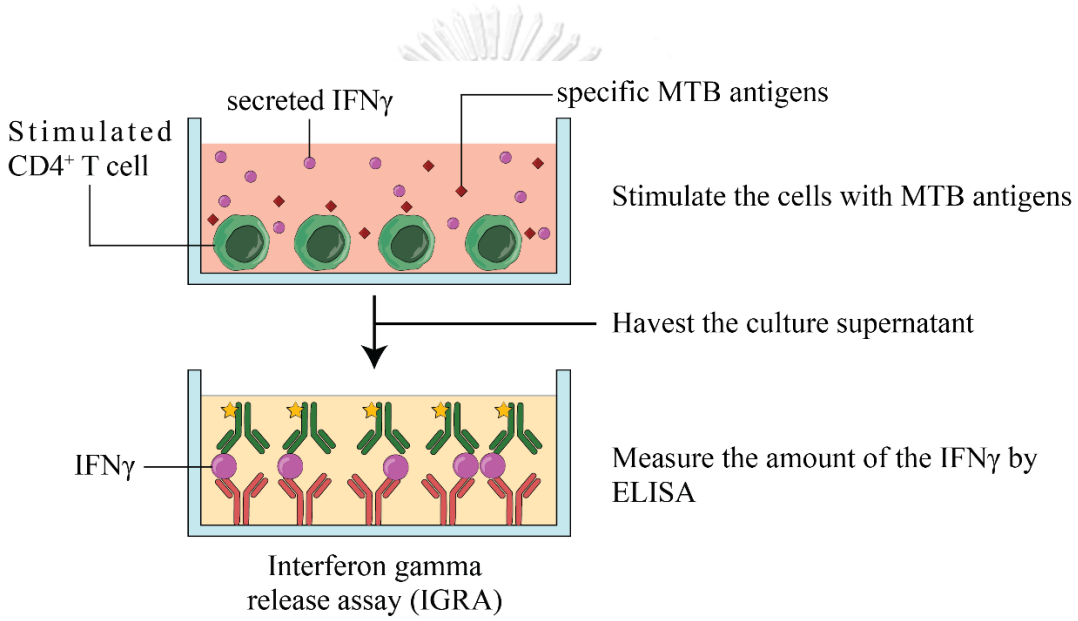


Figure 2 Schematic illustration of IGRA.

The assay can be performed by stimulated the peripheral blood mononuclear cell PBMC such as CD4+ T cell with the MTB specific antigens. If the animal exposed to MTB, specific CD4+ T cells will recognize the antigen and release IFNγ. After the stimulating cells for several days, the culture supernatants are collected to measure the amount of IFNγ by ELISA.

CHAPTER III
MATERIAL AND METHODS

3.1 Peptide antigen selection and antigen preparation

3.1.1 Peptide antigen selection

Synthetic peptide of 18 amino acids in length was selected from the conserved regions of interferon gamma protein sequence from 10 species by using Clustal Omega program (www.ebi.ac.uk). The immunogenic property of the peptide was predicted by using AbDesigner program (https://hpcwebapps.cit.nih.gov/AbDesigner/AbDesignerServlet)[48]. Apart from the immunogenicity, the peptide selection used the following criteria: 1) avoid the asparagine at N-terminal, which can interfere with the removal of the protecting group during the synthesis process, 2) the internal cysteine should be avoided, 3) avoid the presence of multiple oxidation sensitive amino acids such as cysteine, methionine, or tryptophan which can interfere with peptide cleavage and purification, 4) the chosen sequence is found on the surface of the native protein with accessible position [49].

3.1.2 Peptide-carrier protein conjugation

The selected synthetic peptide was conjugated with Keyhole Limpet Hemocyanin (KLH) which is the most widely used carrier protein and has high immunogenicity as a carrier protein in a ratio of 1:2 by using Imject™ EDC mcKLH Spin kit (Thermo Fisher Scientific, USA). According to the manufacturer's protocol, two milligrams of synthetic peptide was dissolved in 450 µl of Imject EDC conjugation buffer and mixed peptide solution with 200 µl of carrier protein solution. Next, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was dissolved with ultrapure water and immediately added 50 µl of this solution to carrier-peptide solution; the reaction was incubated at room temperature for 2 hrs. The conjugate was purified by using spin desalting column (Zeba™ Spin Desalting Columns (7K MWCO), Thermo Fisher Scientific, USA) to eliminate non-reacted

crosslinker and sodium azide. To ensure the success of the conjugation method, indirect ELISA was performed as described below.

3.1.3 Indirect enzyme-linked immunosorbent assay (ELISA)

Ten micrograms per milliliter of the antigen (the conjugated carrier-peptide in fraction 1 and 2) were coated on 96-well ELISA plate (MaxiSorp^{TM}, Nunc, Denmark) and incubated at 37ºC for 1 hr. The unbound proteins were washed with 200 µl of washing buffer (PBS + 0.05% Tween20) three times. Each well was incubated with 200 µl of blocking buffer (3% Skim milk in PBS) for 1 hr at room temperature, then washed the wells three times. One hundred microliters of mouse and rabbit serum specific to recombinant elephant interferon gamma (reIFNγ) (a gift from Dr. Wandee Yindeeyoungyoen from National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand) were used as the primary antibody by diluting with the blocking buffer at 1:2000 and incubated at room temperature for 1 hr. Next, the wells were washed with washing buffer 5 times, and 100 µl of secondary antibody (Goat anti-mouse IgG conjugated with horse-radish peroxidase (HRP), Jackson Immunoresearch, USA and Donkey anti-rabbit IgG conjugated with HRP, GE Healthcare, USA. At a dilution 1:10000 and 1:4000 respectively) was added into the wells. After 30 mins of the incubation at the room temperature all the wells were washed 5 times, then 100 µl of TMB substrate was added into each well. Incubated the reaction in the dark container at the room temperature and stopped the reaction by using 1 M H_2SO_4. Finally, the absorbance was measured by using microplate reader at 450 nm (Multiskan FC, Thermo Fisher Scientific, USA).

3.1.4 Competitive indirect ELISA

One hundred microliters from the conjugated carrier-peptide fraction 1 and 2 (10 µg/ml) were coated onto the wells of ELISA plate by incubated at 37ºC for 1 hr. The excess proteins were washed out three times with 200 µl of washing buffer before blocked the empty spaces with 200 µl of blocking buffer (10% skim milk in PBS) and incubated at the room temperature for

1 hr. In the meantime, the mixture of the diluted mouse serum specific to reIFNγ (dilution at 1:2000 and diluted with PBS) and 4 µg/ml of the synthetic peptide was incubated at 37ºC for 1 hr. After the incubation, the wells were washed three times, and 100 µl of the pre-incubated antigen-antibody were added and incubated at 37ºC for 1 hr. Then, the wells were washed five times and incubated the wells with 100 µl of diluted goat anti-mouse IgG conjugated with HRP for 30 mins at the room temperature. Next, the wells were washed 5 times before adding TMB substrate (100 µl) into each well. The reaction was incubated at the room temperature and stopped with 100 µl of 1 M H_2SO_4. The absorbance was measured by using microplate reader at 450 nm.

3.2 Cell cultures

3.2.1 NSI myeloma cell line

NSI cells mouse myeloma cell line were cultured in completed Roswell Park Memorial Institute (RPMI) 1640 medium (GE Healthcare, USA) which were supplemented with 10% fetal bovine serum (FBS) (Gibco®, Thermo Fisher Scientific, USA), 1% HEPES free acid, 1% sodium pyruvate and 1% penicillin-streptomycin (Thermo Fisher Scientific, USA). Cells were incubated at 37ºC with 5% CO_2 in the CO_2 incubator (Thermo Electron Corporation, USA).

3.2.2 Hybridoma cell

Hybridoma cell lines obtained from fusion were cultured in the complete RPMI 1640 with 1.1 mM glucose and incubated at 37ºC with 5% CO_2.

3.2.3 Peripheral blood mononuclear cell (PBMC)

The isolated PBMC from the blood samples was cultured in the completed RPMI-1640 medium supplemented with 0.05 mM β-Mercaptoethanol (Gibco, USA) and incubated in a CO_2 incubator at 37ºC and 5% CO_2.

3.2.4 Cell preservation

Hybridoma cell and NSI cell line were harvested and resuspended the cell pellets with freezing media (completed RPMI-1640 media with 10% DMSO (v/v) (Merck, Germany)). The cell suspension was transferred into the cryogenic vial (Nunc, Denmark) and kept the cells at -80ºC.

PBMCs were using the FBS containing 10% DMSO (v/v) as freezing media for preservation. After aliquoted cells suspension into the cryogenic vial, then kept the vial at -80ºC

3.2.5 Thawing frozen cell stocks

Placed the vial of cells in the 37ºC water bath and agitated until thawed. The thawed cells were transferred to 10 ml of serum-free RPMI-1640 media and mixed gently by pipetting up and down. The cell suspension was centrifuged for 5 mins at 400 xg. After the centrifugation, the supernatant was discarded and resuspended the pellet with the optimal media.

3.3 Immunization and hybridoma production

3.3.1 Immunization

BALB/c female mice (6 weeks old) were purchased from Nomura Siam International Co, Ltd., (Bangkok, Thailand) were immunized intraperitoneally with a mixture of 50 µl of synthetic peptide solution and 50 µl of complete Freund's adjuvant. The repeated immunization was performed by using antigen with incomplete Freund's adjuvant on day 14, 28 and 42 after the first immunization. Three days before fusion to generate hybridomas, mice were boosted by antigens mixed with PBS. This protocol was approved by Institutional Animal Care and Use Committee (IACUC), with approved animal use protocol no. 1673038.

3.3.2 Measurement of antibody titer by indirect ELISA

After seven days of each immunization, the blood from the facial vein was collected and separated the serum for measuring the antibody titer against reIFNγ by using indirect ELISA as mentioned in 3.1.3. In brief, the indirect ELISA was performed by using 100 µl of reIFNγ at a concentration of 10 µg/ml as an antigen. After the washing and blocking steps, the serial dilutions of the serum (100 µl) were added into the well and incubated at room temperature for 1 hr. The wells were washed 5 times, and 100 µl of secondary antibody (goat anti-mouse IgG conjugated with HRP) was added into the wells. Incubated for 30 mins at the room temperature and washed 5 times. TMB substrate was added and incubated at the room temperature. The reactions were stopped by using 1 M H_2SO_4, and the absorbance was measured by using microplate reader at 450 nm. The limit of detection (LOD) was used as the cutoff to determine the antibody titer (LOD was calculated as described in 3.7.2).

3.3.3 Somatic cell - cell fusion

On day 59, mice were humanely sacrificed, and the splenocytes of the immunized mice were fused with NSI mouse myeloma cells by using 50% polyethylene glycol (PEG, Sigma-Aldrich, USA). First, splenocytes were washed twice by centrifugation at 400 xg for 5 mins in RPMI 1640 medium without serum, for the myeloma cells were washed one time. After the final wash, the two cell pellets were suspended in the serum-free medium and mixed before centrifuged at 400 xg for 5 mins. The supernatant was carefully removed, and 1 ml of PEG (pre-warmed at 37ºC) was added slowly to the cell pellet while resuspending the cells by shaking the tube gently for 50 seconds and continue shaking for another 10 seconds. Third, PEG was washed out of the cells by adding serum-free media and centrifuged at 400 xg for 5 mins and then the cell pellets were resuspended in 50 ml of the hybridoma's complete medium supplemented with hypoxanthine, aminopterin, and thymidine (HAT media supplement, Hybri-Max™, Sigma-Aldrich, USA). Finally, the cells were dispensed into the wells of 96-well plate (100 µl/well) and incubated at

37ºC, 5% CO_2 in the CO_2 incubator until visible colonies developed. The media was change when the media color turned yellowish.

After cultured the cells for approximately 2 weeks, media were changed to hybridoma's complete RPMI 1640 supplemented with hypoxanthine and thymidine (HT media supplement, Hybri-Max™, Sigma-Aldrich, USA) then the cells were cultured with complete media without HT supplement after changing the supplement for 2 weeks.

3.3.4 Limiting dilution to obtain monoclonal hybridomas

Hybridoma cells were sub-cloned until having one cell left in the well by using limiting dilution method. Cells were counted, and the serial dilution method was performed to dilute the cell in the 96-well plate. Cells were monitored with an inverted light microscope and marked the well that contained single cell colony. When cells grew to 25% of the well, the culture supernatants were collected for detecting the specific antibody by using indirect ELISA. This step was repeated three times to ensure the cell in the well was a monoclone.

3.3.5 Monitoring monoclonal antibody produced by hybridoma against reIFNγ using indirect ELISA

Hybridoma cells that produce antibody against reIFNγ were screened by using indirect ELISA as described in 3.1.3. Briefly, one hundred microliters of reIFNγ (1 μg/ml) was coated to the wells of the 96-well ELISA plate and incubated at 37ºC for 1 hr. The uncoated spaces in the wells were blocked with the blocking buffer. Next, one hundred microliters of culture supernatant from the hybridoma cells were added into the well as a primary antibody and incubated at room temperature for 1 hr. The process was continued as mentioned in 3.1.3.

3.4 Characterization of monoclonal antibodies

3.4.1 Isotyping antibody

The monoclonal antibody was subjected to isotyping by using Isotyping kit Reagent (Sigma-Aldrich, USA). According to the manufacturer's instructions, one hundred microliters of each diluted isotype-specific antibodies (diluted at 1:2000 in PBS) were added to 2 well of 96-well ELISA plate and incubated at 37ºC for 1 hr. The coating solution was removed and washed the plate 3 times with washing buffer. The sample (100 µl of culture supernatant) were pipetted into each of the wells then the wells were incubated at room temperature for 1 hr. Next, the wells were washed three times against washing buffer, and each well was incubated with the diluted peroxidase labeled goat anti-mouse IgG (Fab specific) antibody (100 µl of dilution at 1:4000 in washing buffer, Sigma-Aldrich, USA) for 30 mins at room temperature. At the end of the 30 mins incubation, washed the plate three times and one hundred microliters of TMB substrate were added to each well. The plate was incubated at room temperature and the reaction was stopped by adding 100 µl of 1 M H_2SO_4 to each well. The absorbance was measured by microplate reader at 450 nm.

3.4.2 Antibody Purification

3.4.2.1 Ammonium sulfate precipitation

Culture supernatant from the hybridoma cell was harvested by centrifuge at 3000 xg for 30 min at 4ºC. After centrifugation, the supernatant was collected. While the antibody solution was stirring gently with a magnetic stirrer, the equal volume of saturated ammonium sulfate was added to bring the final concentration to 50% saturation and transfer to 4ºC for overnight. Next, the precipitate was centrifuged at 3000 xg for 30 min and carefully removed and discarded the supernatant. The pellet was resuspended in 0.1 volume of the starting volume in PBS before dialysis.

3.4.2.2 Immunoglobulin M (IgM) antibody purification

IgM was purified by using the ammonium sulfate precipitation protocol modified from Victora *et al.*[50]. After the precipitation, the protein pellet was resuspended with 20 mM Tris (pH 8.0). After the precipitation, the antibody solution was dialyzed against 20 mM Tris (pH 8.0) for three days. During dialysis, a white precipitate appeared in the dialysis tube. The precipitate was harvested by centrifuge at 10000 xg, 4ºC for 30 mins then separated the supernatant from the pellet and resuspended the pellet in PBS. The antibody in the supernatant and the resuspended protein pellet were detected by using indirect ELISA as described in 3.1.3.

3.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

3.5.1 Protein measurement

The protein concentration in the samples was measured by BCA™ (bicinchoninic acid) protein assay kit (Pierce, USA), according to the manufacturer's guideline. Bovine serum albumin (BSA) was used as protein standard at the concentration of 0.031, 0.063, 0.125, 0.25, 0.5 and 1.0 mg/ml and the samples were diluted to the desired dilution with sterile type 1 water to make the total volume to 10 µl in 96-well microtiter plate. Two hundred microliters of the mixture of reagent A and reagent B in a ratio 50:1 was added to each well. The plate was incubated at 37ºC for 30 min before measuring the absorbance at 540 nm using microplate reader

3.5.2 SDS-PAGE

The samples were adjusted to the desired amount of protein with the addition of 2x Laemmli sample buffer and boiled at 99ºC for 5 min in the heat box (Thermomixer Compact, Eppendorf, Germany). The samples and a protein standard molecular weight marker (New England Biolabs, USA) were loaded in gel placed in a tank filled with 1x running buffer. The

electrophoresis was performed at 100 volts for 90 min. The gels were stained by Coomassie brilliant blue R-350 (PhastGel™ Blue R, GE Healthcare, USA).

3.5.3 Protein staining with Coomassie Blue R-350

After electrophoresis, gels were removed and stained with Coomassie brilliant blue dye to visualizing proteins for at least 1 hr on a rocker shaker. Next, to make to gel clear, the destain solution was added to the gel to destaining the excess dye and rocked on rocker shaker for 24 hrs or until the gel cleared.

3.5.4 Gel drying

When the gel was completely destained, the gels were rinsed with type 1 water to get rid of leftover destain solution and soaked the gel with a gel drying solution for 30 min. Two cellophane sheets were drenched with gel drying solution before used. One of the cellophane sheets was placed on the glass plate then carefully layered the gel on top of the first cellophane sheet. The second piece of the cellophane was placed carefully to cover the gel. The cellophane papers were clamped to the glass plate and heated in the hot air oven for 24 hrs. Avoid the bubble within the layer of cellophane sheets or nearby the gel which is the cause of gel cracking during the drying process.

3.6 Western blot

After separating protein by SDS-PAGE, gels were equilibrated with transfer buffer for 5 mins. Polyvinylidene fluoride (PVDF) membrane (Immobilon®-P, Merck, Germany) was prepared by soaking in absolute methanol (Merck, Germany), and rinsed with type 1 water twice before soaked in transfer buffer. Six pieces of Whatman filter paper were cut to the gel size and immersed in the transfer buffer. To transfer the protein to the PVDF membranes, 3 pieces of filter paper, PVDF membrane, gel, and 3 pieces of filter paper were layered orderly in the semi-dry transfer instrument (Transfer-Blot® SD, Bio-Rad, USA) and carefully removed the

bubble. The protein transfer was performed using a constant current at 80 mA (for one gel) for 90 mins.

3.6.1 Antibody probing

After transferred the protein onto PVDF membrane, the membrane was blocked with blocking buffer (3%skim milk in PBST) for 5 mins twice on the rocking shaker (Labnet Rocker 25, Labnet International Inc, USA). PVDF membrane was probed with the primary antibody (goat anti-mouse IgM µ-chain specific at the dilution at 1:4000 in blocking buffer, Sigma-Aldrich, USA) for 1 hr at the room temperature on the rocking shaker and transferred to 4ºC overnight. The antibody was removed, and the membrane was washed with PBST for 5 mins and 15 mins twice each before probed with secondary antibody (donkey anti-goat IgG conjugated with HRP diluted 1:6000 with blocking, Santa Cruz Biotechnology Inc, USA) for 1 hr on rocking shaker. The membrane was washed 5 mins two times and 15 mins three times before detection.

In case of using goat anti-mouse, IgG-HRP (dilution 1:10000 in blocking buffer) as the primary antibody; after probed for 1 hr, the membrane was washed with PBST 5 mins two times and 15 mins three times, and then the membranes were ready for the detection.

3.6.2 Protein detection by chemiluminescence

The membrane was incubated with the mixture of solution A and B for 1 min before wrapped with plastic wrap and placed in Hypercassette (Amersham Bioscience, UK) for exposure to High-Performance Chemiluminescence Film (Amersham Hyperfilm^{TM} ECL, Amersham Bioscience, UK) in the dark. The exposed film was developed in X-ray film developer, then washed with tap water and fixed in X-ray film fixer. Finally, the film was washed with tap water to removed fixer solution.

3.7 Development of ELISA to detect elephant IFNγ

3.7.1 Sandwich ELISA by using IgM antibody as the capture antibody

One hundred microliters of purified mouse IgM monoclonal antibody against reIFNγ (5 µg/ml) from hybridoma No. nF1C3#15 was coated onto the well of ELISA plate and incubated at 37ºC for 1 hr. The wells were with washed washing buffer three times and added the blocking buffer (200 µl of 10% FBS in PBS) before incubated at the room temperature for 1 hr. Next, the plate was washed with washing buffer 3 times, and 100 µl of reIFNγ at various concentration (0.0006, 0.0012, 0.0024, 0.0049, 0.0098, 0.019, 0.039, 0.078, 0.16, 0.31, 0.62, 1.25, 2.5, 5, and 10 µg/ml) were added into the wells. Plates were kept overnight at 4ºC. Then, the wells were washed 5 times, and biotinylated mouse IgG monoclonal antibody F1C3#13 at the concentration of 0.2 µg/ml (100 µl) was added to the wells and incubated at 37ºC for 1 hr. The diluted avidin conjugated with HRP (1:4000 in blocking buffer) were added to each well and incubated for 37 ºC at the room temperature. Then, the wells were washed 5 times, and the TMB substrate was added into each well and develop the reaction for 5 mins then stop the reaction with 100 µl of 1 M H_2SO_4. Finally, the absorbance was measured at 450 nm by a microplate reader.

3.7.2 Determination of the sensitivity of the sandwich ELISA (developed in the previous study [51])

Goat anti-rabbit IgG Fc fragment specific antibody (Jackson Immuno-research, USA) was diluted with PBS with ratio 1:1000. One hundred microliters of diluted antibody were coated to the 96-well ELISA plate and incubated at 37ºC for 1 hr. The unbound antibody was washed out of the wells with washing buffer three times then incubated with blocking buffer (200 µl of 10% FBS in PBS) at the room temperature for 1 hr. After the blocking step, the plate was washed with washing buffer 3 time, and 100 µl of diluted rabbit serum specific to reIFNγ (ratio 1:5000 in blocking buffer) was added into the wells. The plate was incubated at 37ºC for 1 hr and washed the excess protein with washing buffer five times. Next, one hundred microliters of reIFNγ were

used as a standard protein at the concentration 0.15, 0.31, 0.62, 1.25, 2.5, 5, and 10 ng/ml. The plates were kept overnight at 4ºC. On the next day, wells were washed 5 times, and 100 µl of mouse monoclonal IgG antibody specific to reIFNγ at the concentration 0.5 µg/ml was added to the wells and incubated at 37ºC for 1 hr. The diluted goat anti-mouse IgG conjugated with HRP (1:10000 in blocking buffer) were added to each and incubated for 30 mins at room temperature. After washed the plate 5 times, 100 µl of TMB substrate was added into each well and develop the reaction for 5 mins then stop the reaction with 100 µl of 1 M H_2SO_4. Finally, the absorbance was measured at 450 nm by a microplate reader. The sensitivity of the assay was determined by using the following formula [52]:

$$\text{Limit of detection (LOD)} = B_0 + 3\ SD$$

Where; B_0 = the mean absorbance at 450 nm of the blank (without reIFNγ)

SD = standard deviation of the blank

3.7.3 Determination of the intra-variation and inter-variation assays of the sandwich ELISA

To determine the intra-assay coefficient of variation (CV), the sandwich ELISA was performed triplicates at the same time with the same reagent and samples, while the inter-assay CV was performed with 3 different times, each time the reagents and samples were prepared separately. The reIFNγ was spiked to the media at the final concentration 0.5, 1, 2.5, and 5 ng/ml. Twelve replicates of each concentration were performed by using the sandwich ELISA mentioned in 3.7.3. The percentage of intra-assay and inter-assay CV (% CV) was calculated using the following formula [53]:

$$\%\ CV = 100 \times (\text{standard deviation/mean})$$

The % CV of the inter and intra-assay should be less than 15 and 10 respectively [54].

3.8 Elephant tuberculosis diagnosis

3.8.1 Interferon gamma release assay (IGRA)

3.8.1.1 Peripheral blood mononuclear cell (PBMC) isolation

Ten milliliters of elephant blood were collected in heparin tube (BD Vacutainer®, Becton Dickinson, USA) and processed within 24 hrs. The PBMCs were isolated by using density centrifugation method. First, the samples were centrifuged at 400 xg for separating the leukocytes and plasma from the erythrocytes before the isolation. The separated leukocytes were diluted with the PBS to adjust the volume to 10 ml before resuspending cell pellets and buffer by inverting the tube several times. After the dilution, carefully overlay the diluted blood sample (10ml) onto 5 ml of the Ficoll®-Paque Premium media solution (GE Healthcare, USA) within 15 ml centrifuge tube and centrifuged at 400 xg for 30 min at 20ºC in this step the break must be turned off. Next, removed the plasma (upper layer) before transferred the mononuclear cells (white color layer) which are the layer between plasma and Ficoll®-Paque Premium media to the new centrifuge tube for washing the cells. The packed volume of the mononuclear cells was estimated and added three volumes of PBS were added to the cells before centrifuged at 400 xg for 10 min after centrifugation the supernatant was discarded and resuspended the cells in 10 ml PBS. The washing step was repeated three times before resuspended the cell pellet in completed RPMI 1640 media supplement with 0.05 mM β-mercaptoethanol.

3.8.1.2 Stimulating PBMCs with mitogen or antigens

PBMCs were seeded at 1×10^5 cells/well in 96 well-plate U-bottom (Thermo Fisher Scientific, USA) with the final volume 150 µl. PBMCs were stimulated with 10 µg/ml of Concanavalin A (ConA) (Sigma Aldrich, USA), 10 µg/ml of PPDB (IDvet, France), 10 µg/ml of PPDA (IDvet, France), ESAT-6 with CFP10 (10 µg/ml each), 10 µg/ml of ESAT-6, 10 µg/ml of CFP-10, 10 µg/ml of Rv3020c

(ESAT-6, CFP-10, and Rv3020c are the recombinant protein gifted from Dr. Wandee Yindeeyoungyoen) for 72 hrs in the CO_2 incubator. Each condition was done with three replicates. After seventy-two hours of the stimulation, culture supernatants were collected for detecting IFNγ by using sandwich ELISA.

3.8.1.3 Measuring the IFNγ by using sandwich ELISA

The secreted IFNγ was measured using sandwich ELISA as described in 3.6.2. The reIFNγ at concentrations of 0.15, 0.31, 0.62, 1.25, 2.5, 5, and 10 ng/ml were used to make the standardize curve and 100 µl of undiluted culture supernatants from unstimulated and stimulated PBMCs were added to each well. The absorbance was calculated as concentrations of IFNγ.

3.8.1.4 Interpretation of IGRA results

IGRA results interpretation was based on the amount of IFNγ that released after stimulated with the specific antigens. The response of PBMC against the stimulating antigens was divided into 3 categories; positive, negative and indeterminate by using LOD as a cutoff between the positive and indeterminate results. If the concentration of the IFNγ within the sample is higher than the cutoff, the result was interpreted as positive. The indeterminate result was determined when the concentration of IFNγ was between the cutoff and the lowest concentration of the standard curve. Finally, the results were determined as negative when the IFNγ within the specimen cannot be detected.

The infection statuses were interpreted by using the results from the responses of PBMCs to each antigen and can be classified into 5 statuses; negative, MTBC infection, MTBC infection suspected, NTM infection and indeterminate. The interpretation criteria of the infection status were summarized in Table 3.

Table 3 Criteria for interpreting the infection status by using developed IGRA assay.

TB infection status		Stimulant antigens			
		ConA	**PPDB**	**PPDA**	**MTB specific antigen**
Negative		+	−	−	−
Positive	**MTBC infection**	+	+ or −	+ or −	+
	MTBC suspected	+	+	N/A or −	−
	NTM infection	+	+ or −	+	−
Indeterminate**		+	I	I	I

* (−) = IFNγ is not detectable within the sample.

(+) = The amount of the IFNγ is higher than the cutoff value

(I) = The amount of the IFNγ is between the cutoff and the lowest concentration of the standard curve

** TB status was determined as indeterminate when the sample gave indeterminate response with no positive response with any stimulating antigens

** The indeterminate response was assumed as negative in case of TB infection positive status.

3.8.2 DPP® VetTB assays for elephants

The DPP® VetTB test kit (Chembio Diagnostic Systems Inc, USA) is a single-use immunochromatographic rapid test for the detection of the antibodies to MTB and *Mycobacterium bovis* in serum. According to the protocol from the manufacturer, five microliters of the plasma was carefully released to the center of the well 1 (sample + buffer well) followed by slowly dropping 2 drops of the buffer from buffer bottle and waited for 5 mins. Next, 4 drops of the buffer were added to the well 2 (buffer well) then waited for 15 mins. The result was read within 5 mins after finished the process.

The results were interpreted as a reactive for TB when the purple color lines were visible in the control area, and in either two test areas (test 1 and 2 area) or only appeared in test 2 area (shown in Figure 3 (B2)). If the color

lines were visible in the test 1 line and the control line means the sample is reactive for TB or mycobacteriosis (shown in Figure 3 (B3)). On the other hand, the result was concluded as nonreactive when the color line appeared in the control area only (shown in Figure 3 (B1)).

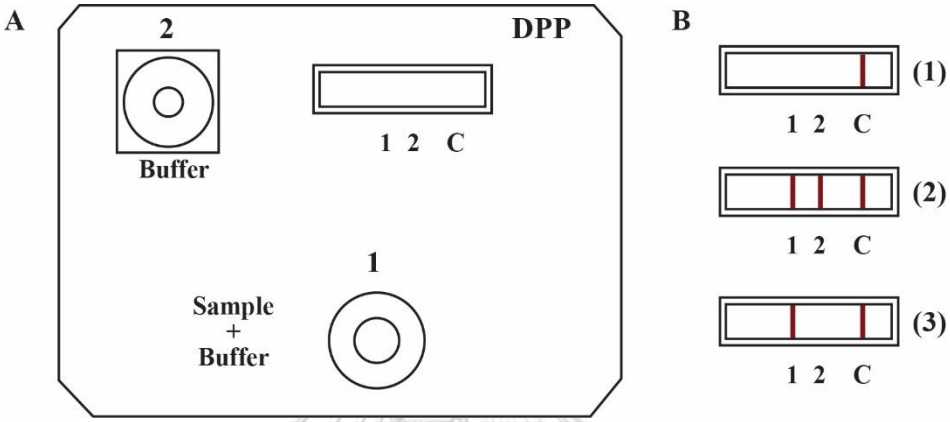


Figure 3 Schematic illustration of DPP® VetTB test kit.

(A) Components of DPP® VetTB test kit.

(B) Possible outcome of the test kit; (1) nonreactive, (2) reactive for TB, (3) reactive for TB or mycobacteriosis

CHAPTER IV
RESULTS

4.1 Production and characterization of elephant IFNγ peptide-specific monoclonal antibody

4.1.1 IFNγ peptide antigen selection for immunization

Amino acid sequence of IFNγ from ten mammalian species was analyzed by using Clustal Omega program as described in 3.1.1 to identify the conserved regions. The length of amino acid sequences of 10 species was in a range of 166-169 amino acids. The amino acid sequence alignments (Figure 4) revealed 70 regions of identical amino acids. The various conserved regions of amino acids were mostly found distributed from the middle of the protein until the C-terminus of the protein.

To predict the potential peptides for used as an immunogen, the immunogenicity was used as another criterion for peptide selection. The immunogenicity of 18 amino acid length peptide from IFNγ sequence of Asian elephants was analyzed by using AbDesigner program [48]. The immunogenicity analysis result was presented in the heat map shown in Figure 5. The result indicated that the highly immunogenic peptides with high immunogenicity score (Ig-score) were located more at the C-terminus of the protein than the peptides in the middle of the sequence. Among ten peptide sequences which predicted to be the best immunogen (Table 4), only two peptides (NGAKRKRRQYSFRGRRAS [148-165] and GAKRKRRQYSRGR RAST [149-166]) contained the most conserved region among 10 species.

However, the peptide GAKRKRRQYSFRGRRAST (ranked 10th according to the Ig-score) was chosen as an immunogen for immunization because another peptide has the asparagine at the N-terminus which must be avoided as stated in 3.1.1. This peptide is referred to as eIFNγ peptide in this study.

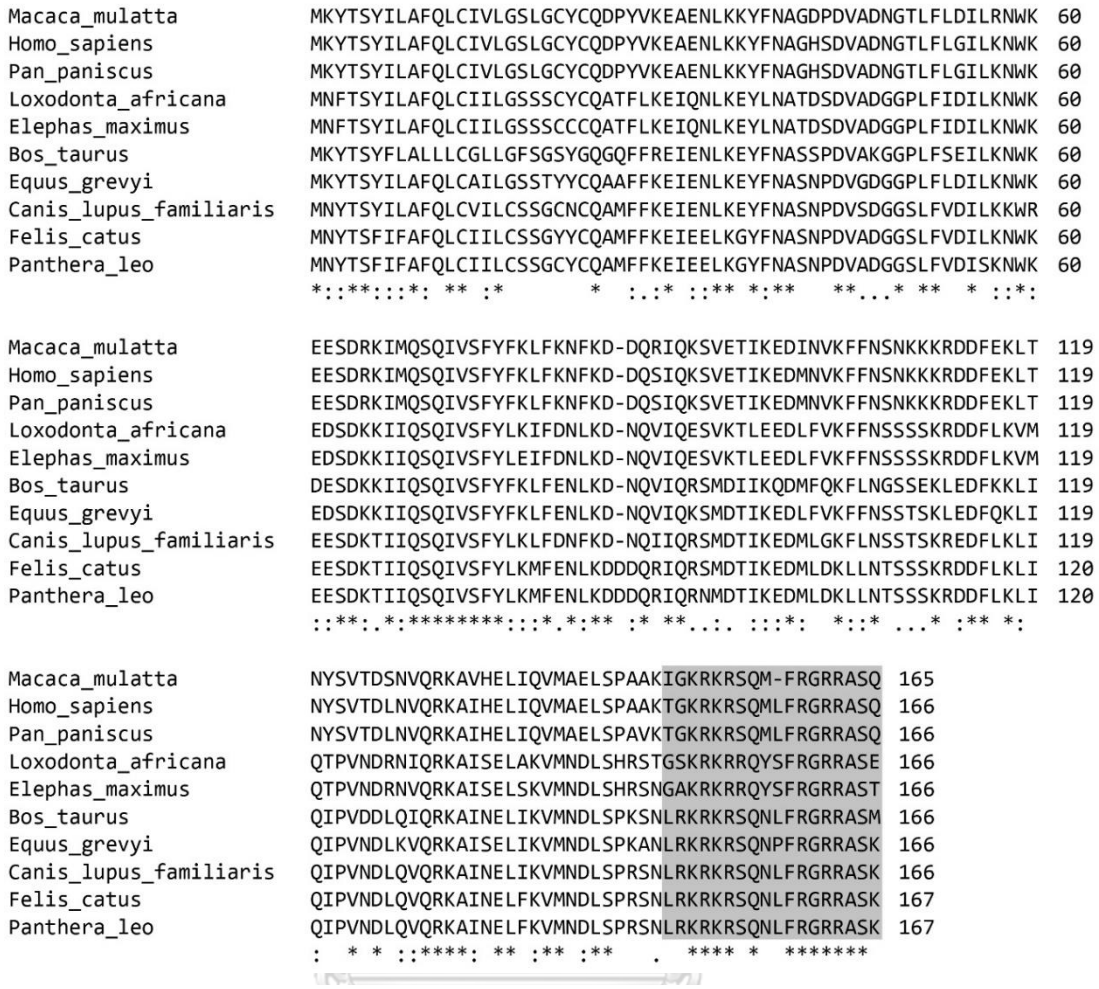


Figure 4 Amino acid alignment of IFNγ from 10 mammal species:

* (asterisk) indicates positions which have a complete conserved residue.

: (colon) indicates positions of amino acid with conservation between groups of strongly similar amino acid properties.

. (period) indicates positions of amino acid with conservation between groups of weakly similar amino acid properties.

Area highlighted in gray indicates peptide selected for immunogens.

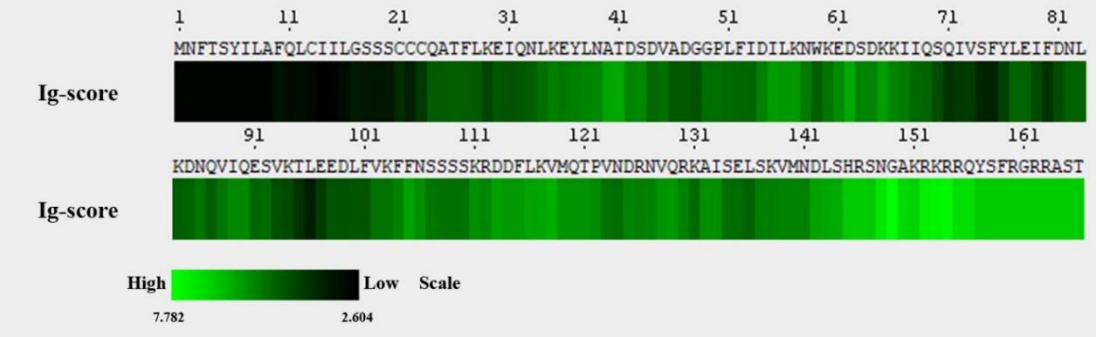


Figure 5 Heat map of immunogenicity analysis of 18 amino acid length peptide from Asian elephant IFNγ amino acid sequence by using AbDesigner program:
The highest and lowest Ig-score were displayed in shade of green and black, respectively.

Table 4 Ig-score rank of the 18 amino acids peptides from Asian elephant IFNγ amino acid sequence.

Rank	Peptide	Amino acid position	Ig-score*
1	NDLSHRSNGAKRKRRQYS	[141-158]	7.782
2	RSNGAKRKRRQYSFRGRR	[146-163]	7.640
3	HRSNGAKRKRRQYSFRGR	[145-162]	7.564
4	SHRSNGAKRKRRQYSFRG	[144-161]	7.515
5	MNDLSHRSNGAKRKRRQY	[140-157]	7.304
6	NGAKRKRRQYSFRGRRAS	[148-165]	7.152
6	SNGAKRKRRQYSFRGRRA	[147-164]	7.152
8	DLSHRSNGAKRKRRQYSF	[142-159]	7.038
9	LSHRSNGAKRKRRQYSFR	[143-160]	6.908
10	GAKRKRRQYSFRGRRAST	[149-166]	6.774

* Ig-score is the predictor of immunogenicity. The higher the score means the greater the predicted immunogenicity.

4.1.2 Peptide-carrier protein conjugation

To prepare immunogen for immunization, eIFNγ peptides were conjugated to carrier protein KLH. After the conjugated peptide was purified by using a desalting column and collected into a fraction, and the conjugation was confirmed with indirect ELISA. The mouse serum [51] and the rabbit serum against reIFNγ (a gift from Dr. Wandee Yindeeyoungyoen from National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand) were used as the primary antibody. The results showed that both sera could detect the antigens within the fraction 1 and 2. The absorbance of fraction 1 was higher than that of fraction 2, indicating that most of the conjugated peptide was in fraction 1 (Figure 6).

Next, the competitive indirect ELISA was used to confirm that the absorbance from the indirect ELISA did not come from the non-specific cross-reaction of the antibodies to carrier protein KLH. Mouse serum was incubated with eIFNγ peptide before adding to the wells that were pre-coated with the conjugated peptide for competition. The result showed that the absorbance was decreased when compared with the condition without competitor eIFNγ peptide (Figure 7). Therefore, the eIFNγ peptide was successfully conjugated to KLH and specifically recognized by polyclonal antibodies against reIFNγ.

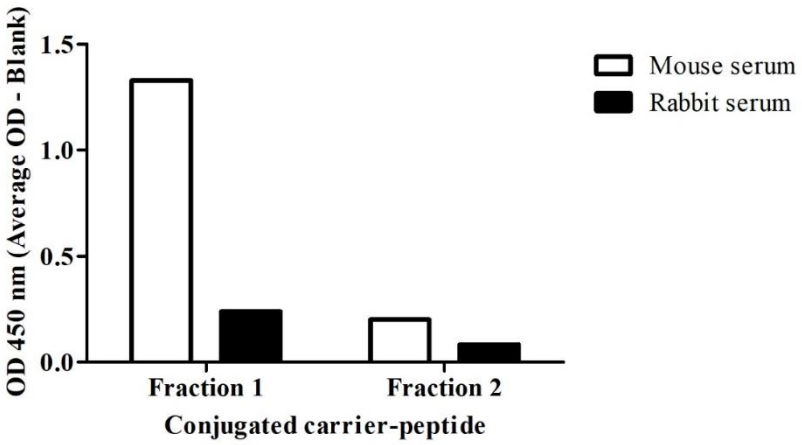


Figure 6 Detection of conjugated peptide by indirect ELISA.
Indirect ELISA was performed by using 10 µg/ml of conjugated peptide from fraction 1 and fraction 2. The mouse and rabbit serum (specific to reIFNγ) at the dilution of 1:2000 were used as detecting antibody. Mouse serum against reIFNγ was from mouse that immunized with reIFNγ in the previous study. Rabbit serum was a gift from Dr. Wandee Yindeeyoungyoen (BIOTEC, Thailand).

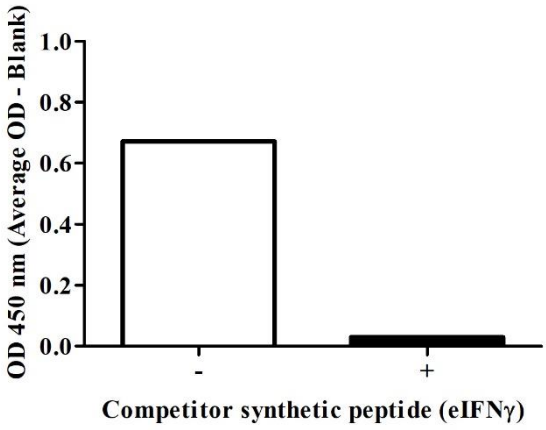


Figure 7 Competitive ELISA to confirm specificity of mouse serum against reIFNγ.
Ten micrograms per milliliter of the conjugation peptide fraction 1 was used as coating antigen, while the eIFNγ peptides (4 µg/ml) were used as the competitor in the competitive ELISA. Mouse serum against reIFNγ (dilution 1:2000) was used as detecting antibody

4.1.3 reIFNγ-specific antibody titer after the 3rd immunization

To determine whether the antibody titer raised by eIFNγ peptide was specific to reIFNγ, the serum from the immunized mice were collected at day seventh after the 3rd immunization and determined the antibody titer by using indirect ELISA. The LOD was calculated by the average absorbance of blank plus three times of standard deviation of the blank (Figure 8). The limit of the detection (LOD) at 0.041 was used as a cutoff to determine the endpoint titer level. Among the 3 mice, the result demonstrated that mouse number 2 showed the highest titer level at 819200, followed by mouse number 1 and 3 with the titer level of 204800, and 51200, respectively (Table 5). Therefore, mouse number 1 and 2 were chosen as spleen donors for hybridoma production.

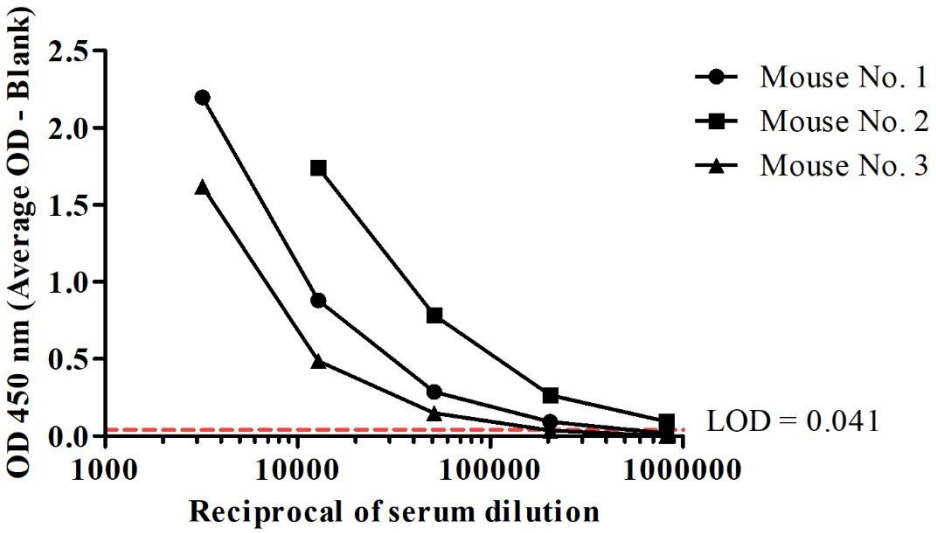


Figure 8 Antibody titer against reIFNγ of the mice after 3rd immunization with conjugated peptide by using indirect ELISA.

Mice sera was serially diluted to monitor the antibody titer using reIFNγ as coating antigen by using indirect ELISA. The LOD was calculated by using following formula; LOD = B_0 – 3SD.

Table 5 reIFNγ specific antibody titer after 3rd immunization

Mouse number	Titer level*
1	204800
2	819200
3	51200

***** Titer level was determined by using LOD at 0.041 as a cutoff.

4.1.4 Testing specificity of serum immunized with eIFNγ peptide

To confirm that antibody obtained from the immunization process in mice with the conjugated peptide did not come from the non-specific binding, the serum of the mouse No.2 was used to detect the reIFNγ at the various concentrations by indirect ELISA. The results showed that by using the serum from mouse No.2 as detecting antibody, the absorbances were correlated with the amount of the reIFNγ and without non-specific signal (Figure 9).

The specificity of the mouse serum against eIFNγ peptide was investigated by competitive indirect ELISA. The eIFNγ peptide was used as a competitor against reIFNγ that was coated onto the wells. The results demonstrated that the mouse serum was specific to the eIFNγ because the absorbance decreased in the presence of the competitor (Figure 10). Taken together, the mouse serum was specific to eIFNγ peptide and did not show the non-specific binding when used in ELISA.

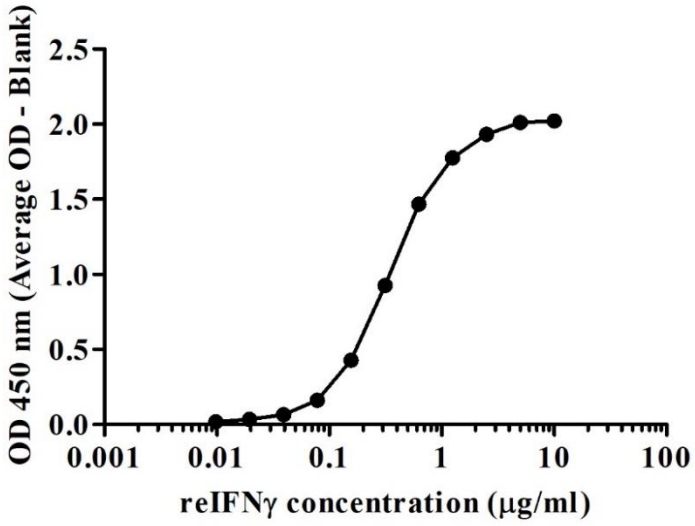


Figure 9 Titration curve of reIFNγ using mouse serum

The reIFNγ at the concentration 0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10 μg/ml was coated onto the wells as antigens. Serum from mouse No.2 was used as detecting antibody in indirect ELISA at the dilution at 1:2000.

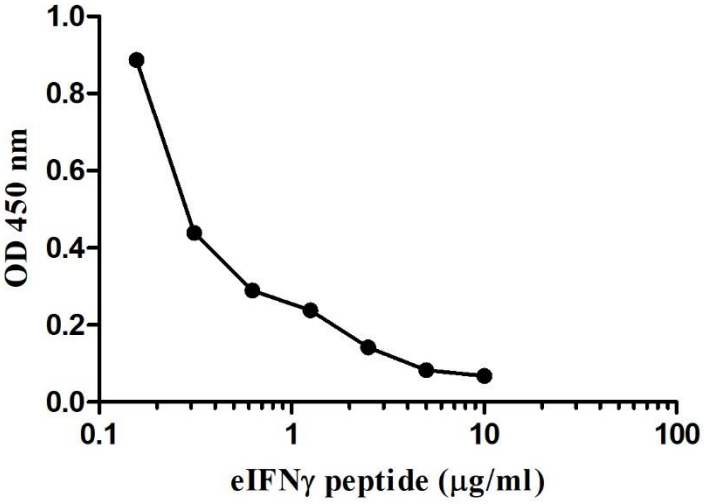


Figure 10 Absorbance of the mouse serum against the competitor by competitive indirect ELISA.

Ten microgram per milliliter of reIFNγ was coated onto the wells. The serum from the mouse No.2 at the dilution at 1:2000 was incubated with various concentrations of eIFNγ peptide as competitors before adding into the wells.

4.1.5 Hybridoma production

4.1.5.1 Somatic cell-cell fusion (Fusion 1)

For somatic cell-cell fusion, the splenocytes from mouse number 2 were fused with NSI myeloma cell line to generate the hybridoma cells. Two weeks after the fusion, the antibody production was screened by using indirect ELISA with reIFNγ as antigen. Out of 1056 wells, 67 wells gave the positive results with indirect ELISA. To obtain monoclones, cells from these sixty-seven wells were subjected to limiting dilution for 3 rounds. After the 3rd round, only 35 clones from 16 original wells (3/H1, 4/B6, 6/H3, 6/A5, 6/A7, 6/C1, 6/H9, 7/A8, 7/D11, 7/F9, 8/H3, 8/H12, 9/A4, 10/H11, 10/E3, and 11/H3) remained positive in ELISA. The hybridomas were classified according to the reactivity to reIFNγ. The reactivity of the monoclonal antibody was categorized into three classes according to the absorbance value at 450 nm i.e. OD450 > 2.5; strong reactivity, 1.5 - 2.5; moderate reactivity and OD450 < 1.5; low reactivity. In Table 6, the results of the reactivity of the monoclonal antibodies showed that 10 hybridomas out of 35 hybridomas produced strong reactive antibody. The hybridoma nF1C3#20 showed the highest absorbance followed by hybridoma nF1C3#16 and nF1C3#15, respectively (Table 7). However, antibody production by the hybridoma nF1C3#16 and nF1C3#20 was lost after recovery from the stock. Therefore, the monoclone hybridoma nF1C3#15 was selected for further study.

Table 6 Reactivity of the monoclonal antibodies produced from hybridomas after the 3rd subcloning.

Reactivity strength of the monoclonal antibodies	Numbers of hybridomas
Strong (OD450 > 2.5)	10
Moderate (OD450 1.5-2.5)	16
Low (OD450 < 1.5)	9

Table 7 Reactivities of antibody produced by selected hybridomas from fusion 1 (strong reactivities) by indirect ELISA.

Hybridoma No.	OD 450 nm
nF1C3#11	2.667
nF1C3#14	2.986
nF1C3#15	3.035
nF1C3#16	3.154
nF1C3#17	2.961
nF1C3#20	3.335
nF1C3#22	3.030
nF1C3#23	2.602
nF1C3#32	2.656
nF1C3#34	3.081

4.1.5.2 Somatic cell-cell fusion (Fusion 2)

To produce mouse hybridomas, the splenocytes from the mouse number 1 were fused with NSI myeloma cell line. The antibody production was screened with indirect ELISA. In the first screening, 104 wells were positive, and 1056 wells were selected for subcloning. After two rounds of subcloning, the reactivity of the monoclonal antibodies was tested and categorized by using indirect ELISA. As shown in Table 8, two monoclonal antibodies were classified as strong reactivity, while 34 monoclonal antibodies were classified as moderate reactivity and 24 monoclonal antibodies were classified as low reactivity. The result in Table 9 demonstrated that the monoclonal antibodies No. nF3C2#43 and nF3C2#46 which were classified as strong reactivity in indirect ELISA came from the same original well (9/G7). Therefore, the monoclonal antibody from these 2 hybridomas were chosen for further study.

Table 8 Reactivity of the monoclonal antibodies produced from hybridomas after the 2nd subcloning.

Reactivity strength of the monoclonal antibodies	Numbers of hybridomas
Strong (OD450 > 2.5)	2
Moderate (OD450 1.5-2.5)	34
Low (OD450 < 1.5)	24

Table 9 Reactivities of antibody produced by selected hybridomas from fusion 2 (strong reactivities) by indirect ELISA.

Hybridoma No.	OD 450 nm
nF3C2#43	2.600
nF3C2#46	2.601

4.1.6 Isotyping of monoclonal antibody

To determine the isotype of individual monoclonal antibodies, the capture ELISA was performed according to the manufacturer's procedure. The antibodies specific for each isotype (IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA) were used to identify the isotype of the monoclonal antibody. Twelve monoclonal antibodies with strong reactivity to reIFNγ from two fusions were chosen for isotyping analysis. The result showed that all monoclonal antibodies have the μ-chain as the heavy chain and determined as IgM (Table 10).

Table 10 Isotyping analysis of selected monoclonal antibodies.

	Hybridoma No.	Isotype (Absorbance)						Determined isotype
		IgG1	IgG2a	IgG2b	IgG3	IgM	IgA	
Fusion 1	nF1C3#11	0.248	0.266	0.264	0.297	1.552	0.184	IgM
	nF1C3#14	0.093	0.093	0.093	0.101	0.731	0.079	IgM
	nF1C3#15	0.126	0.131	0.148	0.162	0.968	0.126	IgM
	nF1C3#16	0.002	0.027	0.000	0.001	0.135	0.002	IgM
	nF1C3#17	0.003	0.026	0.002	0.002	0.105	0.068	IgM
	nF1C3#20	0.081	0.109	0.057	0.069	2.352	0.037	IgM
	nF1C3#22	0.066	0.097	0.049	0.062	2.279	0.034	IgM
	nF1C3#23	0.115	0.165	0.113	0.147	2.381	0.081	IgM
	nF1C3#32	0.229	0.108	0.307	0.098	3.467	0.053	IgM
	nF1C3#34	0.061	0.058	0.188	0.027	3.532	0.016	IgM
Fusion 2	nF3C2#43	0.003	0.007	0.016	0.034	2.039	0.041	IgM
	nF3C2#46	0.002	0.013	0.014	0.002	1.086	0.000	IgM

4.1.7 Partial purification of IgM monoclonal antibody with ammonium sulfate precipitation

Partial purification of IgM was performed as described in 3.4.2. To purify monoclonal antibody, one-hundred milliliters of culture supernatant from hybridoma clone nF1C3#15 was precipitated with saturated ammonium sulfate and dialyzed with 20 mM Tris buffer. After dialysis, the antibody solution was centrifuged to separate the soluble protein and the pellet protein. Ten micrograms of total protein from the culture supernatant, soluble protein, and pellet protein were used for the purity analysis by SDS-PAGE as described in 3.5. The result demonstrated that in the pellet protein sample, the less contaminated protein was detected when compared with the soluble protein sample. Especially the protein with approximate molecular weight of 62.4 kDa appeared in both culture supernatant and soluble protein sample but was mostly disappeared in the pellet protein sample. Moreover, in the pellet

protein lane, there are two high-intensity bands with the approximate molecular weight of 75.9 kDa and 24.0 kDa. These are presumed heavy chain and light chain of the IgM antibody, respectively (Figure 11A).

The heavy chain and light chain of the monoclonal antibody were confirmed by Western blot using goat anti-mouse μ-chain specific antibody to detect the heavy chain and goat anti-mouse IgG (heavy chain and light chain) conjugated with HRP (cross-reacted to the light chain of IgM) to detect light chain of the IgM. The result of the Western blot in Figure 11B confirmed that the protein bands at 75.9 kDa and 24.0 kDa were the heavy chains and light chain of the IgM antibody, respectively.

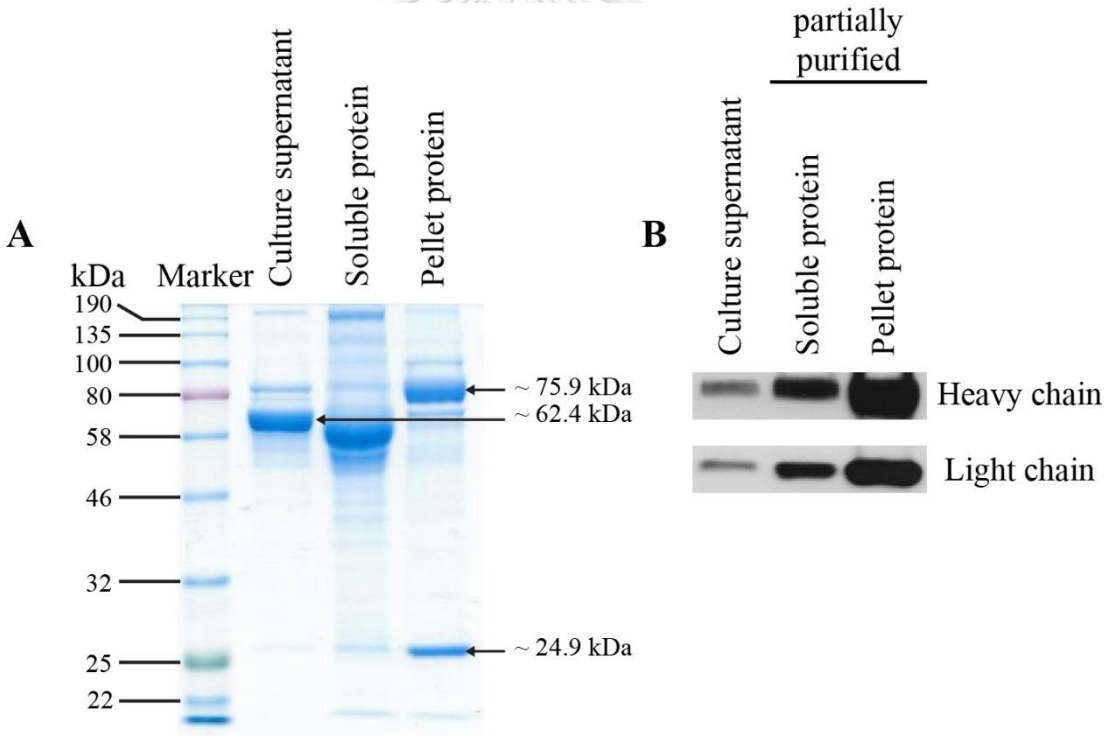


Figure 11 Analysis of the partially purified monoclonal antibody from hybridoma clone nF1C3#15.

A) Characterization of the total protein in the samples by using SDS-PAGE

B) The heavy chain and light chain were detected using Western blot.

4.1.8 Testing sandwich ELISA by using IgM No. nF1C3#15 as the capture antibody

After the partial purification of the monoclonal IgM antibody, five micrograms per milliliter of the purified monoclonal IgM antibody No. nF1C3#15 was used as capture antibody and the biotinylated monoclonal IgG antibody F1C3#13 was used as detecting antibody in the sandwich ELISA format (Figure 12A). The sandwich ELISA was performed as described in 3.7.1. The result showed that the sensitivity in term of the limit of detection of this sandwich ELISA was 190 ng/ml (Figure 12B) which was much higher than the LOD of the sandwich ELISA that developed in previous study using monoclonal antibody obtained with reIFNγ as immunogen (Table 11). Therefore, the IgG sandwich ELISA was chosen to develop IGRA in further study.

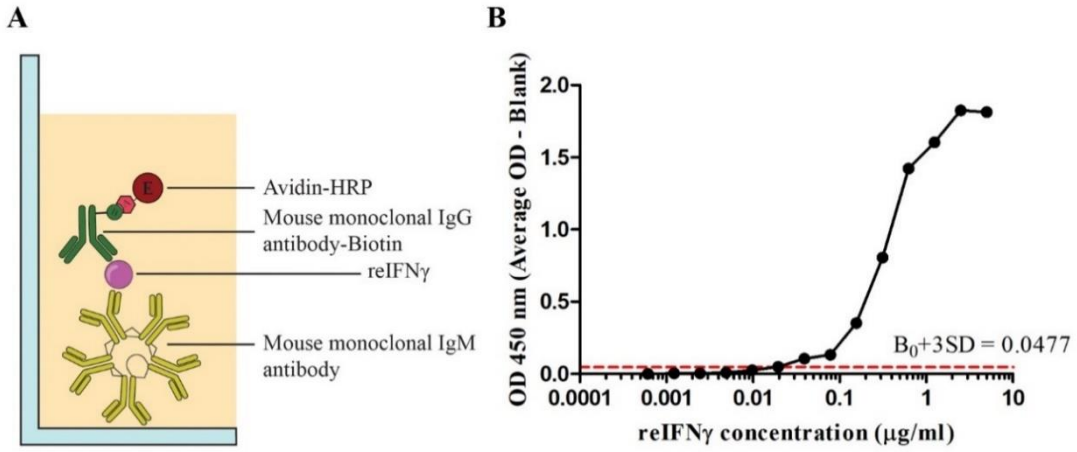


Figure 12 Sandwich ELISA by mouse monoclonal IgM antibody No. nF1C3#15 as capture antibody.

A) Schematic representation of IgM sandwich ELISA. B) The absorbance from the serial dilution of reIFNγ by using purified monoclonal IgM antibody nF1C3#15 and biotinylated monoclonal IgG antibody F1C3#13. The limit of detection was calculated by using the following formula: LOD = B_0 + 3SD

4.2 Characterization of IgG sandwich ELISA to detect eIFNγ

4.2.1 Limit of detection (LOD) of the IgG sandwich ELISA

Previously, we obtained monoclonal IgG (clone No. F1C3#13) specific to whole reIFNγ [51]. To determine the sensitivity in term of the limit of detection (LOD) of the developed sandwich ELISA (mouse monoclonal IgG antibody F1C3#13 as the detecting antibody and rabbit antibody against reIFNγ as capture antibody in this assay), the average absorbance of 20 blank samples (B_0) was used for calculating the LOD by using the formula mentioned in 3.7.4. As shown in Figure 13 and Table 11, the LOD was 0.257 ng/ml.

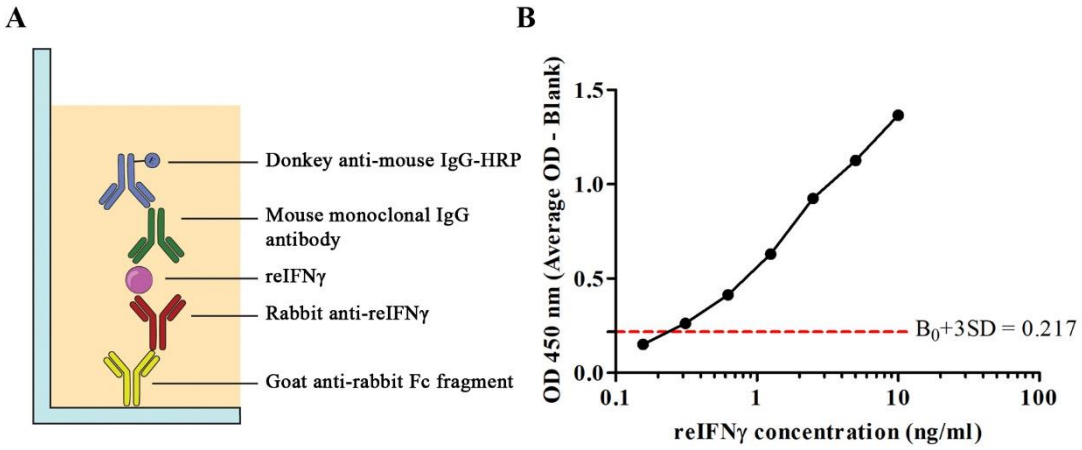


Figure 13 Standard curve of reIFNγ using IgG sandwich ELISA.

A) Schematic representation of developed IgG sandwich ELISA. B) Standard curve of reIFNγ using IgG sandwich ELISA. The reIFNγ at the concentration of 0.16, 0.31, 0.62, 1.25, 2.5, 5, and 10 ng/ml were used to generate standard curve with mouse monoclonal IgG antibody No. F1C3#13 (0.5 µg/ml) as detecting antibody and rabbit serum against reIFNγ as capture antibody.

Table 11 LOD of IgG sandwich ELISA.

B_0 (n=20)	SD	B_0 + 3SD	Linear equation	LOD (ng/ml)
0.160	0.0191	0.217	y = 0.3031ln(x) + 0.6284	0.257

* SD = Standard deviation

4.2.2 Precision of the developed sandwich ELISA

Sandwich ELISA was performed to determine the precision of the assay in term of the percentage of coefficient of variation (%CV) between inter and intra-assay. The reIFNγ at the concentration of 0.5, 1.0, 2.5, and 5.0 ng/ml were spiked to the RPMI 1640 media and the amount of reIFNγ was measured by the sandwich ELISA. The %CV of intra-variation and the inter-variation assay were in the ranges of 0.78-2.50 and 5.65-12.31, respectively (Table 12). From these results, the assay has a low range of the %CV of the intra-variation assay (less than 10) which implied that the assay has a low variation of the results within the data set obtained from one performance. The %CV of the inter-variation assay was less than 15 which indicated that the assay has the consistency of the results in each performance at different times. Taken together, the sandwich ELISA is an assay with reproducibility and acceptable variation.

Table 12 %CV of IgG sandwich ELISA.

Intra assay (n=12)	**Spiked reIFNγ concentration (ng/ml)**			
	0.5	**1**	**2.5**	**5**
	%CV			
I	2.26	1.76	1.88	0.97
II	2.09	2.14	1.75	0.78
III	2.49	2.5	1.67	1.43
Inter assay (N=3)	12.31	5.65	6.51	7.49

4.3 Optimization of an in-house IGRA for diagnosis of elephant TB

4.3.1 Optimization of the mitogens to stimulate elephant PBMC

To determine the suitable mitogen for elephant PBMC as a positive control in ELISA, the PBMCs from blood samples of 12 elephants were stimulated with Concanavalin A (ConA) or pokeweed mitogen (PWM) at the concentration of 10 µg/ml for 72 hours. After the stimulation, culture supernatants were collected and subjected to a sandwich ELISA. The result in

Figure 14 demonstrated that the mean of the elephant IFNγ (eIFNγ) concentration ± SEM of the sample group, when stimulated with ConA was 0.380 ± 0.071 ng/ml with the highest concentration of eIFNγ was 0.735 ng/ml. When PWM was used for stimulating the PBMCs, the mean concentration of eIFNγ ± SEM was 0.315 ± 0.058 ng/ml and the highest concentration was at 0.679 ng/ml. The statistical analysis also showed that there was no significant difference in the values obtained from the PBMCs that stimulated with ConA and PWM. However, the concentration of eIFNγ in ConA stimulated PBMCs were higher than using PWM. Therefore, ConA was chosen as mitogen for PBMCs stimulation as the positive control in IGRA.

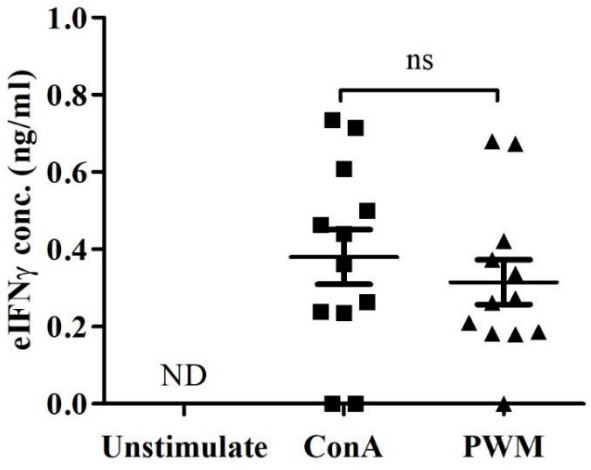


Figure 14 Stimulation of elephant PBMCs by two mitogens.
PBMCs from 12 elephants were stimulated with ConA (10 µg/ml) and PWM (10 µg/ml) for 72 hrs. The amount of the eIFNγ was measured by sandwich ELISA. ns: not significance with *p-value* > 0.05.
*ND = Not detected

4.3.2 Testing stimulating antigens in an in-house IGRA

To choose best combination of stimulation antigens. Five samples were subjected to IGRA twice using PBMC from two successive blood samples. In the preliminary test of the developed IGRA, the combination of recombinant ESAT-6 and CFP10 was used as an only stimulating antigen in this diagnosis

method. Among 5 samples, two samples were found negative and 3 samples were determined as MTBC infection positive (Figure 15).

Using the follow up PBMC, the blood samples from elephant No. 1 and 5 were subjected to IGRA again over 6 and 9 months after the first examination, respectively. While the elephant No. 2, 3, and 4 were subjected to IGRA again on the forth months after the first examination. In addition to the stimulating antigen that used in the first diagnosis, the PPDB, single recombinant ESAT-6 and CFP10 were added as stimulating antigen. The result in Figure 15 showed that sample No. 2, 3, and 5 were consistently positive with MTBC infection after the second examination. However, the results of the sample No. 1 and 4 changed from negative to MTBC infection suspected when stimulated with PPDB in the second round of diagnosis. However, the response of the PBMCs when stimulated with the combination of ESAT-6 and CFP10 in the first and the second results were almost the same in all five samples. Based on this result, the developed IGRA have a potential to be used as diagnostic tool to diagnose and confirm the TB infection in elephants by using combination of stimulating antigens. This developed method was reproducible because the response of the PBMCs against the combination of ESAT-6 and CFP10 showed the consistent results.

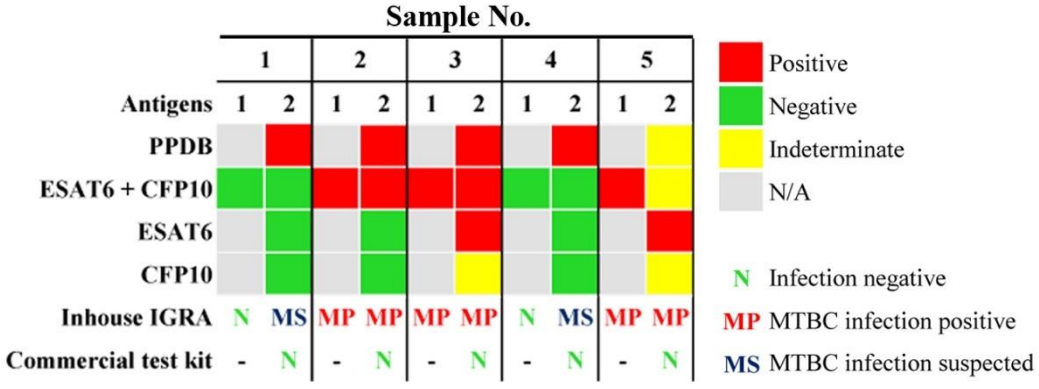


Figure 15 Follow up test using in-house IGRA and commercial test kit (DPP® VetTB test kit) in 5 elephant samples.

The response of PBMC against the stimulated antigens was categorized into 3 categories; positive (red box), negative (green box), and indeterminate (yellow box).

4.3.3 Elephant TB diagnosis by in-house IGRA

To diagnose tuberculosis in elephants, PBMCs from blood samples of the elephant were separated and stimulated with the mycobacterial specific antigens or a mitogen. The PPDB was used as a standard control for MTBC infection in the assay. To specify the response of the cells, the recombinant MTBC specific proteins (e.g. ESAT-6, CFP10) were used as a stimulating antigen. This stimulation was used to confirm the MTBC infection results in the in-house IGRA assay. The responses of the PBMCs against stimulating antigens were classified into three categories; negative, positive, and indeterminate as described in 3.8.1.4.

In this experiment, the TB status of 61 elephants was determined by in-house IGRA according to the responses of the PBMCs against stimulating antigens (Figure 16). The TB status was determined by using the criteria as described in 3.8.1.4. By using in-house IGRA, the results demonstrated that out of 61 samples, 23 samples (37.7%) were diagnosed as negative, 3 samples (4.9%) were found as indeterminate, and 35 samples (57.4%) showed positive results as potential TB infection (MTBC infection 19 samples and MTBC infection suspected 16 samples which accounting for 31.1% and 26.2% of the total numbers of the elephant samples, respectively). The results from Table 13 illustrated that the PBMCs were most responsive to PPDB which accounting for 55.7% of the total numbers of the elephant samples. In addition, when ESAT-6 was used in combination with CFP10 for stimulating the cells, the response of the PBMCs was greater than using either ESAT-6 or CFP10 alone, accounting for 31.1%, 21.3%, and 8.2%, respectively. Among 61 samples tested, sample No. 66 was from elephants previously recorded as MTB culture positive. In our assay it was also found positive by responding to all the MTBC specific antigens.

Among 61 samples which were subjected to diagnosis by in-house IGRA, thirty-two samples were randomly selected to diagnose of the TB infection with DPP® VetTB test kit which is a commercial test kit for comparison. This test kit contains printed ESAT-6 / CFP10 fusion protein and

MBP83 onto the nitrocellulose membrane for using as a representative antigen of MTBC and NTM, respectively. The results from Figure 16 demonstrated that out of 32 samples, only 11 samples that showed the same results with the IGRA and DPP VetTB assay, accounting for 34.4% of the numbers of the samples that test with both IGRA and DPP® VetTB assay (6 samples found the negative results and 5 samples found as TB infection positive results in both assays (Table 15)). The response of the PBMC against the specific antigen by IGRA demonstrated that the sample No. 16, 22, 27, 59, and 66 that found positive in both assays were positive with PPDB, and ESAT-6 with CFP10 (except sample No.59 that gave the indeterminate response) which consistent with the antigens that used in DPP® VetTB assay.

The results from DPP® VetTB assay showed that the antibody from these five samples were reactive to ESAT-6/CFP10 fusion protein that printed on the test band No.2. (Sample No. 66 was the only sample that gave strong reactive band against ESAT6/CFP10 antigens (Table 14)). Apart from 11 samples that gave consistent results in both assays, the remaining. Therefore, the TB status of these samples was determined random to the IGRA results because the non-reactive result of the DPP® VetTB assay did not preclude the possibility of exposure to TB or infected with TB.

Taken together, the developed in-house IGRA can be used to diagnose TB infection with the same or better accuracy than using a commercial test kit. Moreover, IGRA showed higher sensitivity than DPP® VetTB test kit because it can detect TB infection in the samples that DPP® VetTB test kit showed negative result.

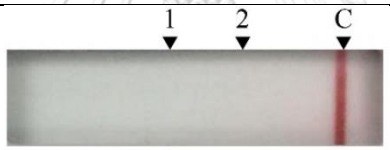
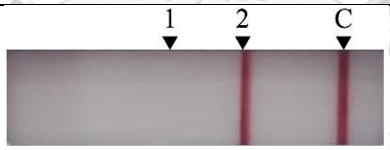
Table 13 Percentage of the response of PBMCs in in-house IGRA.

Response (%)	Stimulating antigens			
	PPDB	ESAT-6 + CFP10	ESAT-6	CFP10
Negative	37.7 (23)	62.3 (38)	72.1 (44)	85.2 (52)
Indeterminate	6.6 (4)	6.6 (4)	6.6 (4)	6.6 (4)
Positive	55.7 (34)	31.1 (19)	21.3 (13)	8.2 (5)

* The numbers in the blanket are the number of the samples that responded to each antigen.

Table 14 Antibody detection by DPP VetTB assay.

The images represent the results obtained for non-reactive and reactive for TB samples. (All the DPP® VetTB assay results were showed in Supplement Table 6)

Sample No.	Results	TB status
63		Non-reactive
66		Reactive for TB (ESAT-6/CFP10 test band)

* Test band No.1 was coated with mycobacterial antigen (MPB83). Test band No.2 was coated with specific MTBC antigen (ESAT-6/CFP10). Test band C was the control of the test kit.

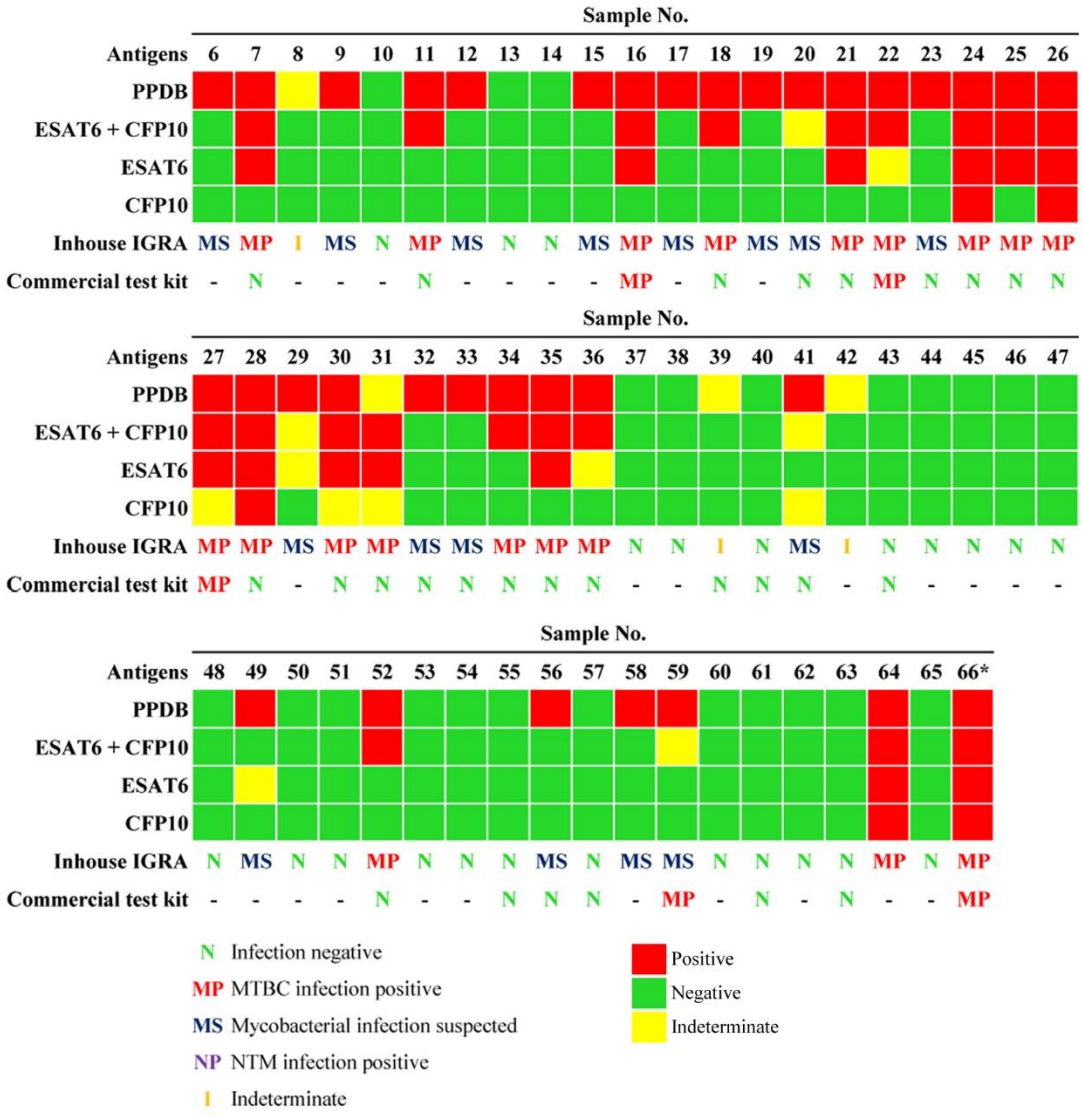


Figure 16 Summary of PBMC response to stimulation antigens in in-house IGRA and commercial test kit.

The response of PBMC against the stimulated antigens was categorized into 3 categories; positive (red box), negative (green box), and indeterminate (yellow box). (*) bacterial culture positive sample. Sixty-one elephant samples were tested with in-house IGRA. Among 61 samples, 32 samples were tested with commercial test kit parallel with in-house IGRA.

Table 15 Comparison of TB infection status by in-house IGRA and DPP® VetTB assay (n=32).

TB status	In-house IGRA	DPP® VetTB assay	Result matching	Result unmatching
Positive	26	5	5	21
Negative	6	27	6	21

4.3.3.1 Improvement of the accuracy of inhouse-IGRA by addition of stimulating antigens

To improve the accuracy to identify the TB status by using in-house IGRA, the MTBC specific antigens Rv3020c and NTM specific antigen PPDA from *M. avium* were tested as stimulating antigens in in-house IGRA apart from existing antigens. Forty-eight samples were stimulated with the Rv3020c in addition to existing antigens. The result from Figure 17A demonstrated that when using Rv3020c as an additional MTBC specific antigen, the TB status of the samples No. 20, 23, 39, and 57 were changed to positive for MTBC infection and the sample No. 50 was changed from negative to indeterminate when considered the response of the cells against Rv3020c. Moreover, to specify the NTM infection by using in-house IGRA, twenty-eight samples were stimulated with PPDA apart from MTBC specific antigens. The results demonstrated that 7 samples were changed to positive for NTM infection and the sample No.61 was changed from negative to indeterminate when considered the response of the cells against PPDA (Figure 17B).

Therefore, the accuracy of the developed in-house IGRA can be increased to identify differences in TB status by using additional antigens Rv3020c and PPDA as stimulating antigens in in-house IGRA.

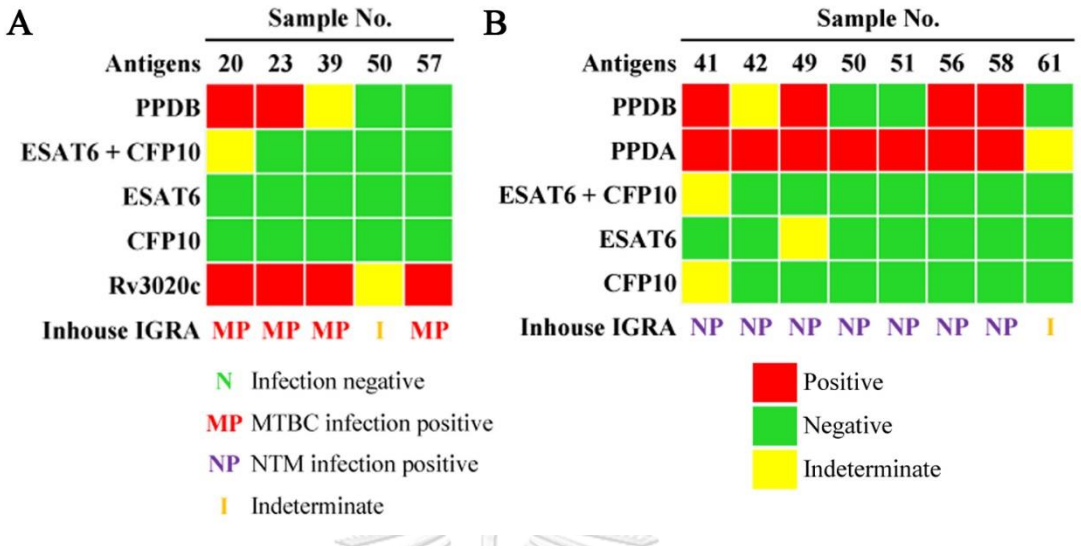


Figure 17 Response of PBMC against TB antigens by using in-house IGRA.

Among 61 samples, 48 samples were stimulated with the Rv3020c and 28 samples were stimulated with the PPDA in addition to existing antigens. The response of PBMC against the stimulated antigens was categorized into 3 categories; positive (red box), negative (green box), and indeterminate (yellow box).

4.3.4 Cross-reactivity of IgG sandwich ELISA against tapir IFNγ

To determine the cross-reactivity of IgG sandwich ELISA against tapir IFNγ, the PBMCs from four tapirs were separated and stimulated with TB specific antigens (PPDB, ESAT-6, and CFP10) or mitogen for 72 hrs. The results in Table 16 showed that the tapir IFNγ could not be detected with the developed sandwich ELISA.

Table 16 Tapir IFNγ detection by IgG sandwich ELISA.

Antigens		**Sample No.**			
		1	2	3	4
Unstimulated	IFNγ conc.	ND	ND	ND	ND
ConA	IFNγ conc.	ND	ND	ND	ND
PPDB	IFNγ conc.	ND	ND	ND	ND
ESAT-6	IFNγ conc.	ND	ND	ND	ND
CFP10	IFNγ conc.	ND	ND	ND	ND

***** ND: Not detected

The absorbance values were converted to concentrations of IFNγ by the linear equation from standard curve; $y = 0.5123\ln(x) + 1.3016$

CHAPTER V
DISCUSSION

Tuberculosis is a zoonotic disease that transmitted from infected humans to animals such as Asian elephant, African elephant, cattle, non-human primate, and tapir [2-5]. The infection has an impact on animal welfare, humans in close contact, tourist industry which can affect the economy. TB in animals is difficult to diagnose with clinical signs alone, especially, the latent stage of TB infection when the clinical signs are not visible. Therefore, accurate diagnosis methods are necessary for the surveillance and control of the epidemic of the disease. In this study, we aimed to develop the diagnostic method for diagnosing TB in animals, especially in elephants using a monoclonal antibody specific to eIFNγ by sandwich ELISA.

In this study, we generated the monoclonal antibody against IFNγ by using conserved eIFNγ peptide (GAKRKRRQYSFRGRRAST; [149-166] conjugated with carrier protein) as an immunogen for immunization. This peptide is the conserved region in ten mammal species and predicted to be highly immunogenic. After somatic cell-cell fusion, the hybridomas were screened to find the monoclonal antibody that specific to the reIFNγ by using indirect ELISA method. Among the 62 clones from 2 fusions, only 12 clones that produced the monoclonal antibodies that were categorized as strong reactivity against reIFNγ. In addition, the isotype of all monoclonal antibodies was determined as IgM. The reasons why only IgM were obtained were not clear but it is possible that the frequency of immunization was not sufficient to induce isotype switching.

The obtained monoclonal IgM antibody No. nF1C3#15 showed the strongest reactivity among the surviving clones was used as a capture antibody in sandwich ELISA format. The LOD of this format was 190 ng/ml which was higher than the LOD of the IgG sandwich ELISA format developed in our previous study which has the LOD at 0.257 ng/ml. The IgG sandwich ELISA used rabbit antibody against the reIFNγ as capture antibody which is a polyclonal antibody. The polyclonal antibody is a mixture of the antibody produced by many clones of B lymphocytes that may bind

with each different epitope of an antigen [55], while the monoclonal antibody is an identical antibody molecule with specific to a single epitope on the antigen. Thus, by using polyclonal antibody in ELISA it can offer greater sensitivity for detecting the antigen when using as capture antibody than using monoclonal antibody.

In this study, our developed IGRA has used the antibody obtained by using the reIFNγ as an immunogen for stimulating the production of specific antibody in mouse and rabbit. The use of reIFNγ as an antigen to produce a specific antibody for elephant IGRA development has been reported in previous studies [14,15]. Although the reIFNγ has been used in the immunization process to produce the antibody used in IGRA development, the formats used for detecting the eIFNγ were different. In the study by Angkawanish *et al.*, they developed elephant IGRA using mouse monoclonal-based sandwich ELISA to detect the reIFNγ which have the limit of detection (LOD) at 1 pg/ml [14], while in the study by Paudel *et al.* reported that the LOD of the IGRA using rabbit polyclonal antibody-based sandwich ELISA was 100 pg/ml [15]. In contrast, our developed IGRA which used the combination of the monoclonal and polyclonal antibody to detect the reIFNγ in sandwich ELSA system has the limit of detection of reIFNγ of 0.257 ng/ml. The sensitivity of our developed sandwich ELISA was sufficient for detecting the eIFNγ secreted from the Concanavalin A stimulated PBMCs which have the mean concentration at 0.380 ± 0.071 ng/ml. In addition, the sandwich ELISA showed the high accuracy and precision.

PPDB and PPDA were used as a standard control for MTBC infection and NTM infection in the assay, respectively. PPD is the mixture of the proteins [18], which can be used to increase the chance to recall the memory of the PBMCs in the TB exposed animals. In this study, most of the TB infected samples were found positive with PPDB stimulation. However, the PPDB also shared some of the antigenic components with the PPDA [18]. Therefore, the PPDB alone is not sufficient to discriminate the NTM exposed from the MTBC exposed animals[56]. Therefore, the specific antigens that present only in MTBC are necessary to distinguish the MTBC infection from NTM infection. In the present study, we used ESAT-6 and CFP10 which are potent T-cell antigens and were strongly recognized by T cells, resulting in

the IFNγ release from PBMCs from human TB patients [20] ESAT-6 and CFP10 were used as MTBC-specific antigens for diagnosing TB infection by IGRA in many animal species [12,14,56]. Interestingly, our data showed that the use of the combination of ESAT-6 and CFP10 to stimulate the PBMCs increased the chance of the PBMCs response to MTBC, resulting in increased sensitivity of the diagnostic performance than using either ESAT-6 or CFP10 as a single antigen. Similar results have been reported that the combination of ESAT-6 and CFP10 increase the diagnostic performance of IGRA than using as a single antigen in human and cattle [57].

Rv3020c is an ESAT-6 like protein which has been reported to be a potential diagnostic antigen for skin-testing, and IGRA (Bovigram® assay) in cattle [58,59]. The current study showed that the use of the Rv3020c improved the accuracy to identify the MTBC infection. This antigen is recognized by PBMCs from elephants that did not respond to the other MTBC-specific antigens. Similar results were reported in case of *M. bovis* infected-red deer when using Rv3020c as one of the stimulating antigens in Bovigam assay [60]. Moreover, seven of the elephant samples were classified as NTM infection positive by responding to PPDA antigen and unresponsive to MTBC specific antigen, indicating that PPDA can be used to discriminate between the NTM infection and other TB infection status.

The trunk wash culture method has several limitations such as poor sensitivity, time-consuming and can detect only the active TB stage [7]. In addition, currently, there is no gold standard to diagnose latent TB infection. In our study, only sample No.66 was confirmed to be culture positive. Therefore, we decided to use the DPP® VetTB assay which is the only available commercial test kit for diagnosing TB infection in the elephants for comparison with in-house IGRA. This assay is based on the immunochromatographic principle to detect the antibody specific to the ESAT-6/CFP10 fusion protein and MPB83 that printed on the membrane [3 8]. The advantages of the assay are user-friendly and easy to perform on site. However, a non-reactive result from this assay does not preclude the possibility of exposure to TB or infection with TB.

This study is the first attempt to elucidate the TB diagnostic performance of the developed in-house IGRA in a large population of Asian elephants in Thailand.

We found that by comparison of both IGRA and DPP® VetTB assay in thirty-two samples, 11 samples yielded consistent results in both assays. The five samples reactive to the ESAT-6/CFP10 test band in DPP® VetTB assay also responded to PPDB and the combination of ESAT-6 and CFP10 in in-house IGRA. In particular, in the elephant No.66 that previously recorded as MTB culture positive was found strongly reactive to the ESAT-6/CFP10 test band on DPP® VetTB assay and strongly responsive to all the MTBC specific antigens stimulation as well as PPDA, due to the cross-reactive response to NTM [61]. This indicated that our developed assay can be used to diagnose the active TB infection similar result to DPP® VetTB assay.

Interestingly, by using the IGRA we found that twenty-one samples responded to MTBC specific antigens stimulation, but the antibody from these samples were not reactive with the antigens that printed on the membrane of the DPP® VetTB test kit. This is the first report which IGRA shows the contradictory results against DPP® VetTB assay in elephant. We suspected that these elephants were already exposed to the MTBC or might be in the latent stage of the MTBC infection which resulted in the production of IFNγ in response to MTBC antigens [62]. However, there is currently no gold standard method for diagnosing the latent TB infection in the elephant. Most of the TB infected elephant does not show the clinical sign, even in the active stage of the infection which makes it difficult to diagnose TB in the elephant [7,8]. Therefore, the TB status of these elephants should be monitored carefully and periodically diagnosed with the IGRA and DPP® VetTB assay and confirmed the results with trunk wash culture method.

In this study, the IGRA was developed to use as an alternative method for diagnosis of TB infection in the elephant because IGRA has many advantages of due to the high sensitivity and specificity [43,44]. As described above, IGRA detects the response of the cellular immunity towards the MTBC specific antigens such as ESAT-6, and CFP-10, which are known to be absent in most of the NTM. Therefore, this method can reduce the cross-reactivity to most NTM and all BCG vaccine strains [44]. Although IGRA can be used to detect the latent stage of TB infection, it still cannot distinguish between latent and active TB infection alone by itself [45]. Moreover, IGRA is not user-friendly when compared to the DPP® VetTB assay, as it required sample

processing in the laboratory, trained personnel to perform, and the blood must be processed within 8-30 hours after collection because the delay of the blood processing can affect the performance of the test. [46,47]. Therefore, easier to use IGRA should be developed on the current platform.

Taken together, IGRA shows the promising diagnostic performance for using as an alternative approach from the trunk wash culture method in latent and active TB infection samples due to the specificity, sensitivity, and reproducibility of the assays.

CHAPTER VI
CONCLUSION

6.1 The use of the conjugated eIFNγ as an immunogen elicited antibody specific to the eIFNγ peptide and reIFNγ in mice.

6.2 The monoclonal antibody No. nF1C3#15 was chosen as a capture antibody in IgM sandwich ELISA which yields the LOD of 190 ng/ml.

6.3 The developed IgG sandwich ELISA have the LOD at 0.257 ng/ml with reproducibility and acceptable variation.

6.4 IGRA can be used for diagnosis of TB infection in elephant with better or equivalent sensitivity as DPP® VetTB test kit.

6.5 The in-house IGRA developed in this study is promising test kit to diagnose TB in the elephant.

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VITA

NAME	Songkiat Songthammanuphap
DATE OF BIRTH	7 April 1993
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	2016, Bachelor degree of Science, Chulalongkorn University, Thailand
HOME ADDRESS	50 Charoennakorn Rd., Klongtonsai, Klongsan, Bangkok, 10600, Thailand
PUBLICATION	Proceeding at the 11th Srinakharinwirot University Research Conference

APPENDICES

APPENDIX A
LIST OF PREPARING REAGENTS

1. 1x Phosphate buffer saline (PBS), pH 7.4 (1000 ml)

-	NaCl	8.0	g
-	KCl	0.2	g
-	Na_2HPO_4	1.44	g
-	KH_2PO_4	0.24	g
-	Type 1 water	1000	ml

Adjusted the pH to 7.4 and made to final volume to 1000 ml. Sterile the solution by autoclaved at 121ºC, 15 psi for 15 minutes.

2. 10x Phosphate buffer saline (PBS), pH 7.4 (1000 ml)

-	NaCl	80	g
-	KCl	2	g
-	Na_2HPO_4	14.4	g
-	KH_2PO_4	2.4	g
-	Type 1 water	1000	ml

Adjusted the pH to 7.4 and made to final volume to 1000 ml. Sterile the solution by autoclaved at 121ºC, 15 psi for 15 minutes.

3. Washing buffer (PBST) (1000 ml)

-	10x PBS	100	ml
-	Tween20	0.5	ml
-	Type 1 water	900	ml

Diluted 10x PBS ten-fold with type 1 water and added 0.5 ml of Tween20 to the diluted PBS. The solution should not be used after 1 week, during this period keep the solution at 4ºC.

4. 0.2 M 3,3',5,5'-Tetramethylbenzidine (TMB) buffer, pH 4 (1000 ml)

- Tri-potassium citrate monohydrate ($C_6H_5K_3O_7 \cdot H_2O$)	0.0665	g
- Citric acid	39.38	g
- Type 1 water	500	ml

Adjusted the pH to 4.0 and autoclaved at 121ºC, 15 psi for 15 minutes. Buffer was kept at 4ºC.

5. TMB substrate solution (10ml)

- TMB	0.003	g
- Dimethylsufoxide (DMSO)	0.300	ml
- 0.2 M TMB buffer, pH 4	10	ml
- 30% Hydrogen peroxide (H_2O_2)	0.0025	ml

Dissolved TMB with 0.3 ml of DMSO and added 0.250 ml of dissolved TMB into 10 ml of 0.2 M TMB buffer, pH 4. After adding H_2O_2, mix the regent well and used immediately.

6. 1 M H_2SO_4 (1000 ml)

- 96% H_2SO_4	56	ml
- Type 1 water	944	ml

7. Completed RPMI 1640 media (100 ml)

- RPMI 1640	87	ml
- FBS	10	ml
- 1 M HEPES	1	ml
- 100 mM Sodium Pyruvate	1	ml
- 100x Penicillin-Streptomycin	1	ml

8. Freezing media (10% DMSO)

- Completed RPMI 1640 media	0.9	ml
- DMSO	0.1	ml

9. Saturated ammonium sulfate (4.1M $(NH_4)_2SO_4$) (1000 ml)

- $(NH_4)_2SO_4$	541.77	g
- Type 1 water	1000	ml

After completely dissolved ammonium sulfate, the volume of the solution was adjusted to 1000 ml with type 1 water and autoclaved at 121ºC, 15 psi for 15 minutes.

10. 20 mM Tris-HCl buffer, pH 8.0 (1000 ml)

- Tris	2.4	g
- Type 1 water	500	ml

Adjust the pH to 8.0 with 1 M HCl and sterile by autoclaved at 121ºC, 15 psi for 15 minutes.

11. 2x Laemmli Sample buffer (SDS-dye) (10 ml)

- 1 M Tris-HCl, pH 6.8	1	ml
- 10% SDS	4	ml
- 99.5% Glycerol	2.01	ml
- Bromophenol blue	0.001	g
- Type 1 water was added to adjust volume to	10	ml

The buffer was aliquoted into 900 µl/tube and keep in -20ºC. One hundred microliters of β-mercaptoethanol was added before used.

12. 1.5 M Tris-HCl buffer, pH 8.8 (500 ml)

- Tris 90.855 g
- Type 1 water 500 ml

Adjusted the pH to 8.8 with 1 M HCl and autoclaved at 121ºC, 15 psi for 15 minutes.

13. 1 M Tris-HCl buffer, pH 6.8 (1000 ml)

- Tris 60.57 g
- Type 1 water 500 ml

Adjusted the pH to 8.8 with 1 M HCl and autoclaved at 121ºC, 15 psi for 15 minutes.

14. SDS-polyacrylamide gel preparation

14.1. 10% Separating gel (8 ml)

- Type 1 water 3.836 ml
- 40% Acrylamide and Bis-acrylamide solution 2.000 ml
- 1.5 M Tris-HCl buffer, pH 8.8 2.000 ml
- 10% SDS 0.080 ml
- 10% Ammonium persulfate (APS) 0.080 ml
- TEMED 0.004 ml

Adjusted the surface of the gel with type 1 water by overlayered the water to the surface of the gel

14.2. 5% Stacking gel (2 ml)

-	Type 1 water	1.204	ml
-	40% Acrylamide and Bis-acrylamide solution	0.250	ml
-	1 M Tris-HCl buffer, pH 6.8	0.504	ml
-	10% SDS	0.020	ml
-	10% Ammonium persulfate (APS)	0.020	ml
-	TEMED	0.002	ml

15. 5x Running buffer (1000 ml)

-	Tris	15.1	g
-	Glycine	94	g
-	SDS	5	g
-	Type 1 water	1000	ml

Diluted 5x Running buffer to 1x before use.

16. Transfer buffer for semidry transfer system (1000 ml)

-	Tris	5.08	g
-	Glycine	2.9	g
-	SDS	0.37	g
-	Type 1 water	800	ml
-	Absolute Methanol	200	ml

Brought volume to 800 ml before adding absolute methanol 200 ml.

17. 100mM Tris-HCl buffer, pH 8.5 (500 ml)

-	Tris	6.057	g
-	Type 1 water	500	ml

Adjusted pH to 8.5 and make to final volume to 500 ml before autoclaved at 121^{o}C, 15 psi for 15 minutes.

18. ECL substrate

18.1. 90 mM Coumaric acid

- Coumaric acid	0.0148	g
- DMSO	1	ml

18.2. 250 mM Luminol

- Luminol	0.0443	g
- DMSO	1	ml

18.3. Solution A

- 100 mM Tris-HCl buffer, pH 8.5	2.5	ml
- 90 mM Coumaric acid	0.011	ml
- 250 mM Luminol	0.025	ml

18.4. Solution B

- 100 mM Tris-HCl buffer, pH 8.5	2.5	ml
- 30% H_2O_2	0.0015	ml

19. Film developer and fixer

Film developer and fixer were diluted in tap water at dilution 1:4.

20. Coomassie brilliant blue staining solution

20.1. PhastGel Blue R (stock) (200 ml)

- PhastGel Blue R-350	1	tablet
- Type 1 water	80	ml
- Absolute Methanol	120	ml

Dissolved a tablet of PhastGel Blue R-350 with 80 ml of type 1 water and stirred for 5 to 10 minutes. Then gently added absolute methanol and stirred until all the dye is dissolved. After completely dissolved, filtrated the solution with Whatman paper number 1 and keep the stock solution at 4ºC.

20.2. Coomassie Blue dye (working)

-	Phost Gel Blue R (stock)	1	ml
-	Destain solution	9	ml

21. Destaining solution (1000 ml)

-	Absolute Methanol	300	ml
-	Acetic acid	100	ml
-	Type 1 water	600	ml

22. Gel drying solution (1000 ml)

-	Absolute Methanol	300	ml
-	Glycerol	50	ml
-	Type 1 water	650	ml

23. Red blood cell lysis buffer (1000 ml)

-	NH_4Cl	8.3	g
-	0.5 M EDTA	0.2	ml
-	$NaHCO_3$	1	g

Brought volume to 1000 ml and filtrated with 0.2 µm filter and keep buffer was at 4ºC.

APPENDIX B
SUPPLEMENT

Supplement Table 1 Ig-score rank of the 18 amino acids peptides from Asian elephant IFNγ amino acid sequence.

Ig-score rank	Peptide	Amino acid position	Ig-score
1	NDLSHRSNGAKRKRRQYS	[141-158]	7.782
2	RSNGAKRKRRQYSFRGRR	[146-163]	7.640
3	HRSNGAKRKRRQYSFRGR	[145-162]	7.564
4	SHRSNGAKRKRRQYSFRG	[144-161]	7.515
5	MNDLSHRSNGAKRKRRQY	[140-157]	7.304
6	NGAKRKRRQYSFRGRRAS	[148-165]	7.152
6	SNGAKRKRRQYSFRGRRA	[147-164]	7.152
8	DLSHRSNGAKRKRRQYSF	[142-159]	7.038
9	LSHRSNGAKRKRRQYSFR	[143-160]	6.908
10	GAKRKRRQYSFRGRRAST	[149-166]	6.774
11	KVMNDLSHRSNGAKRKRR	[138-155]	6.770
12	VMNDLSHRSNGAKRKRRQ	[139-156]	6.738
13	SKVMNDLSHRSNGAKRKR	[137-154]	6.722
14	SKRDDFLKVMQTPVNDRN	[110-127]	6.186
15	LSKVMNDLSHRSNGAKRK	[136-153]	6.106
16	DILKNWKEDSDKKIIQSQ	[54-71]	6.074
17	NLKEYLNATDSDVADGGP	[33-50]	6.035
18	ELSKVMNDLSHRSNGAKR	[135-152]	5.989
19	SSKRDDFLKVMQTPVNDR	[109-126]	5.986
20	QNLKEYLNATDSDVADGG	[32-49]	5.986
21	EEDLFVKFFNSSSSKRDD	[97-114]	5.932
22	SELSKVMNDLSHRSNGAK	[134-151]	5.924
23	SSSKRDDFLKVMQTPVND	[108-125]	5.922
24	FNSSSSKRDDFLKVMQTP	[105-122]	5.867

Ig-score rank	Peptide	Amino acid position	Ig-score
25	KNWKEDSDKKIIQSQIVS	[57-74]	5.851
26	DGGPLFIDILKNWKEDSD	[47-64]	5.807
27	SSSSKRDDFLKVMQTPVN	[107-124]	5.751
27	NSSSSKRDDFLKVMQTPV	[106-123]	5.751
29	GPLFIDILKNWKEDSDKK	[49-66]	5.747
30	GGPLFIDILKNWKEDSDK	[48-65]	5.697
31	TPVNDRNVQRKAISELSK	[121-138]	5.677
32	QTPVNDRNVQRKAISELS	[120-137]	5.647
33	KRDDFLKVMQTPVNDRNV	[111-128]	5.612
34	DDFLKVMQTPVNDRNVQR	[113-130]	5.573
34	RDDFLKVMQTPVNDRNVQ	[112-129]	5.573
36	NDRNVQRKAISELSKVMN	[124-141]	5.548
37	KFFNSSSSKRDDFLKVMQ	[103-120]	5.533
38	EDLFVKFFNSSSSKRDDF	[98-115]	5.522
39	DRNVQRKAISELSKVMND	[125-142]	5.517
40	DFLKVMQTPVNDRNVQRK	[114-131]	5.464
41	IDILKNWKEDSDKKIIQS	[53-70]	5.457
42	NLKDNQVIQESVKTLEED	[82-99]	5.405
42	DNLKDNQVIQESVKTLEE	[81-98]	5.405
44	IQNLKEYLNATDSDVADG	[31-48]	5.376
45	KEYLNATDSDVADGGPLF	[35-52]	5.373
46	NWKEDSDKKIIQSQIVSF	[58-75]	5.351
47	LKNWKEDSDKKIIQSQIV	[56-73]	5.340
48	FFNSSSSKRDDFLKVMQT	[104-121]	5.332
49	KAISELSKVMNDLSHRSN	[131-148]	5.317
50	ILKNWKEDSDKKIIQSQI	[55-72]	5.311

Supplement Table 2 The absorbance of monoclonal antibodies produced from hybridomas fusion 1 after 3rd subcloning by indirect ELISA.

Hybridoma No.	Well Code	OD 450 nm
nF1C3#1	3/H1/E11/B3/C3	1.249
nF1C3#2	3/H1D12/F5/B6	1.003
nF1C3#3	4/B6/A4/C3/G8	1.831
nF1C3#4	4/B6/A4/E2/D6	1.088
nF1C3#5	6/H3/A9/F5/G11	1.809
nF1C3#6	6/H3/A9/D6/G3	1.407
nF1C3#7	6/H3/A10/H7/G6	1.627
nF1C3#8	6/A5/A1/F12/G11	1.700
nF1C3#9	6/A5/A1/F12/F12	2.363
nF1C3#10	6/A7/A3/E5/E6	1.171
nF1C3#11	6/A7/A4/F8/H9	2.667
nF1C3#12	6/C1/D5/G10/F1	1.792
nF1C3#13	6/C1/D5/G11/G6	1.165
nF1C3#14	6/H9/A11/D1/F11	2.986
nF1C3#15	6/H9/A11/E3/F3	3.035
nF1C3#16	6/H9/A12/F4/G6	3.154
nF1C3#17	7/A8/D4/F11/G10	2.961
nF1C3#18	7/A8/D4/E12/G2	2.026
nF1C3#19	7/A8/D4/F12/D6	1.550
nF1C3#20	7/A8/C3/C3/G9	3.335
nF1C3#21	7/D11/A3/D10/E10	1.603
nF1C3#22	7/F9/D5/F2/G6	3.030
nF1C3#23	7/F9/D5/E3/G7	2.602
nF1C3#24	7/F9/D5/E9/F12	2.436

Hybridoma No.	Well Code	OD 450 nm
nF1C3#25	8/H3/B7/E11/F2	1.191
nF1C3#26	8/H12/C9/E3/E11	1.556
nF1C3#27	8/H12/B9/H4/G2	1.899
nF1C3#28	8/H12/B9/H6/H6	1.416
nF1C3#29	9/A4/A11/C12/E11	0.977
nF1C3#30	9/A4/A11/C12/D12	1.926
nF1C3#31	10/H11/A9/E10/H5	2.398
nF1C3#32	10/H11/A9/G10/G8	2.656
nF1C3#33	10/E3/D6/H5/F6	2.458
nF1C3#34	10/E3/C5/E8/G12	3.081
nF1C3#35	11/H3/D3/C2/F3	1.578

Supplement Table 3 The absorbance of monoclonal antibodies produced from hybridomas fusion 2 after 2nd subcloning by indirect ELISA.

Hybridoma No.	Well Code	OD 450 nm
nF3C2#1	1/F1/G5/F1	1.803
nF3C2#2	1/F1/G5/G2	1.995
nF3C2#3	1/F1/G5/E3	2.004
nF3C2#4	1/F11/G4/G4	1.799
nF3C2#5	1/F11/G4/F5	1.874
nF3C2#6	1/F11/G4/F6	1.687
nF3C2#7	1/H11/E2/C7	2.346
nF3C2#8	1/H11/E2/D8	2.212
nF3C2#9	1/H11/E2/B9	2.225
nF3C2#10	2/A3/E4/D10	1.650
nF3C2#11	2/A3/E4/E11	1.790
nF3C2#12	2/A3/E4/D12	1.540
nF3C2#13	2/A3/E3/F1	1.989
nF3C2#14	2/A3/E3/F2	2.038
nF3C2#15	2/A3/E3/G2	1.317
nF3C2#16	3/B4/B3/C7	0.969
nF3C2#17	3/B4/B3/B8	2.278
nF3C2#18	3/B4/B3/C8	1.904
nF3C2#19	3/F9/F8/F7	2.162
nF3C2#20	3/F9/F8/E8	2.179
nF3C2#21	3/F9/F8/E7	2.146
nF3C2#22	3/F9/F6/E10	1.180
nF3C2#23	3/F9/F6/F11	1.750
nF3C2#24	3/F9/F6/E12	1.958
nF3C2#25	4/C9/D7/E2	2.333
nF3C2#26	4/C9/D7/E3	1.074

Hybridoma No.	Well Code	OD 450 nm
nF3C2#27	4/C9/D7/F3	2.072
nF3C2#28	4/C9/D6/G4	2.409
nF3C2#29	4/C9/D6/F5	2.490
nF3C2#30	4/C9/D6/F6	2.390
nF3C2#31	5/D2/H4/C10	0.738
nF3C2#32	5/D2/H4/B12	0.688
nF3C2#33	5/D2/H4/C12	0.806
nF3C2#34	11/C9/E6/A7	1.422
nF3C2#35	11/C9/E6/B7	0.927
nF3C2#36	11/C9/E6/A8	0.428
nF3C2#37	8/F9/H1/D6	1.077
nF3C2#38	8/F9/H1/E5	0.989
nF3C2#39	8/F9/H1/D5	0.975
nF3C2#40	9/B11/B2/A10	2.000
nF3C2#41	9/B11/B2/A11	2.011
nF3C2#42	9/B11/B2/A12	1.904
nF3C2#43	9/G7/E4/F1	2.600
nF3C2#44	9/G7/E4/F2	2.005
nF3C2#45	9/G7/E4/F3	1.142
nF3C2#46	9/G7/E3/D4	2.601
nF3C2#47	9/G7/E3/D5	2.140
nF3C2#48	9/G7/E3/E6	2.140
nF3C2#49	10/B3/B5/C7	0.750
nF3C2#50	10/B3/B5/A8	0.704
nF3C2#51	10/B3/B5/C8	0.878
nF3C2#52	10/C8/C5/B1	1.930
nF3C2#53	10/C8/C5/B2	1.290
nF3C2#54	10/C8/C5/A3	2.002

Hybridoma No.	Well Code	OD 450 nm
nF3C2#51	10/B3/B5/C8	0.878
nF3C2#52	10/C8/C5/B1	1.930
nF3C2#53	10/C8/C5/B2	1.290
nF3C2#54	10/C8/C5/A3	2.002
nF3C2#55	10/E12/D6/F5	1.270
nF3C2#56	10/E12/D6/G5	0.900
nF3C2#57	10/E12/D6/F6	1.200
nF3C2#58	10/E12/D4/E7	1.500
nF3C2#59	10/E12/D4/F8	0.700
nF3C2#60	10/E12/D4/D9	1.000

Supplement Table 4 Follow up test using in-house IGRA in 5 elephant samples. The PBMC responses was shown as measured concentration of eIFNγ by IgG sandwich ELISA.

Sample No.	eIFNγ concentration (ng/ml)					Determined TB status
	Stimulating antigens					
	ConA	PPDB	ESAT-6 + CFP10	ESAT 6	CFP10	
1	0.823	N/A	ND	N/A	N/A	Negative
	0.630	0.646	ND	ND	ND	MTBC Suspected
2	1.065	N/A	0.238	N/A	N/A	MTBC Positive
	0.431	0.692	0.282	ND	ND	MTBC Positive
3	1.008	N/A	0.228	N/A	N/A	MTBC Positive
	0.533	0.591	0.451	0.254	0.184	MTBC Positive
4	0.483	N/A	ND	N/A	N/A	Negative
	0.203	0.291	ND	ND	ND	MTBC Suspected
5	1.293	N/A	0.863	N/A	N/A	MTBC Positive
	0.494	IND	IND	0.310	IND	MTBC Positive

* N/A = Not available, ND = Not detected, IND = Indeterminate,
MTBC = Mycobacterium tuberculosis complex

Supplement Table 5 Summary of PBMC response to stimulating antigens and in in-house IGRA. The PBMC responses was shown as measured concentration of eIFNγ by IgG sandwich ELISA.

Sample No.	eIFNγ concentration							Determined TB status
	Stimulating antigens							
	ConA	PPDB	PPDA	ESAT-6 + CFP10	ESAT6	CFP10	Rv3020c	
6	0.448	0.343	N/A	ND	ND	ND	N/A	MTBC Suspected
7	0.899	1.197	N/A	0.504	0.789	ND	N/A	MTBC Positive
8	0.637	IND	N/A	ND	ND	ND	N/A	Indeterminate
9	0.978	0.227	N/A	ND	ND	ND	N/A	MTBC Suspected
10	0.642	ND	N/A	ND	ND	ND	N/A	Negative
11	0.366	0.333	N/A	0.324	ND	ND	N/A	MTBC Positive
12	0.640	0.291	N/A	ND	ND	ND	N/A	MTBC Suspected
13	0.393	ND	N/A	ND	ND	ND	N/A	Negative
14	0.257	ND	N/A	ND	ND	ND	N/A	Negative
15	0.479	0.475	N/A	ND	ND	ND	N/A	MTBC Suspected
16	0.492	0.505	N/A	0.445	0.224	ND	N/A	MTBC Positive
17	0.233	0.310	N/A	ND	ND	ND	N/A	MTBC Suspected
18	0.392	0.256	N/A	0.238	ND	ND	N/A	MTBC Positive
19	0.634	0.644	N/A	ND	ND	ND	ND	MTBC Suspected
20	0.701	0.367	N/A	IND	ND	ND	0.380	MTBC Positive

* N/A = Not available, ND = Not detected, IND = Indeterminate,

MTBC = Mycobacterium tuberculosis complex, NTM = Non-tuberculous mycobacterium

Sample No.	eIFNγ concentration							Determined TB status
	Stimulating antigens							
	ConA	PPDB	PPDA	ESAT-6 + CFP10	ESAT6	CFP10	Rv3020c	
21	0.344	0.523	N/A	0.447	0.417	ND	ND	MTBC Positive
22	0.317	0.531	N/A	0.313	IND	ND	0.440	MTBC Positive
23	0.538	0.499	N/A	ND	ND	ND	0.464	MTBC Positive
24	0.436	0.673	N/A	0.643	0.661	0.573	0.628	MTBC Positive
25	0.411	0.452	N/A	0.324	0.330	ND	IND	MTBC Positive
26	0.380	0.519	N/A	0.515	0.506	0.535	0.402	MTBC Positive
27	0.361	0.518	N/A	0.467	0.502	IND	ND	MTBC Positive
28	0.452	0.559	N/A	0.533	0.467	0.525	0.532	MTBC Positive
29	0.330	0.672	N/A	IND	IND	ND	ND	MTBC Suspected
30	0.522	0.662	N/A	0.686	0.519	IND	0.644	MTBC Positive
31	0.968	IND	N/A	0.617	0.453	IND	IND	MTBC Positive
32	0.639	1.191	N/A	ND	ND	ND	ND	MTBC Suspected
33	0.981	1.043	N/A	ND	ND	ND	ND	MTBC Suspected
34	1.724	1.635	N/A	0.905	ND	ND	ND	MTBC Positive
35	0.925	1.191	N/A	1.037	1.024	ND	ND	MTBC Positive

* N/A = Not available, ND = Not detected, IND = Indeterminate,

MTBC = Mycobacterium tuberculosis complex, NTM = Non-tuberculous mycobacterium

Sample No.	eIFNγ concentration							Determined TB status
	Stimulating antigens							
	ConA	PPDB	PPDA	ESAT-6 + CFP10	ESAT6	CFP10	Rv3020c	
36	1.153	1.364	N/A	1.222	IND	ND	ND	MTBC Positive
37	0.913	ND	N/A	ND	ND	ND	ND	Negative
38	0.847	ND	N/A	ND	ND	ND	ND	Negative
39	0.894	IND	IND	ND	ND	ND	0.490	MTBC Positive
40	2.232	ND	ND	ND	ND	ND	ND	Negative
41	2.616	0.990	0.655	IND	ND	IND	IND	NTM positive
42	4.351	IND	1.378	ND	ND	ND	IND	NTM positive
43	4.506	ND	ND	ND	ND	ND	ND	Negative
44	1.678	ND	ND	ND	ND	ND	ND	Negative
45	0.768	ND	ND	ND	ND	ND	ND	Negative
46	0.711	ND	ND	ND	ND	ND	ND	Negative
47	0.315	ND	ND	ND	ND	ND	ND	Negative
48	0.543	ND	ND	ND	ND	ND	ND	Negative
49	0.376	0.181	0.226	ND	IND	ND	ND	NTM positive
50	0.476	ND	0.345	ND	ND	ND	IND	NTM positive
51	0.217	ND	0.309	ND	ND	ND	ND	NTM positive

* N/A = Not available, ND = Not detected, IND = Indeterminate,

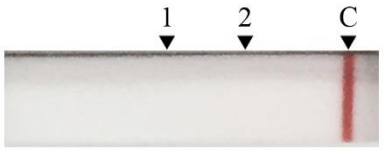
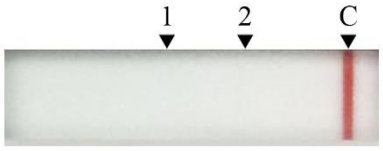
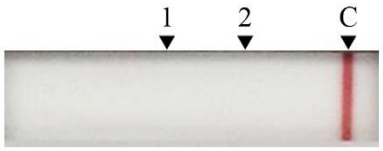
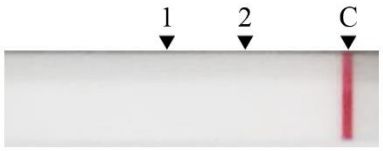
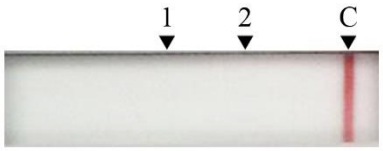
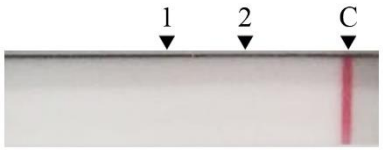
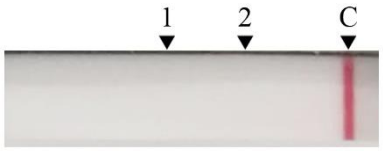
MTBC = Mycobacterium tuberculosis complex, NTM = Non-tuberculous mycobacterium

Sample No.	eIFNγ concentration							Determined TB status
	Stimulating antigens							
	ConA	PPDB	PPDA	ESAT-6 + CFP10	ESAT6	CFP10	Rv3020c	
52	0.383	0.218	ND	0.215	ND	ND	ND	MTBC Positive
53	0.221	ND	ND	ND	ND	ND	ND	Negative
54	0.225	ND	ND	ND	ND	ND	ND	Negative
55	0.518	ND	ND	ND	ND	ND	ND	Negative
56	0.762	0.708	1.227	ND	ND	ND	ND	NTM Positive
57	0.419	ND	ND	ND	ND	ND	0.537	MTBC Positive
58	0.661	0.331	0.299	ND	ND	ND	ND	NTM Positive
59	0.473	0.282	ND	IND	ND	ND	ND	MTBC Suspected
60	0.407	ND	ND	ND	ND	ND	ND	Negative
61	0.318	ND	IND	ND	ND	ND	ND	Negative
62	0.524	ND	ND	ND	ND	ND	ND	Negative
63	0.455	ND	ND	ND	ND	ND	ND	Negative
64	1.083	0.609	0.204	0.815	0.228	0.384	0.359	MTBC Positive
65	0.629	ND	ND	ND	ND	ND	ND	Negative
66	0.771	2.485	0.442	1.303	0.762	1.303	ND	MTBC Positive

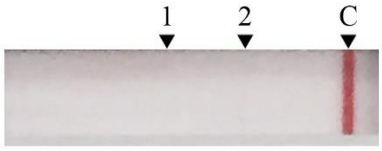
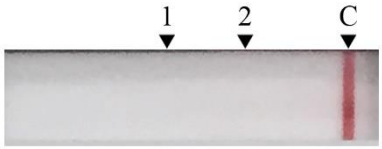
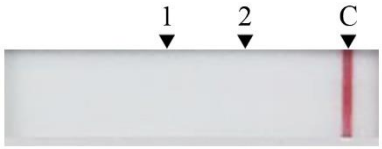
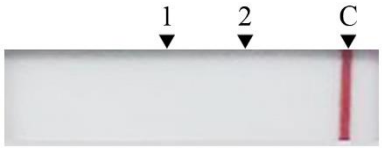
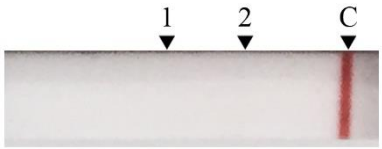
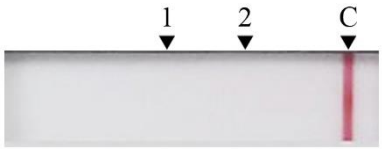
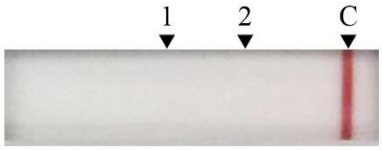
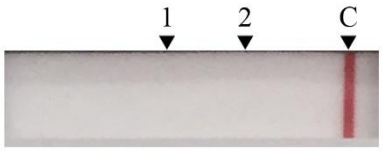
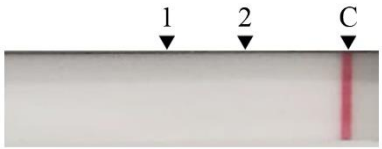
* N/A = Not available, ND = Not detected, IND = Indeterminate,

MTBC = Mycobacterium tuberculosis complex, NTM = Non-tuberculous mycobacterium

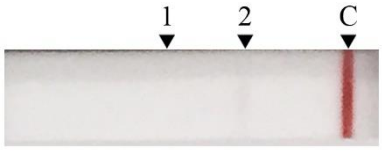
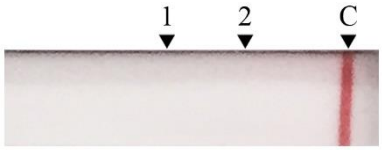
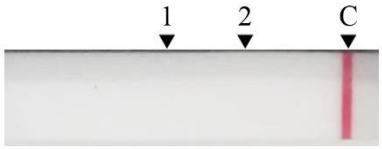
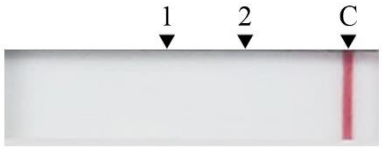
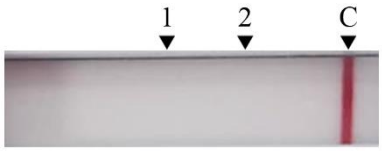
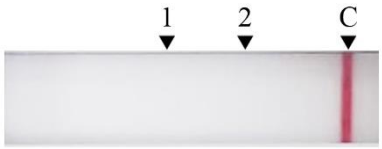
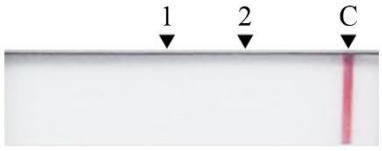
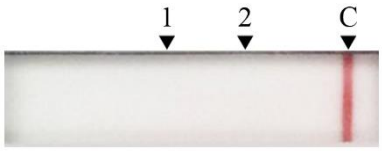
Supplement Table 6 Antibody detection by DPP VetTB assay in 32 samples.
Test band No.1 was coated with mycobacterial antigen (MPB83). Test band No.2 was coated with specific MTBC antigen (ESAT-6/CFP10). Test band C was the control of the test kit.

Sample No.	Results	TB status
1		Non-reactive
2		Non-reactive
3		Non-reactive
4		Non-reactive
5		Non-reactive
7		Non-reactive
11		Non-reactive

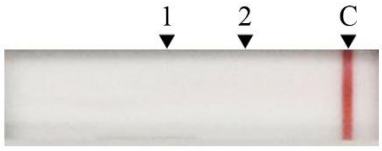
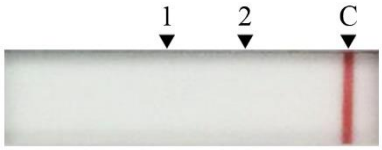
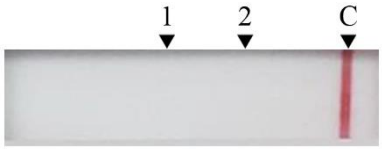
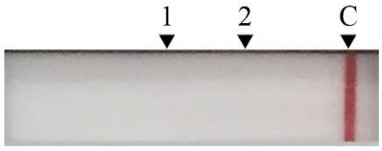
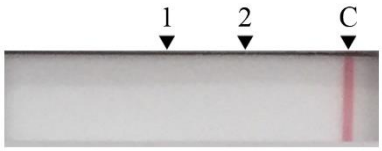
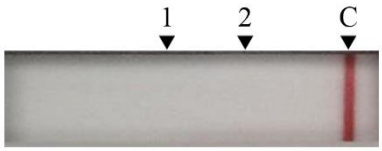
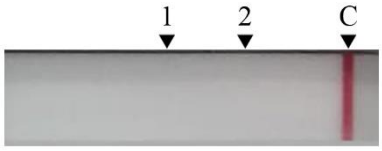
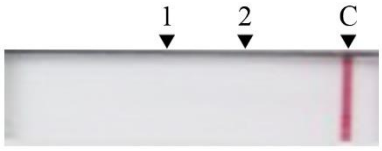
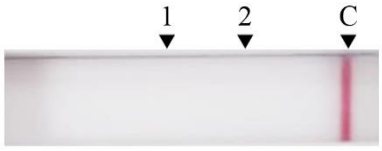
* Pink color on test line 2 has faint color, which can be read only with the eyes.

Sample No.	Results	TB status
16		Reactive for TB* (ESAT-6/CFP10 test band)
18		Non-reactive
20		Non-reactive
21		Non-reactive
22		Reactive for TB (ESAT-6/CFP10 test band)
23		Non-reactive
24		Non-reactive
25		Non-reactive
26		Non-reactive

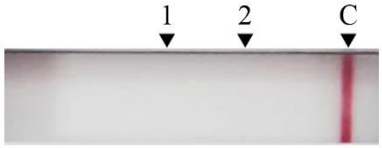
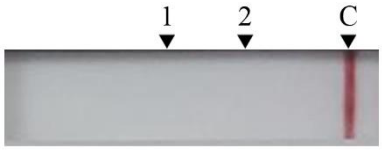
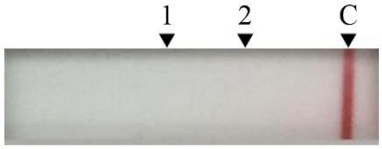
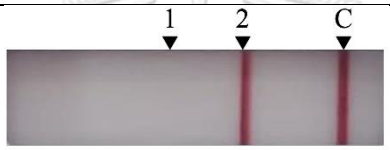
* Pink color on test line 2 has faint color, which can be read only with the eyes.

Sample No.	Results	TB status
27		Reactive for TB* (ESAT-6/CFP10 test band)
28		Non-reactive
30		Non-reactive
31		Non-reactive
32		Non-reactive
33		Non-reactive
34		Non-reactive
35		Non-reactive

* Pink color on test line 2 has faint color, which can be read only with the eyes.

Sample No.	Results	TB status
36		Non-reactive
39		Non-reactive
40		Non-reactive
41		Non-reactive
43		Non-reactive
52		Non-reactive
55		Non-reactive
56		Non-reactive
57		Non-reactive

* Pink color on test line 2 has faint color, which can be read only with the eyes.

Sample No.	Results	TB status
59		Reactive for TB* (ESAT-6/CFP10 test band)
61		Non-reactive
63		Non-reactive
66		Reactive for TB (ESAT-6/CFP10 test band)

* Pink color on test line 2 has faint color, which can be read only with the eyes.