PRODUCTION OF HUMAN MONOCLONAL ANTIBODIES AGAINST RABIES VIRUS USING PHAGE DISPLAY TECHNOLOGY

Natcha Pruksametanan

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2013

การผลิตโมโนโคลนอลแอนติบอดีของมนุษย์ที่จำเพาะต่อไวรัสพิษสุนัขบ้า ด้วยเทคโนโลยีเฟจ

นางสาวณัชชา พฤกษาเมธานันท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2556

PRODUCTION OF HUMAN MONOCLONAL ANTIBODIES AGAINST RABIES VIRUS USING PHAGE DISPLAY TECHNOLOGY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

_____

(Prof. Dr. Neung Teaumroong)

Chairperson

_____

(Assoc. Prof. Dr. Montarop Yamabhai)

Member (Thesis Advisor)

_____

(Dr. Pakamatz Khawplod)

Member

_____

(Dr. Nanthanit Jaruseranee)

Member

_____

(Prof. Dr. Sukit Limpijumnong)

Vice Rector for Academic Affairs and Innovation

_____

(Asst. Prof. Dr. Suwayd Ningsanond)

Dean of Institute of Agricultural Technology

นางสาวณัชชา พฤกษาเมธานันท์ : การผลิตโมโนโคลนอลแอนติบอดีของมนุษย์ที่จำเพาะต่อไวรัสพิษสุนัขบ้า ด้วยเทคโนโลยีเฟจ (PRODUCTION OF HUMAN MONOCLONAL ANTIBODIES AGAINST RABIES VIRUS USING PHAGE DISPLAY TECHNOLOGY) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.มณฑารพ ยมาภัย, 159 หน้า.

โมโนโคลนอลแอนติบอดีของมนุษย์ที่จำเพาะต่อเชื้อพิษสุนัขบ้า ถูกคัดเลือกจากคลังแอนติบอดี (scFv) มนุษย์ที่ไม่ถูกกระตุ้นด้วยไวรัสก่อโรคพิษสุนัขบ้า (คลัง YAMO-I) และคลังที่ถูกกระตุ้นด้วยไวรัส (คลัง Yamo-Rb) ด้วยเทคโนโลยีเฟจ โดยทำการคัดเลือก (ไบโอแพนนิ่ง) จำนวน 1-5 รอบ โดยใช้เชื้อไวรัสก่อโรคพิษสุนัขบ้าที่หมดฤทธิ์แล้ว (inactivated virus) 2 ชนิด คือ PCEC และ PVRV และไกลโคโปรตีนจากผิวของไวรัสก่อโรคพิษสุนัขบ้า (RVG) เป็นเป้าหมายในการคัดเลือก ซึ่งสามารถคัดเลือกโคลนที่สามารถจับจำเพาะต่อเชื้อพิษสุนัขบ้าได้จำนวน 16 โคลน ได้แก่ IRA7c (IVB4cv), IIIRC2c, IYC11c, IYC12c, IYD1c, IYF5c, IIRD5v, IIYB5v, IRC3c, IIYG4v, IIYE5v, IIYG8v, IIYD4v, R1, R4 และ Y6 ผลจากการทดสอบโดยใช้วิธีการอีไลซ่าพบว่า โคลนเหล่านี้มีความสามารถในการจับจำเพาะได้ดีต่อเชื้อหรือไกลโคโปรตีนที่ใช้ในการคัดเลือก อย่างไรก็ตามบางโคลนสามารถจับจำเพาะกับทั้งเชื้อและไกลโคโปรตีน โคลนที่ได้จากการคัดเลือกโดยใช้เฉพาะไกลโคโปรตีนทั้งหมดเป็นชิ้นส่วนของแอนติบอดีแบบเส้นเดี่ยว (scFv) และไม่มีความสามารถที่จะจับเชื้อไวรัส (PCEC และ PVRV) ได้ ลำดับเบสของดีเอนเอได้ถูกวิเคราะห์โดยวิเคราะห์ลำดับเบสอัตโนมัติ จากนั้นได้ทำการศึกษาลำดับกรดอะมิโนของแอนติบอดี รวมทั้งได้ทำนายโครงสร้างสามมิติของแอนติบอดีโคลน IYF5c, IRA7c และ IIIRC2c ด้วย นอกจากนั้นแล้วยังได้นำแอนติบอดีบางตัวไปสร้างให้เป็นแอนติบอดีเส้นเดี่ยวที่เชื่อมต่อกับเอนไซม์อาลคาไลฟอสฟาเตส (scFv-AP) เพื่อให้สะดวกในการตรวจจับด้วยวิธีอีไลซ่า อีกทั้งได้ทำการนำไปทดสอบความสามารถในการยับยั้งการก่อโรคพิษสุนัขบ้า ซึ่งพบว่า โคลน IRA7c และ IIIRC2c มีความสามารถในการยับยั้งการก่อโรคพิษสุนัขบ้าได้ หลังจากนั้นได้นำแอนติบอดีเส้นเดี่ยวบางโคลนมาผลิตเป็นแอนติบอดีเส้นเดี่ยวที่ไม่ติดอยู่บนผิวเฟจ โดยให้ผลิตจากแบคทีเรียสายพันธุ์ อีโคไล เอช บี 2151 หรือ โคลนเข้าเวคเตอร์ pET27b แล้วผลิตแอนติบอดีเส้นเดี่ยวจากแบคทีเรียสายพันธุ์บีแอล 21 ดีอี 3 จากนั้นนำแอนติบอดีเหล่านี้ไปทดสอบความสามารถในการจับเชื้อไวรัสก่อโรพิษสุนัขบ้าโดยใช้วิธีการอีไลซ่า สุดท้ายแอนติบอดีบริสุทธิ์ IYF5c, IRA7c และ IIIRC2c ถูกนำมาทดสอบความสามารถในการยับยั้งการก่อโรคพิษสุนัขบ้า ผลจากการทดสอบพบว่า IYF5c ซึ่งแสดงความสามารถในการจับได้เป็นอย่างดีกับเชื้อไวรัสชนิดตาย (PCEC) ไม่มีความสามารถยับยั้งการก่อโรคได้เลย มีเพียง IRA7c และ IIIRC2c ซึ่งได้รับการคัดเลือกมาจากคลังแอนติบอดีมนุษย์ที่ได้รับ

การกระตุ้นด้วยเชื้อพิษสุนัขบ้าเท่านั้น ที่มีความสามารถยับยั้งการก่อโรคพิษสุนัขบ้าได้ โดยมีความสามารถยับยั้งการก่อโรคพิษสุนัขบ้า 4.54 IU/mg และ 0.20 IU/mg ตามลำดับ ดังนั้นจึงสามารถนำแอนติบอดีทั้งสองไปพัฒนาเพื่อประยุกต์ใช้ในการรักษาและวินิจฉัยโรคพิษสุนัขบ้าได้ต่อไปในอนาคต

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

NATCHA PRUKSAMETANAN : PRODUCTION OF HUMAN MONOCLONAL ANTIBODIES AGAINST RABIES VIRUS USING PHAGE DISPLAY TECHNOLOGY. THESIS ADVISOR : ASSOC. PROF. MONTAROP YAMABHAI, Ph.D., 159 PP.

PHAGE DISPLAY/RABIES VIRUS/SINGLE-CHAIN FRAGMENT (scFv)/ HUMAN MONOCLONAL ANTIBODY

Human monoclonal antibodies against the rabies virus were selected from non-immunized human scFv library (YAMO-I library) and immunized library (Yamo-Rb library) by using phage display technology. The affinity selection (Bio-panning) was performed for 1-5 rounds by using two types of inactivated rabies vaccines as targets. These were purified vero cell rabies vaccine (PVRV) and purified chick embryo cell vaccine (PCEC). In addition, a viral coat protein (RVG) was also used as a target for biopanning. A total of 16 positive clones from various methods of biopanning that can bind specifically to rabies virus, i.e.; IRA7c (IVB4cv), IIIRC2c, IRC3c, IYC11c, IYC12c, IYD1c, IYF5c, IIRD5v, IIYB5v, IIYG4v, IIYE5v, IIYG8v, IIYD4v, R1, R4 and Y6 were isolated and their genes were sequenced. The ELISA results showed that the positive clones always bind strongly to the targets that were used for the biopanning. However, some clones can cross-react to the related virus. All of the clones isolated using viral glycoprotein contained only partial scFv fragments and none of them could bind to the whole inactivated virus (PCEC and PVRV). The amino acid sequence analysis of the selected clones was performed using information obtained from automated DNA sequencing. The 3D structure of clones IYF5c, IRA7c

and IIIRC2c were predicted. In addition, selected scFv clones were engineered to create scFv- alkaline phosphatase (scFv-AP) fusions and used as reagents for one-step detection in the ELISA format. *In vitro* neutralization assay using different phage clones showed that clones IRA7c and IIIRC2c could inhibit viral infection. The selected scFv antibody fragments were expressed as soluble forms using *E. coli* non-suppressor strain, HB2151, or cloned into pET27b vectors and expressed in *E. coli* BL21 (DE3). Then selected soluble antibody fragments were tested for their ability to bind the rabies virus by using an enzyme-linked immunosorbent assay (ELISA). Finally, purified soluble scFv antibody fragments of clones IYF5c, IRA7c and IIIRC2c were tested for neutralization activity. While clone IYF5c interacted specifically and strongly to the PCEC target, it did not neutralize the virus. Only clones IRA7c and IIIRC2c, which were derived from immunized library showed neutralization of the rabies virus *in vitro* at 4.54 IU/mg and 0.20 IU/mg, respectively. These isolated recombinant scFv antibodies could be further developed to be used for diagnostic and therapeutic purposes in the future.

School of Biotechnology
Academic Year 2013

Student's Signature_____
Advisor's Signature_____
Co-advisor's Signature _____

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to Assoc. Prof. Dr. Montarop Yamabhai, my advisor. I appreciate her encouragement, forgiveness, patience and support during this project.

I am grateful to my co-advisor, Dr. Pakamaz Khawplod for her advice on rabies and her valuable suggestion.

I would like to express my sincere thanks to Dr. Nanthanit Jaruseranee for her sharing, not only her scientific and technical knowledge, but also spirited prodding has kept me go through many years of work.

This thesis would not have been possible unless kind help from people in Queen Saowabha Memorial Institue, Thai Red-Cross Society, Thailand.

I would like to thank all Biotechnology lecturers. Assoc. Prof. Dr. Neung Teaumroong, Assoc. Prof. Dr. Mariena Ketudat-Cairns, Asst. Prof. Dr. Chokchai Wanapu, and Dr. Kaemwich Jantama, Dr. Panlada Tittabutr. Dr. Parinya Noisa, etc. for their knowledge and patient teaching me.

I would like to thank Prof. Dr. Toshiyuki Tanaka and Dr. Nakao Nomura for his support in everything while I was in Japan.

I do appreciated my truly friend; Fairy, Otto, Air, Goft, Kae, Dear, Lemon and my sister who never leave me when I was down.

I would like to express my special thanks to Kirana Yoohat and Suchintana chumsang for their concern and soul support.

I would like to thank all of my friends and colleagues in Biotechnology school especially MY lab members, Suranaree University of technology for their kind help, inspiration and support in many things.

I would like to express my deepest appreciated for Asst. Prof. Dr. Ian Spence and Dr. Shaun Galbraith, for their kind help in checking my writhing.

Finally, my special thanks are given to my beloved family for love and support without limitation.

Natcha Pruksametanan

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

AP	=	Alkaline phosphatase
ABTS	=	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
BSA	=	Albumin from bovine serum
bp	=	base pair
DNA	=	deoxyribonucleic acid
ELISA	=	Enzyme linked immunosorbance assay
et al.	=	Et alia (and other)
g	=	gram
h	=	hour
HRP	=	Horse radish peroxidase
IMAC	=	Immobilized metal affinity chromatography
IPTG	=	Isopropyl -D-1-thiogalactopyranoside
IU	=	International unit
Kda	=	Kilo daltal
l	=	litre
MPBS	=	Skim Milk in PBS
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar

LIST OF ABBREVIATIONS (Continued)

ng	=	nanogram
PCEC	=	purified chick embryo cell rabies vaccine
PVRV	=	purified vero cell rabies vaccine
PBS	=	Phosphate buffer saline
PBST	=	Phosphate buffer saline supplemented with Tween 20
rpm	=	revolution per minute
scFv	=	Single-chain variable fragment
v/v	=	volume per volume
w/v	=	weight per volume
x *g*	=	times gravity
YAMO-I	=	YAMO-I library
Yamo-Rb	=	Yamo-Rabies library
°C	=	degree Celsius
µg	=	microgram
µl	=	microlitre

CHAPTER I

INTRODUCTION

1.1 Significance of this study

Rabies is a fatal zoonotic central nervous system (CNS) disease that is transmitted by both wild and domestic animals. Globally, it is estimated that at least 55,000 people die of rabies each year. This disease is an endemic disease, which usually occurs in Africa and Asia (Zhao et al., 2008; Matsumoto et al., 2010). In Asia, over 30,000 people die due to rabies and more than 3 million people in developing countries in Asia are exposed to rabid dog every year (Yousaf et al., 2012). Nowadays, pre-exposure and post-exposure prophylaxes are used in human for prevention and treatment of rabies. According to the World Health Organization (WHO), people who expose to rabies virus (category III) are treated by post-exposure prophylaxes which consisting of thorough wound cleaning and immediate administration of rabies immune globulin (RIG) together with a full course of rabies vaccination. There are about 10 millions people who receive post-exposure vaccination annually (Zhao et al., 2008; Houimel and Dellagi, 2009a). Currently available RIG for clinical use are Equine Rabies Immunoglobulin (ERIG) and Human Rabies Immunoglobulin (HRIG). These plasma-derived, polyclonal products are obtained from rabies vaccinated horses or human, and can only be produced in limited amounts (Bakker et al., 2005; Matsumoto et al., 2010). Moreover, HRIC is too costly, not easily available; suffer from potential disadvantages, such as limited capacity,

batch-to-batch variation and possible contamination with blood borne adventitious agents. ERIG also has drawbacks of animal origin that carries a risk of occasional adverse reaction, including anaphylaxis (Kramer et al., 2005; Kruif et al., 2007; Houimel and Dellagi, 2009a; Matsumoto et al., 2010). Therefore, utilization of Phage display technology for the production of human antibody specific to rabies virus is attractive and suitable alternative strategy for the prevention, treatment and diagnostic of rabies.

1.2 Research objective

1.2.1 To select phage displayed-scFv antibodies against Rabies virus by biopanning from both naïve and immunized human scFv antibody library.

1.2.2 To produce scFv antibody that can neutralize Rabies virus.

1.2.3 To develop a specific scFv antibody for the detection of Rabies virus.

1.3 Scope and limitation of the study

scFv antibody fragments from human will be investigated only by various *in vitro* analyses.

CHAPTER II

LITERATURE REVIEW

2.1 Rabies

2.1.1 Rabies and rabies virus

Rabies is a fatal neurological infectious disease caused by infection of rabies virus (RABV) (Duana et al., 2012 and Kaku et al., 2011). This disease has been threatening the lives of mankind for more than 4,000 years (Liu et al., 2011; Schnell et al., 2010). The virus is transmitted among mammals through close contact with saliva from infected animals (Okumura and Harty, 2011). This virus belongs to the genus *Lyssavirus* in the *Rhabdoviridae* family (Ando et al., 2005; Schnell et al., 2010). The virions or virus particles have a bullet-shaped structure (75 nm diameter and 100-300 nm length), a single-stranded and negative-sense RNA genome of about 12 kb nucleotide (Liu et al., 2011; Houimel and Dellagi., 2009b). Rabies virus encodes five subgenomic mRNAs that encodes five structural proteins. The viral proteins include (i) the nucleoprotein (N), which encapsidates the genomic and antigenomic RNA to form the ribonucleoprotein (RNP) complex ; (ii) the phospoprotein (P), which is the noncatalytic subunit of the RNA polymerase complex ; (iii) the viral polymerase protein (L), which transcribes and replicates the RNA genome; (iv) the transmembrane glycoprotein (G), which is the surface spike protein involved in attachment to host cell; and (v) the matrix protein (M), which is the major structural protein involved in virion assembly and egress (Okumura and Harty, 2011;

Liu et al.,2011). All rhabdoviruses have two major structural components i.e., a helical riboneucleoprotein core (RNP) and a surrounding envelope. The capsid is surrounded by the host cell-derived membrane that interacts with two viral proteins, the matrix protein and glycoprotein. The N, P and L together with the genomic RNA form the riboneucleoprotein complex (RNP) (Ando et al., 2005; Okumura and Harty, 2011; Yousaf et al., 2012). Among these, the rabies virus glycoprotein (G) is the only one that is exposed on the viral particle surface and can mediate viral entry into the host cell (Duana et al., 2012). The G protein is a key protein for both virus infectivity and eliciting protective immunity as an antigen. Nevertheless, the nucleoprotein (N) is also a significant rabies virus antigen (Yang et al., 2013).

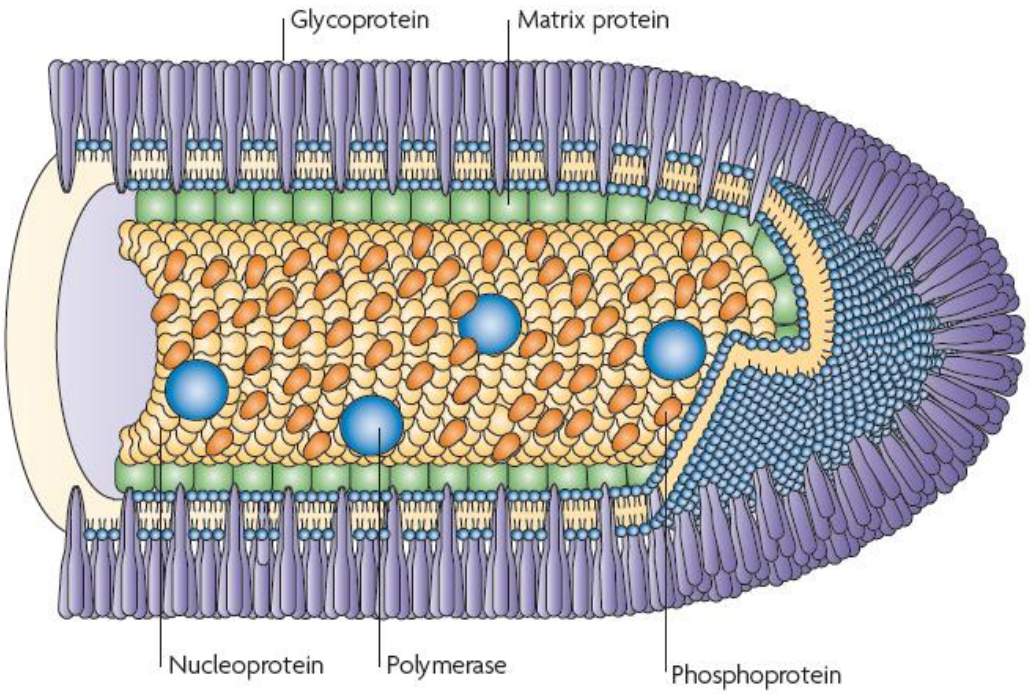


Figure 1. The rabies virus viron (Schnell et al., 2010).

RABV G protein (RVG), a typical type I membrane glycoprotein, forms a homo-trimer structure projecting from the surface of the virion. G protein not only has a pivotal role in virion attachment to the host cell, but is also a crucial component of immune resistance against RABV. As a result, eight antigenic sites (I-VI, minor "a", and GI) have been identified. Two of these eight antigenic sites, antigenic site II and III, have been defined as major conformational sites. Rabies virus glycoprotein (RVG) antigenic site I (226-KLCGVL-231) (Sun et al, 2012), II (34-GCTNLSGFS-42) (198-KRA-200) (Masumoto et al., 2010; Aavula et al, 2011) or III (330-KSVRTWNEI-338) (Houimel et al, 2009).

Rabies caused by rabies virus (RABV) genotype 1 is one of the most common fatal infections worldwide. However, the existence of lyssaviruses that are closely related to rabies virus and that can also causes clinical diseases has been known for several decades. There are 7 major species within the genus: classical rabies virus (RABV), Lagos bat virus (LBV), Mokola bat virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus type 1 (EBLV-1), European bat lyssavirus type 2 (EBLV-2), and Australian bat lyssavirus (ABLV). Currently, more than 7 strains have been isolated worldwide as shown in Table 1 (Both et al., 2012).

Table 1. Global distribution of lyssaviruses (Both et al., 2012).

	Phylogroup	Distribution	Host
Rabies virus	1	Worldwide (although a number of countries are rabies free)	Dogs, cats, wild carnivores, insectivorous bats, haematophagous bats, livestock, man
Lagos bat virus	2	Central African Republic, Ethiopia, Nigeria, Senegal, South Africa, Zimbabwe	Dogs, cats, mongoose, frugivorous bats
Mokola virus	2	Central African Republic, Cameroon, Ethiopia, Nigeria, South Africa, Zimbabwe	Dogs, cats, shrews, rodents, man
Duvenhage virus	1	Kenya, South Africa, Zimbabwe	Insectivorous bats, man
European bat lyssavirus-1	1	Denmark, France, Germany, Netherlands, Poland, Russia, Slovakia, Spain	Insectivorous bats, man, sheep, stone martens, cats
European bat lyssavirus-2	1	Germany, Netherlands, Switzerland, UK	Insectivorous bats, man
Australian bat lyssavirus	1	Australia	Insectivorous bats, frugivorous bats, man
Aravan virus	1	Southern Kyrgyzstan	Insectivorous bats (lesser mouse-eared bat)
Khujand virus	1	Northern Tajikistan	Insectivorous bats (whiskered bat)
Irkut virus	1	Eastern Siberia	Insectivorous bats (greater tube-nosed bat)
West Caucasian bat virus	3†	Caucasus mountains	Insectivorous bats (common bent-winged bat)
Shimoni bat lyssavirus	2	Kenya	Insectivorous bats (Commerson's leaf-nosed bat)
Bokeloh bat lyssavirus*	1	Germany	Insectivorous bats (Natterer's bat)
Ikoma lyssavirus*	3†	Tanzania	African civet

*Species are unclassified. †Tentative classification only

2.1.2 Pathogenesis and symptoms of rabies

In 1802, George Zinke showed that rabies could be transmitted from saliva of a rabid dog to a healthy dog and cause disease (Schnell et al., 2010). All mammals, but mainly carnivores and bats, are susceptible and can transmit rabies

virus (Both et al., 2012), though the companion animal, the dog, is the major vector in most developing countries. Infection is usually acquired from transcutaneous or mucosal exposure to virus-laden saliva of a rabid animal (Dodet et al., 2008; Ullas et al., 2012; Wang et al., 2012). A person or animal can become a victim of rabies in many ways including bites, non-bite exposure and human to human transmission. Rabies is an acute infection of the central nervous system (CNS) (Kaku et al., 2011; Yousaf et al., 2012). The clinical stages of rabies are: incubation, prodrome, acute neurological signs, and death. The incubation period or eclipse phase can vary from weeks to years, but lasts 1-2 months on average (Hemachudha et al., 2013). Clinical manifestations of rabies in humans have two forms: the furious (classical) form (80% of infections), and the numb (non-classical or paralytic) form (20% of infections). When the first prodromal symptoms such as fever, flu like symptoms and gastrointestinal disturbances appear, the virus is already widely disseminated throughout the CNS. The furious form of rabies is characterized by hydrophobia: terror and excitation with spasm of inspiratory muscles, larynx and pharynx precipitated by attempts to drink, and episodes of hallucinations and excitement are common (Both et al., 2012). Animals often present with extreme aggression and randomly attack objects, other animals or humans. These behavioral changes occur simultaneously with the shedding of large amounts of rabies virus in the saliva, which facilitates the spread of the virus to a new host. If the virus successfully infected an exposed individual and causes clinical disease, death is almost inevitable. The numb form of rabies is characterized by weakness and flaccid paralysis, which sometimes causes misdiagnosis at the onset of this clinical form of rabies (Bakker et al., 2008; Schnell et al., 2010; Both et al., 2012).

2.1.3 Rabies treatment

Rabies vaccines have evolved from the first crude nerve tissue vaccines developed by Louis Pasteur and his colleagues in 1885 (Smith et al., 2011; Schnell et al., 2010). Nowadays, the use of inactivated cell culture rabies vaccines, i.e. purified chick embryo cell rabies vaccine (PCEC), purified vero cell rabies vaccine (PVRV), purified duck embryo cell vaccine (PDEV) is increasing around the world (Briggs et al., 2000; Quiambao et al., 2005). In the early 1890s, Behring and Kitasato entertained the possibility of passive antibody therapy protecting against a bacterial toxin. After that, development of antibody therapies against disease for which no antimicrobial therapy was available led to a renewed interest in passive antibody therapy in the second half of the 20th century. Administration of virus-neutralizing antibodies represents a proven and effective method for the prevention or treatment of several infections, including rabies. In the 1930s, Shortt, Proca, and Hoyt provided evidence that crude anti-rabies serum increased rabies virus incubation period and contributed to survival. Subsequent studies showed that anti-rabies serum combined with vaccination was more efficient than vaccination or serum alone in the prevention of rabies in several animal models (Both et al., 2012; Kramer et al., 2005). Nowadays, the administration of rabies immune globulin (RIG) or antibody against rabies as soon as possible after exposure is essential to inhibit viral spread in the interval before sufficient immunity is developed in response to vaccination. The recommended treatment for individuals exposed to rabies virus is the combined administration of rabies vaccine and rabies immune globulin (RIG) (Dodet et al., 2008; Both et al., 2012; Bakker et al., 2008; Kruif et al., 2007). According to the categorization of exposure defined by WHO (Table 2) , the most severe case (category III) require

wound cleaning, rabies vaccination, and direct infiltration around the wound site with specific antibodies against rabies virus or rabies immunoglobulin (RIG) (Dodet et al., 2008; Bakker et al., 2008 ; Zhao et al., 2009). Currently, there are 2 types of RIG for rabies treatment; Human rabies immunoglobulin (HRIG) and equine rabies immunoglobulin (ERIG) (Bakker et al., 2008; Zhao et al., 2009). In Thailand, the Queen Saovabha Memorial Institute (QSMI) of The Thai Red Cross Society is the principal rabies diagnostic centre for the central region of Thailand and also manages most rabies post-exposure treatment of humans. For RIG administration, Human rabies immunoglobulin (HRIG) is administrated at 20 IU/kg of body weight while equine rabies immunoglobulin (ERIG) is administrated at 40 IU/kg of body weight (Mitmoonpitak et al., 1998; Smith et al., 2011)

Table 2. WHO post-exposure prophylaxis (PEP) guidelines based on the category of exposure to a rabid animal **(**Both et al., 2012).

	Exposure to rabid animal*	**Treatment**
Category I	Touching or felling; licked unbroken skin	None
Category II	Nibbled uncovered skin; minor scratches or abrasions without bleeding	Local treatment of the wound† and immediate vaccination
Category III	One or more transdermal bites or scratches; licked broken skin; contamination of mucous membrane with saliva from lick; any degree of exposure to potentially rabid bats	Local treatment of the wound†, immediate vaccination and administration of rabies immunoglobulin

*Exposure to a confirmed or suspected rabid animal or to an animal unavailable for testing. †Wound cleansing with soap, water, and a virucidal agent is a cheap and effective part of post-exposure prophylaxis and has been shown to substantially increase survival rates.

2.2 Making monoclonal antibody by phage display technology

2.2.1 Phage and phage display technology

Phage display technology is an interesting technology that was established and developed for more than twenty years for the selection of polypeptides with desired properties. This technology was established by George P. Smith in 1985 (Smith, 1985). Phage is a virus that infected bacteria (Figure 2). This virus is a filamentous bacteriophage. The M13 bacteriophage which belongs to Ff phage family are widely used as vehicle for phage display.The native phage particle is a thin, cylindrical shape, usually 900 nm long and 6-7 nm in diameter (Willats, 2002 ; Carmen and Jermutus, 2002). Ff phage (fl, fd, and M13) has a single standed DNA genome (ssDNA). Each genome encodes 11 genes, the products of which are listed in Table 2. The unique advantage of this technology is that both the gene and its protein are selected by the specific interaction with the interested target *in vitro* (Kay et al., 2001a; Kehoe et al., 2005; Rodi et al., 2002; Smothers et al., 2002). The protein may be a short peptide (Kay et al., 2001; Kay et al., 1996; Schumacher et al., 1996), or various sizes of proteins of approximately up to 500 amino acid long (Kehoe et al., 2005; Kay et al., 1996), including antibody fragments (Clackson et al., 1991; O'Brien et al., 2002). Therefore, there are many reports on the applications of this technology for the study of the interactions between many types of proteins, such as interaction between specific peptide and binding domains of protein (Yamabhai et al., 1998) or for selecting protein that can interact with interested protein from cDNA library (Rodi et al., 2002). Moreover, phage peptide can also be a source of enzyme's inhibitor and activator (Betra et al., 2002; Kay et al., 2001b), as well as drug leads (Hancock and

Sahl, 2006; Ladner et al., 2004; Latham, 1999; McCarron et al., 2005; Sato et al., 2006).

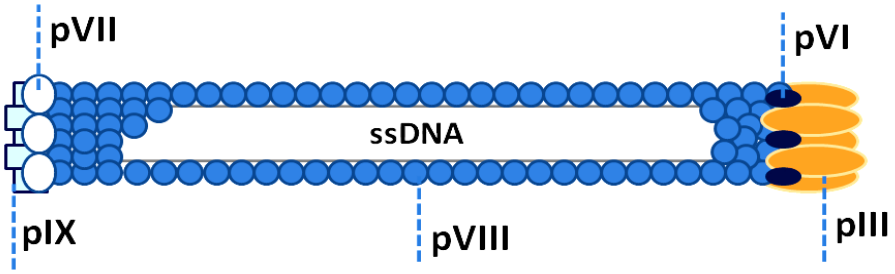


Figure 2. Schematic view of a filamentous phage particle. Most of viral capsid consists of about 2,700 molecules of the major coat protein (pVIII or g8p). The minor coat proteins pVII (g7p), pIX (g9p), pIII (g3p) and pVI (g6p) are present in approximately five copies each. On one end, the particle is capped by proteins pVII and pIX. The minor coat proteins pIII and pVI are on the other end of the particle.

Table 3. Genes and gene product of bacteriophage (Webster, 1996).

Gene	Function	Amino acid
I	Assembly	348
II	DNA replication	410
III	Minor capsid protein	406
IV	Assembly	405
V	Binding of ssDNA	87
VI	Minor capsid protein	112
VII	Minor capsid protein	33
VIII	Major capsid protein	50
IX	Minor capsid protein	32
X	DNA replication	111
XI	Assembly	108

2.2.2 Phagemid vector and phage display antibody library

The most commonly used antibody phage display systems for phage display library construction are based on phagemid vectors (Figure 3), which produce the fusion coat protein pIII (Type III) or pVIII (Type VIII). A phagemid is a plasmid that bears a phage-derived origin of replication, a selective marker, an intergenic (IG) region, a gene of a phage coat protein, restriction enzyme recognition sites, a promoter, and a DNA segment encoding a signal peptide. The IG region usually contains the packing sequence and replication origin of minus and plus strands. Additionally, a molecular tag can be included to facilitate screening of phagemid-based library (Carmen and Jermutus, 2002; Paschke, 2006; Qi et al., 2012).

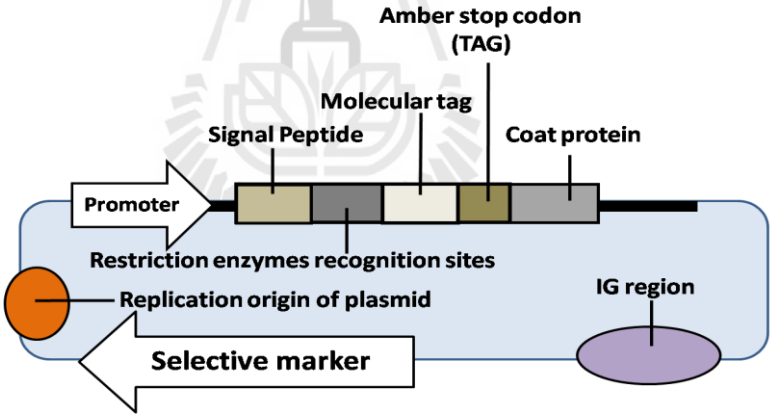


Figure 3. The scheme of phagemid vector.

Phagemids usually encode no or only one kind of coat proteins. Other structural and functional proteins necessary to accomplish the life cycle of the phagemid are provided by the helper phage as indicated in Table 4 (Schirrmann et al., 2011; Qi et al., 2012).

Table 4. Helper phage and their features (Paschke., 2006).

Helper phage	**Features**
Ex-phage pIII	carries amber stop codon
CT-Phage	N1–N2 deleted
Hyper-phage	pIII deletion, special packaging strain
R408d3	pIII deletion
M13Δ3.2	pIII deletion
Phaberge	pIII carries amber stop codon
KM13	protease site in pIII
M13 K07	replication-deficient
VCS M13	derivative of M13 K07

In order to express the antibody fragments on the surface of the filamentous bacteriophage as shown in Figure 4, phagemids must be converted to filamentous phage particles with the same morphology as Ff phage by co-infection (super-infecting) phagemid-carrying cells with the helper phages, such as R408, M13K07, KM13 and VCSM13 (Paschke., 2006; Qi et al., 2012; Ahmad et al., 2012). In this procedure, often called "phage rescue", the helper phage provides all the proteins and enzymes required for phagemid replication, ssDNA production and packaging, and also the structural proteins forming the phage coat (Paschke, 2006).

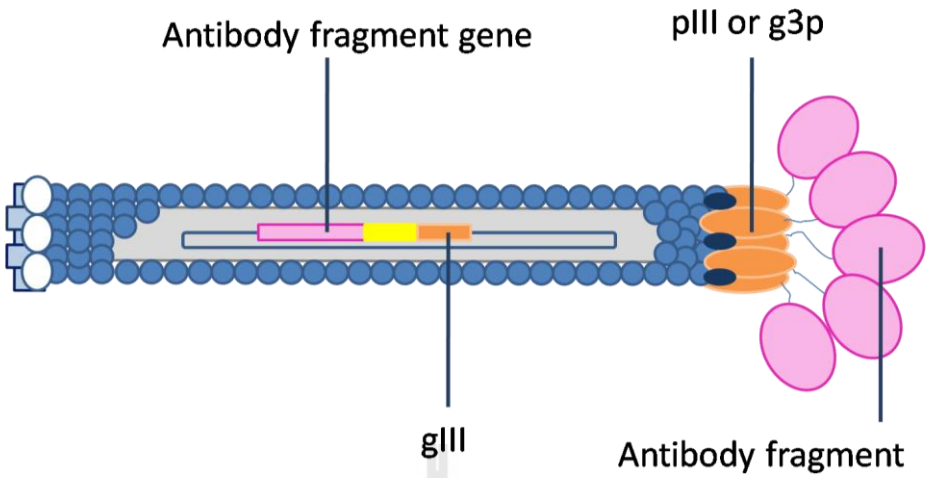


Figure 4. Phage displayed antibodies. The antibody fragments are fused to the minor coat protein pIII.

The antibody genes can be fused either to the pVIII gene or the pIII gene. The most commonly used antibody phage display systems are based on phagemid vectors encoding the antibody library (as Fv, scFv or Fab fragment libraries) fused to the minor coat protein pIII or its C-terminal (CT) domain. In a phage displaying system using type III phagemids harboring the phage gene III, there are three to five copies of fusion proteins in the progeny phages due to structural properties of the Ff phages. The fused antibody fragment coat protein pIII are located on the top of the phage, which is responsible for attaching the phage to its bacterial host during infection. The single-stranded DNA genome of the filamentous phage is coated with approximately 2,700 copies of the major coat protein (pVIII) and can be fused together with peptide to result in multivalent display of the fragment (Smith, 1985). However, due to the *E. coli* folding machinery, only antibody fragments (Figure 5) like single chain fragment variable (scFv), Fragment antigen binding

(Fabs), the variable heavy domain of camels (V_{HH}) or single domain antibodies human (sdAbs), which bind specifically without a corresponding light chain variable domain, are routinely use for antibody phage display by fusing with pIII (Schirrmann et al., 2011).

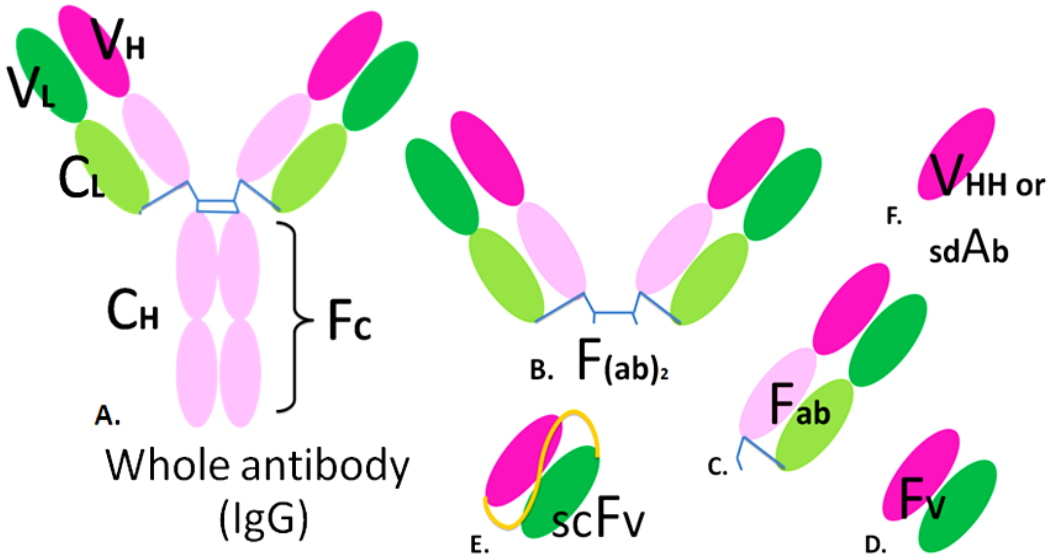


Figure 5. Antibody structure and derived fragments. (A) Immonoglobulin subtype G (IgG), ~150 kilodaltons (kD); (B) Two Fab molecule ($F(ab)_2$) , ~100 kD; (C) Fragment of antigen binding or Fab fragment, ~50 kD; (D) Fv fragment, ~25 kD; (E) Single-chain Fragment of variation or Fv with 15-17 amino acid long linker (scFv), ~25-30 kDa; (F) The single-domain antibody (sdAb) is avoid of light chains and can be derived from camelids V_{HH} antibody, ~15 kD;. V_H: variable heavy chain; V_L: variable light chain; C_H: constant heavy chain; C_L: constant light chain (Carmen and Jermutus, 2002)

Phagemid vectors can also be engineered for display and secretion of free antibody fragments from infected bacteria (Figure 6). By incorporating an amber

stop codon between the antibody fragment gene and pIII gene, the antibody fragments are fused to pIII and displayed on phage when the amber stop codon is suppressed and secreted when it is not. Thus, the growth of phage in suppressor bacteria represent phage displayed antibody fragments while the free soluble antibody fragment is produced when the phage is growth in non-suppressor bacteria (Winter et al., 1994).

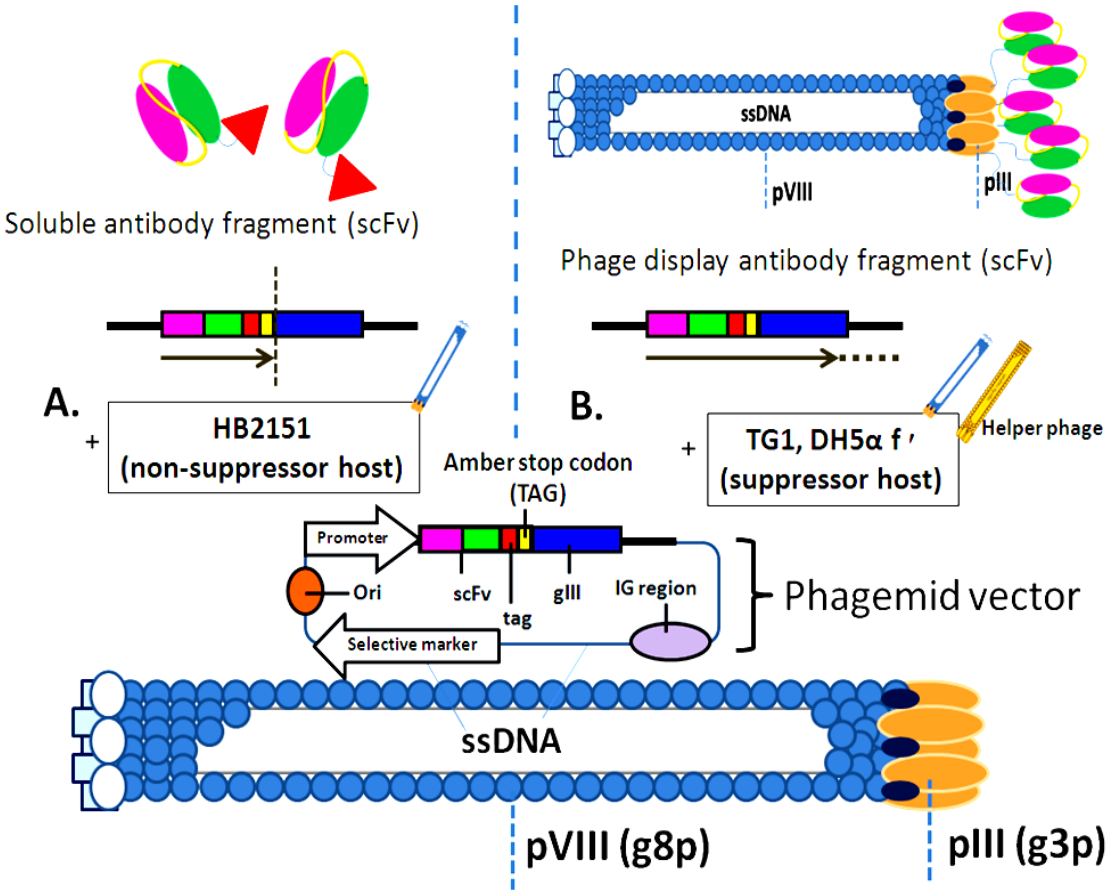


Figure 6. Antibody fragments can be characterized and used as free soluble fragment or as phage. (A) The free soluble antibody fragment is produced when the phage is grown in a non-suppressor host. (B) Phage displayed antibody fragments are produced by growing phage in a suppressor host (*E. coli* strain TG1F' or DH5αF').

Phage display technology has been used to produce monoclonal antibodies since 1990 (McCafferty et al., 1990). At first, the libraries were constructed from animals that were immunized by antigens (immunized library). In these libraries, only the specific binding part of antibody fragment , Fab antibody (Chang et al., 1991; Hoogenboom et al., 1991), or the single chain variable fragments, scFv antibody (McCafferty et al., 1990), were displayed on the surface of phage minor coat protein (pIII site). Those antibody libraries could be successfully used to produce monoclonal antibodies against many antigens (Bugli et al., 2008; Burton et al., 1991; Cai *and* Garen, 1995; Graus et al., 1997; Hof et al., 2008; Lee et al., 2008; Persson et al., 2008; Shaw et al., 2008). Nevertheless, this method is limited only to the antibody against the antigen that is used to immunize animal, a time-consuming process. However, the success in using the immunized library indicated that phage display technology can be used as an alternative method for the production of specific monoclonal antibody against various antigens.

Phage display antibody technology has become popular in the past ten years after the non-immunized library or naïve library was constructed (Marks et al., 1991). This library can be used to select specific antibodies against a wide variety of antigens because it contains various combinations of the antibody repertoire from the animal in nature. The selected monoclonal antibodies have high affinity (1-200 mM) and the specificity is the same as monoclonal antibodies from hybridoma technology (Nissim et al., 1994; Sheets et al., 1998; Winter et al., 1994).

2.2.3 Affinity selection (Bio-panning) of antibody by phage display technology

In vitro isolation of antibody fragments from antibody libraries by using their affinity binding activity is called “Bio-panning” (Figure 7), referring to the gold washer’s tool (Clackson et al., 1991; Schirrmann et al., 2011).

The antigen is immobilized on a solid surface such as nitrocellulose, magnetic beads, column matrices, and plastic surfaces like polystyrene tubes or 96 well microtitre plates, and incubated with phage displayed antibody library. During this incubation step, physical (e.g., temperature), chemical (e.g., pH) and biological (e.g., competitor) parameters can be controlled to select for antibodies that are able to bind the antigen under these defined conditions. In this “panning” process, binding phages are captured, whereas, nonbinding ones are washed off. In the next step, the bound phages are eluted (e.g., by trypsin or pH shift) and amplified by re-infection of *E. coli* cells. Subsequently, the phagemid bearing *E. coli* are super-infected with a helper phage that provides all the necessary proteins for packaging the phagemid. The amplified phage population can be subjected to the next round of panning until a significant enrichment of antigen specific phage is achieved. Usually two or three panning rounds, and sometimes up to six, are necessary to select specifically binding antibody fragments. After panning, soluble monoclonal antibody fragments or antibody phages are produced and specific antigen binding is analyzed by phage ELISA to identify individual binders. Afterwards, these individual binders can be sequenced, further biochemically characterized, or re-engineered to fit various purposes (Paschke, 2006; Schirrmann et al., 2011).

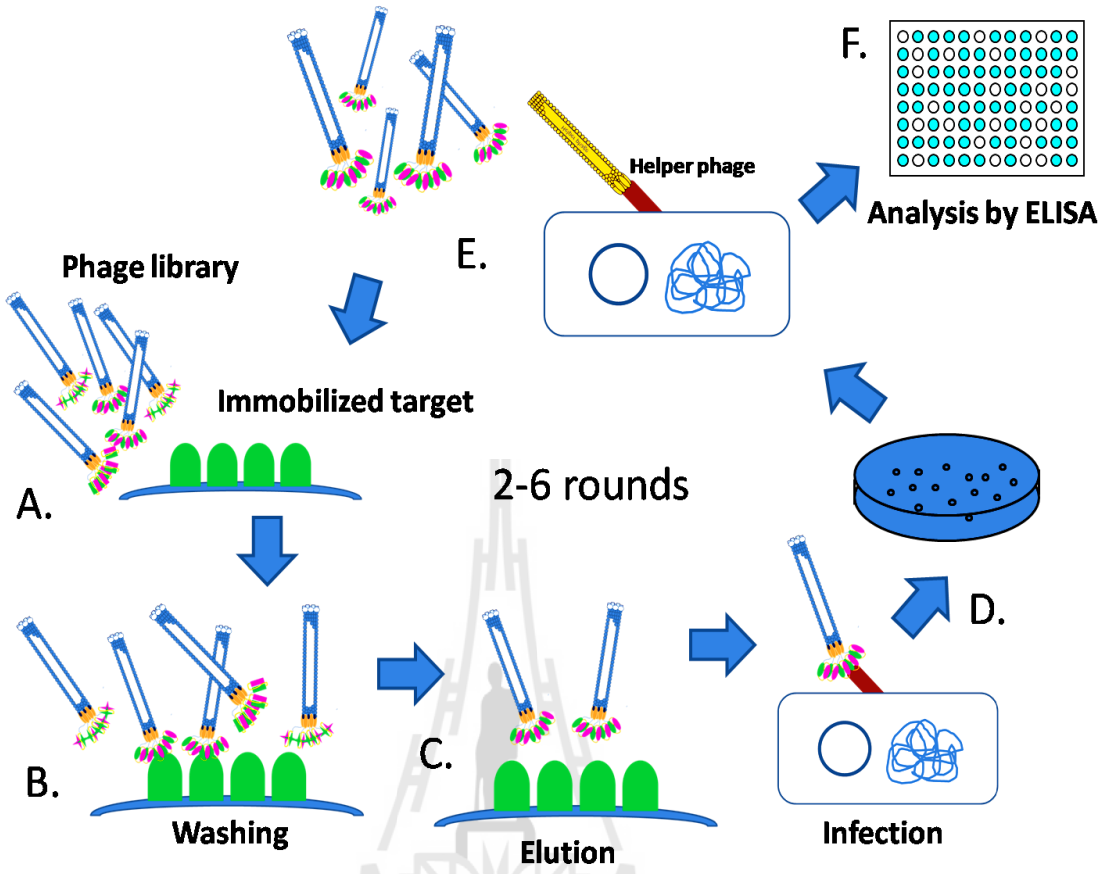


Figure 7. Schematic overview of the selection of antibodies ("Biopanning") by phage display technology. The phage displayed antibodies are exposed to immobilized target (A). Bound phages are captured whereas nonbinding ones are washed off (B). After that, bound phages are eluted. (C) The eluted phage are use to infect *E. coli* for amplification after super infection with helper phage (D). The amplified phage population can be subjected to the next round of panning (E). If the bio-panning steps are repeated, the populations of the bound phage are enriched and the phage population becomes less diverse, but the binding affinity tends to be higher.

The specificity and affinity of the recombinant antibody obtained from phage display technology can be further improved by genetic engineering approach, based on directed evolution technique (O'Brien et al., 2002; Hoogenboom et al., 1997) such as error prone PCR (Fromant et al., 1995; Martineau, 2002) or chain or DNA shuffling (Crameri et al., 1996; Fermer et al., 2004; Korpimaki et al., 2003; Proba et al., 1998; Zhang et al., 2003). Especially for chain shuffling, previous study has shown that this method can be successfully used to improve the binding affinity of certain antibodies as much as 1,000 folds, or up to 10^{11} M^{-1} (Schier et al., 1996a; Schier et al., 1996b; Yang et al., 1995).

In addition to binding affinity, this technology can also be used to improve other desired properties of antibodies such as stimulation of a specific cellular signaling pathway, (Burtrum et al., 2003; Dauvillier et al., 2002; Gejima et al., 2002; Kovaleva et al., 2006; Li et al., 2004; Paz et al., 2005; Piloto et al., 2005; Willemsen et al., 2005), enhancement or antagonism of the protein or enzyme (Rowley et al., 2004; Xie et al., 1997), tolerance of the antibody towards acidic, basic, or reducing conditions (Kristensen and Winter, 1998; Pedersen et al., 2002), proteinase resistance (Kristensen and Winter, 1998), development for therapeutic uses (Almagro and Fransson, 2008; Booy et al., 2006; Hale, 2006; Jolliffe, 1993; Mitra et al., 2006; Reilly, 2006; Santos and Padlan, 1998; Stowell, 2006), or tags for diagnosis (Buscomb, 2006; Carter and Merchant, 1997; Filpula, 2007; Howard and Kaser, 2006; Teillaud et al., 2005; Van et al., 2004).

In conclusion, application of phage display technology for the production of monoclonal antibodies has several adventage over the conventional method as follows (Ahmad et al., 2012);

1. This method does not require animals, and is less expensive.

2. This method is easier, saving time and labors.

3. Many types of antigens can be used by this technique, including toxic antigens, whole cell, nonimmunogenic antigens, or an antigen that is similar to the proteins of the animal.

4. This method can be used to produce both polyclonal and monoclonal antibodies.

5. Human antibodies, which are suitable for therapeutic purposes, can be produced using this method.

6. There are unlimited supplies of the antibody because the gene can be stored in the phage or bacteria.

7. Phages are more stable and can be stored for several years at 4 ^{o}C, and forever at -80 ^{o}C.

8. This technology can be applied to produce monoclonal antibodies on an industrial scale.

9. Higher affinity mutants of scFv (single chain fragment variable) can be generated through site directed or random mutagenesis.

2.3 Current research on recombinant antibodies against rabies

As recommended by the world health organization (WHO), effective post-exposure prophylaxes (PEP) treatment of rabies requires the prompt use of human or equine anti-rabies immunoglobulin (RIG) and the administration of rabies vaccine. The need to replace these hyper-immune serum preparations is widely recognized. Monoclonal antibodies (mAbs) that neutralize rabies virus (RABV) have been recognized as one alternative to overcome the limitations of RIG and offer the opportunity to replace RIG. The WHO has strongly encouraged the use of mAbs technology to develop novel treatment to replace RIG for PEP against rabies. The advantages of mAbs include the possibility of large-scale production, increase specificity and decrease risk of blood borne diseases (Zhaoet al., 2009; Smith et al., 2011; Duanet al., 2012). Nowadays, phage display technology has been used for the generation of monoclonal antibodies against rabies virus (Bakker et al., 2005; Goudsmit et al., 2006; Kramer et al., 2005). Antibody fragments such as single chain fragment variable (scFv) (Ray et al., 2001; Kramer et al., 2005; Zhao et al., 2008; Kaku et al., 2011; Aavula et al., 2011), the fragment antigen binding (Fab) (Ando et al., 2005; Houimel et al., 2009; Liu et al., 2011; San et al., 2012), the variable heavy domain (V_{HH}) of camels or Single-domain antibodies (dAbs) (Hultberg et al., 2011; Boruah et al., 2013) have been displayed on phage for selection of antibody fragments against rabies (See summary in Table 4). Ray and colleagues (2001) have described the selection of anti-GPRV scFvs from a human synthetic scFv phage display library. scFvs were cloned into a mammalian expression vector carrying the human IgG1 Fc region. Two of the scFv-Fc fusion proteins have been shown to neutralize the PV11 strain in a standard *in vivo* neutralization assay (Ray et al., 2001).

In 2005, Crucell has shown the successful construction and isolation of scFvs from a human immune library (Kramer et al., 2005). Selections were performed by using three different antigen formats of the RV glycoprotein (gp) resulting in the identification of 147 unique antibody fragments specific for the RV gp. Their work was continued and improved in an antibody cocktail aimed for use in rabies post-exposure prophylaxis. The cocktail antibody derived from phage display technology developed by Crucell is now in clinical trials (Kramer et al., 2005; de Kruif et al., 2007). This antibody is a combination of two types of monoclonal antibodies. One is from hybridoma and the other is from phage display technology. Ando *et al*. have reported 132 Fabs selected from a human Fab immune library. Among 132 selected clones, two Fab preparations revealed neutralizing activities against RV strains. Thus, there are many reports on isolation of neutralizing Fab and scFv against rabies virus from human immune libraries by phage display technology (Zhao et al., 2008; Houimelet al., 2009; Liu et al., 2011; Kaku et al., 2011; San et al., 2012). Heavy chain antibody fragments (V_{HH}) from llama has become an interesting alternative for treatment of viral infections including rabies (Hultberg et al., 2011). These recombinant antibodies were selected from a llama immune library by phage display technology. By fusion of V_{HH} with variable linker lengths, multimetic constructs were made with improved neutralization potencies up to 1,500-fold. Moreover, ribosome display technology for selection of human antibodies against rabies virus from an scFv library has been shown. After five rounds of selection, sequence analysis showed that mutations were introduced at random by PCR among the round of selection and variants with high affinity were isolated (Zhao et al., 2009). Expression, purification and characterization of a human scFv antibody fragment targeting a rabies antigen

fused with the Fc of an IgG1 in yeast (*Pichiapastoris*) were reported (Wang et al., 2012). A human scFv antibody fragment was fused with the Fc of an IgG to develop its use as a component of PEP cocktail. The production of scFv-Fc was optimized and scaled up in an 80 L fermenter with yields exceeding 60 mg/L and 95% purity of scFv-Fc (Wang et al., 2012). To enhance the scFv fragment stability and neutralizing potency, recombinant antibody (scFv) was engineered by introduction of cysteines at VH44 and VL100. The cysteine at VL85 was mutated to serine to construct the recombinant antibody in order to avoid potential mis-formed disulfide bonds which would alter the affinity of the scFv. The stability and activity of the scFv expressed in *Escherichia coli* were evaluated. The results with mutant scFv showed that the stability of scFv was notably improved, and its *in vitro* neutralizing potency against RV infection was enhanced. The findings from this stabilization modification study support the feasibility of developing scFvs for PEP treatment of rabies (Duan et al., 2012).

Table 5. Monoclonal antibody against Rabies virus by phage display technology.

Target	Bio-panning	Library	Country	Reference
The purified PV11 virus	4 rounds	scFv library (Human)	India	Ray et al., 2001
Inactivated virus,	40 rounds	scFv library (Human)	Natherland	Kramer et al., 2005 (Crucell)
Purified rabies virus glycoprotein, and cell surface expressed rabies virus glycoprotein				
RV viron or the purified RV Glycoprotein	5 rounds	Fab library (Human)	Japan	Ando et al., 2005
Peptide G5-24 (PPDQLVNLHAFVRSDEIEHLVVEE)	4 rounds	scFv library (Human)	China	Zhao et al., 2008
Inactivated virus	4 rounds	Fab library (Human)	Tunisia	Houimel et al., 2009
Inactivated virus	5 rounds	Fab library (Human)	China	Liu et al., 2011
Rabies glycoprotein (G protein)	-	VHH library (Llama)	Spain	Hultberg et al., 2011
Recombinant RABV-P protein	3 rounds	scFv library (Human)	Japan	Kaku et al., 2011
Purifyed native glycoproteins from Pasteur virus (PV GP)	-	scFv library (Mice)	India	Aavula et al., 2011
Purified RV virions (Chinese vaccine strain aG) from Vero cell culture	3-4 rounds	Fab library (Human)	China	San et al., 2012
Rabies virus G protein (RABV-G protein)	3 rounds	VHH library (Llama)	China	Boruah et al., 2013

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER III

METERIAL AND METHODS

3.1 Materials

3.1.1 Bacteria

3.1.1.1 *Eschericia coli* strain TG1 (suppressor host) (S.M. Chemical)

(lac-proAB) Sup E thi hrd D5*/F' tra D36 pro* A+B $lacI_q$ *lacZΔM15*

3.1.1.2 *E. coli* strain HB2151 (non suppressor host) were kindly provided by Prof. Greg Winter and Dr. Ian Tomlinson.

K12 *ara Δ* (*lac-proAB*) *thi*/F'*pro*A+B $lacI_q$ *lacZΔM15*

3.1.1.3 *E. coli* strain DH5αF' (suppressor host) (NEB, USA)

F'/*end*A1 *hsdR17* (rK- mK +) *supE44 thi-1 recA1 gyrA* (*Nalr*) *relA1* (*LacZYA-argF*)*U169 deoR* (80 dlac *(lacZ) M15)*

3.1.1.4 *E. coli* BL21(DE3)

fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5

3.1.2 Phage

3.1.2.1 Phage display human scFv libraries

Two types of phage display libraries, i.e., non-immunized (YAMO-I) (Pansri et al., 2009) and immunized libraries (Yamo-Rb) (Yamabhai M. unpublished) were used.

3.1.2.2 KM 13 helper phage from the MRC, Cambridge Centre for Protein Engineering, Cambridge England were kindly provided by Prof. Greg Winter and Dr. Ian Tomlinson, respectively.

3.1.3 Primer

3.1.3.1 Yamo primer

5’CAGGAAACAGCTATGACC3’

3.1.3.2 96 GIII primar

5’CCCTCATAGTTAGCGTAACG3’

3.1.4 Instruments

Autoclave:	Hiclave HA-3000MIV, Hirayama, Japan
Balance:	Precisa 205A, Precisa Instruments, Switzerland
	Precisa 3000C, Precisa Instruments, Switzerland
Centrifuge machine:	Thermoscientific, Sorvall legend XTR centrifuge, USA
	Eppendrof centrifuge 5810 R, Eppendrof, USA
	Eppendrof minispin plus, wiswspin® feedback control digital timer function, Eppendrof, USA
Deep freezer -70 ^{o}C:	Thermosciencetific, forma 900 series, USA.
ELISA reader:	Sunrise, TECAN, Austria
Electroporator :	Eppendrof 2510, Eppendrof, USA
Freezer -20 ^{o}C:	Heto, HLLF 370, Denmark.
	Haier, China.
Gel Document set:	White/Ultraviolet Transilluminator

	GDS7500, UVP, USA
Gel dryer:	Digital Graphic Printer UP-D890, Sony, Japan.
	Drygel sr. SLAB GEL Dryer model SE1160, Hoefer Scientific Instruments, USA
Gel electrophoresis apparatus:	Mini Protean® 3 cell, BioRad, USA
Heat Box:	HB1, Wealtee Corp., USA
Incubator shaker:	Innova 4230 refrigerated incubator shaker, New Brunswick Scientific, USA
	Innova® 42 incubator shaker series, USA
Incubator:	Memmert, BE 500, WTB Binder BD115,
Laminar hood:	Thermoscientific 1300 series A2, USA
	BH 2000 Series ClassII Biological Safety Cabinets
	BHA120 & BHA180, Clyde-Apac
Membrane transfer machine:	Semi Phor, Hoefer Scientific instruments, USA
Microcentrifuge:	Mini spin plus, Eppendrof, USA
	Eppendorf 54154, Eppendorf, Germany
pH meter:	Ultra Basic pH meter, Denver Instruments, Germany
PCR machine:	DNA Engine PTC 200 peltier Thermal cycler,
	MJ Research, USA
Rotator:	Certomat TCC, B. Braun Biotech International, Germany

Sequencing machine:	ABI prism model 310 Genetic Analyzer, Applied Biosystems, USA
Shaker:	Innova 2300 platform shaker, New Brunswick Scientific, UK Certomat TC2, B. Braun Biotech International, Germany
Sonicator:	Waken GE100 Ultrasonic processor, Japan
Spectrophotometer:	Ultrospec 2000, Pharmacia biotech, UK
Stirrer:	Variomag Electronicrührer Poly 15, Germany Magnetic stirrer MSH300, USA
Thermomixer:	Thermomixer compact, Eppendrof, USA
Vortex:	Vortex-Genie 2 G506, Scientific Industries, USA

3.1.5 Cell line

BHK-21, C13 cells were kindly provided by Dr. Pakamatz Khawplod from Queen Saowabha Memorial Institue, Thai Red-Cross Society.

3.1.6 Rabies virus

CVS-11 was kindly provided by Dr. Pakamatz Khawplod from Queen Saowabha Memorial Institue, Thai Red-Cross Society.

3.1.7 Target

3.1.7.1 PCEC (LEP-Flury strain, Rabipur, Chiron, India) Potency 7.97 IU/dose/1 ml

3.1.7.2 PVRV (VeroRab, Pitman-more/ W138-153-3M strain, Sanofi-Pasteur, Lyon, France) Potency 9.2 IU/ dose/0.5 ml

3.1.7.3 Rabies G protein pre-coated 8-well strips (BioRad, USA)

3.2 Methods

3.2.1 Library construction (Yamo-Rb library)

Yamo-Rb library was constructed from four human donors immunized with PVRV (VeroRab, Pitman-more/ W138-153-3M strain, Sanofi-Pasteur, Lyon, France) or PCEC (LEP-Flury strain, Rabipur, Chiron, India) according to previously plublished method(Pansri et al, 2009). Peripheral blood from four human donors including 2 males and 2 females were collected into four pools. Total RNA was prepared from the B lymphocytes and pooled together before being used as templates for the construction of V-genes repertoire. A mixure of oligo-dT18 and random hexamers was used to synthesize cDNA, so that all five antibody isotypes could potentially be represented. There were 75 independent PCR reactions to amplify V gene segments using all possible combinations within a primer set (Table 6). The primer sequences, which in theory encompass the entire repertoire of human antibody genes, were obtained from V BASE. All primers containing either *Sfi*I or *Not*I recognition sites or linker sequence are listed in Table 6. A total of 33 primers were used for the construction of the scFv library. The final pull-through PCR could be done with two primers (PTfw & PTrv), compatible with the 5' *Sfi*I or 3'*Not*I segments of the heavy and light chain gene repertoires. After the final scFv gene repertoires had been sequentially digested with the restriction enzymes *Sfi*I and *Not*I, they were ligated directly into pre-digested and dephosphorylated phagemid pMODI Vector.

Finally, an immunized scFv library (Yamo-Rb library) consisting of 1.5×10^6 different scFv molecules could be obtained.

Table 6. Primers for the construction of human scFv phage display library (Pansri et al, 2009).

Primer	Sequence
VH5'Sfi	5' CCTTTCTATGC*GGCCCAGCCGGCC***ATGGCCCAGGTGCAGCTGGTGCAGTCTGG** 3'
	5' CCTTTCTATGC*GGCCCAGCCGGCC***ATGGCCGAGGTACAGCTGCAGCAGTCAGG** 3'
	5' CCTTTCTATGC*GGCCCAGCCGGCC***ATGGCCCAGGTCAACTTAAGGGAGTCTGG** 3'
	5' *GCCCAGCCGGCC***ATGGCCGAGGTGCAGCTGGTGGAGTCTGG** 3'
	5' *GCCCAGCCGGCC***ATGGCCCAGGTGCAGCTGCAGGAGTCGGG** 3'
	5' *GCCCAGCCGGCC***ATGGCCGAGGTGCAGCTGTTGCAGTCTGC** 3'
VH3'link	5' *ACCAGAGCCGCCGCCGCCGCTACCACCACCACC***TGAGGAGACGGTGACCAGGGTGCC** 3'
	5' *ACCAGAGCCGCCGCCGCCGCTACCACCACCACC***TGAGGAGACGGTGACCGTGGTCCC** 3'
	5' *ACCAGAGCCGCCGCCGCCGCTACCACCACCACC***TGAAGAGACGGTGACCATTGTCCC** 3'
	5' *ACCAGAGCCGCCGCCGCCGCTACCACCACCACC***TGAGGAGACGGTGACCAGGGTTCC** 3'
VL5'link-κ	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***GACATCCAGATGACCCAGTCTCC** 3'
	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***GAAATTGTGCTGACTCAGTCTCC** 3'
	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***GATGTTGTGATGACTCAGTCTCC** 3'
	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***GAAATTGTGTTGACGCAGTCTCC** 3'
	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***GACATCGTGATGACCCAGTCTCC** 3'
	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***GAAACGACACTCACGCAGTCTCC** 3'
VL5'link-λ	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***AATTTTATGCTGACTCAGCCCCA** 3'
	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***CAGTCTGTGTTGACGCAGCCGCC** 3'
	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***CAGTCTGCCCTGACTCAGCCTGC** 3'
	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***TCCTATGTGCTGACTCAGCCACC** 3'
	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***TCTTCTGAGCTGACTCAGGACCC** 3'
	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***CACGTTATACTGACTCAACCGCC** 3'
VL3'NotI-κ	5' *AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCC***CAGGCTGTGCTCACTCAGCCGTC** 3'
VL3'NotI-λ	5' CAGTCATTCTCGACTT*GCGGCCGC***ACGTTTGATTTCCAGCTTGGTCCC** 3'
	5' CAGTCATTCTCGACTT*GCGGCCGC***ACGTTTAATCTCCAGTCGTGTCCC** 3'
	5' CAGTCATTCTCGACTT*GCGGCCGC***ACGTTTGATCTCCAGCTTGGTCCC** 3'
	5' CTCGACTT*GCGGCCGC***ACGTTTGATATCCACTTTGGTCCC** 3'
	5' CTCGACTT*GCGGCCGC***ACGTTTGATCTCCACCTTGGTCCC** 3'
PTfw	5' CAGTCATTCTCGACTT*GCGGCCGC***ACCTAAAACGGTGAGCTGGGTCCC** 3'
	5' CTCGACTT*GCGGCCGC***ACCTAGGACGGTGACCTTGGTCCC** 3'
	5' CTCGACTT*GCGGCCGC***ACCTAGGACGGTCAGCTTGGTCCC** 3'
PTrv	5' CCTTTCTATGC*GGCCCAGCCGGCC*ATGGCC 3'
	5' CAGTCATTCTCGACTT*GCGGCCGC*ACG 3'

*Bold fonts indicate sequence complementary to the V-gene segments. Recognition sites for restriction enzymes (*Sfi*I/*Not*I) and linker sequence are italicised.

3.2.2 Amplification of phage library for bio-panning

Two types of phage display libraries, i.e., non-immunized (YAMO-I) (Pansri et al., 2012) and immunized libraries (Yamo-Rb) were constructed using antibody genes isolated from the peripheral blood of human donors. The YAMO-I library was constructed from 140 non-immunized (naïve) donors; whereas, Yamo-Rb library was constructed from four human donors immunized with PVRV (VeroRab, Pitman-more/ W138-153-3M strain, Sanofi-Pasteur, Lyon, France) or PCEC (LEP-Flury strain, Rabipur, Chiron, India). To rescue phagemid library, 500 µl of library stock was add into 50 ml of pre-warmed 2xYT containing 100 µg/ml ampicillin (Emresco, USA) and 1% w/v glucose (Carlo erba, Italy) and grown with shaking at 200 rpm, 37 ^{o}C until the cell reached to the mid-log phase (OD_{600} about 0.4). Then, $2x10^{11}$ KM13 helper phage was added into 50 ml cultures and was incubated at 37 ^{o}C without shaking for 30 min. The culture was centrifuged at 4,000 rpm for 10 min following by resuspension of the cell pellet in 100 ml of 2xYT containing 100 µl/ml ampicillin, 50 µg/ml kanamycin (Fluka, Switzerland) and 0.1% glucose. After that, the culture was incubated with shaking at 30 ^{o}C overnight. The overnight culture was centrifuged at 4,000 rpm for 30 min. The 40 ml supernatant was precipitated by mixing with 10 ml PEG (Fluka, Switzerland) /NaCl (Emresco, USA) (20% Polyethylele glycol 6000, 2.5 M NaCl) for 1 h on ice followed by centrifugation at 4,000 rpm for 30 min. All supernatant was then removed, and the pellet was resuspended in phosphate buffered saline (PBS, 137 mM NaCl, 3 mM KCl (Emresco, USA), 8 mM Na_2HPO_4 (Merck, German), 1.5 mM KH_2PO_4 (AnalaR, England), pH 7.4). The phage solution was centrifuged at 10,000 rpm for 10 min, to remove any pellet. The phage library solution was transferred to the new tube and stored at 4 ^{o}C

before biopanning. For long term storage, phage supernatant was kept in 20% glycerol (Emresco, USA)/PBS and stored at -70 ^{o}C. To determine the phage titer, the PEG precipitate phage was diluted by making six 100-fold serial dilutions and adding 100 µl of diluted phage into 900 µl of mid-log *E. coli* TG1. The infected *E. coli* TG1 cells were incubated at 37 ^{o}C for 30 min followed by plating on separate 2xYT agar containing 100 µg/ml ampicillin and 1% w/v glucose. The plates were incubated at 37 ^{o}C for overnight, before numbers of colonies were count.

3.2.3 Biopanning procedures

3.2.3.1 Using PCEC/PVRV as targets

Selection was performed using inactivated rabies vaccines (PVRV or/and PCEC) as a target. Two to five rounds of selection were carried out. A Maxisorp Immuno tube (Nunc, Denmark) was pre-coated with 0.35-1.4 IU of inactivated rabies virus, at 37 ^{o}C for 3 h followed by 4 ^{o}C overnight, in 100 mM $NaHCO_3$, pH 8.5. After that, the immuno tube was stabilized with 5% w/v sucrose (Carlo erba, Italy), 0.3% w/v BSA (Emresco, USA), and 50 mM $NaHCO_3$ (Merck, German) for 45 min. Then the tube was washed three times with phosphate buffered saline (PBS) and blocked with PBS containing skimmed milk (HiMedia, India) (2%, w/v, MPBS) for 1 h. For each round of biopanning, the phage library (~10^{10} phages) was incubated with pre-coated inactivated rabies virus in 4%, w/v, MPBS for 2 h at room temperature. Unbound phage was washed away 10 times with PBST (PBS supplemented with 0.05% (v/v) Tween 20) and with PBS 10 times. Phage antibody against rabies viruses was recovered with trypsin buffer (10 µl of 10 mg/ml trypsin stock in 90 µl of PBS) and 0.2 M glycine HCl, pH 2.0. The eluted phage was infected into *E. coli* suppressor host by infecting 2 ml of *E. coli* mid-log phase with 150 µl

eluted phage and incubated at 37 ^{o}C for 30 min. For the output tittering, the infected *E. coli* was diluted with 2xYT media. Three 10-fold serial dilutions were performed. Then the three dilutions, non-infected *E. coli* and undiluted infected *E. coli* were separately spread on to 2xYT agar containing 100 µg/ml ampicillin and 1% w/v glucose. The plates were incubated at 37 ^{o}C for overnight. The recovered phage was used to infect *E. coli* in order to amplify for the next round of selection. For each round of selection, the specificities of individual phage scFv clones were determined by the enzyme-linked immunosorbent assay (ELISA).

3.2.3.2 Using glycoproteins as target

Rabies G protein pre-coated 8-well strips from BioRad were used for biopanning. Three rounds of selection were carried out. For each round of biopanning, the phage library (~10^{10} phages) was incubated with pre-coated inactivated rabies virus in 4%, w/v, MPBS for 2 h at 37 ^{o}C (Biorad, USA). Unbound phage was washed away with PBS supplemented with 0.05% (v/v) Tween 20 (PBST) and PBS. Phage antibody against rabies viruses was recovered with trypsin buffer (10 µl of 10 mg/ml trypsin stock in 90 µl of PBS) and 0.2 M glycine HCl, pH 2.0. The eluted phage was infected into *E. coli* strain TG1 to obtain individual phage clone and the output titer was checked as previously described. The recovered phage was infected into *E. coli* strain TG1 in order to amplify for further rounds of selection. For each round of selection, the specificities of individual phage scFv clones were identified by the enzyme-linked immunosorbent assay (ELISA).

3.2.3.3 Further rounds of selection

The infected *E. coli* TG1 or *E. coli* DH5αF' colonies on 2xYT agar containing 100 µg/ml ampicillin and 1% w/v glucose were scraped and kept as 20% glycerol stock at -70 ^{o}C. Ten µl of scraped bacteria was inoculated into 10 ml of 2xYT containing 100 µg/ml ampicillin and 1% w/v glucose. The culture was grown at 37 ^{o}C until the cells reached mid-log phase. After that, $2x10^{11}$ KM13 helper phage was added and the culture was incubated at 37 ^{o}C without shaking for 30 min. After spinning at 3,300 x g for 10 min, the supernatant was discarded and the pellet was resuspened in 10 ml of 2xYT containing 100 µl/ml ampicillin, 50 µg/ml kanamycin and 0.1% glucose. After that the culture was incubated with shaking at 30 ^{o}C for 18-20 h. The next day the overnight culture was centrifuged at 3,300 x g for 30 min. The supernatant was mixed with PEG/NaCl (20 % Polyethylele glycol 6000, 2.5 M NaCl) for 1 h on ice, and then centrifuged at 3,300 x g for 30 min. The pellet was resuspended in PBS buffer. This precipitated phage was used for the next round of selection. For the input titering of the next round, the PEG precipitate phage was diluted by making six 100-fold serial dilutions and adding 100 µl of diluted phage into 900 µl of mid-log *E. coli* TG1. The infected *E. coli* TG1 cells were incubated at 37 ^{o}C for 30 min followed by plated on separate 2xYT agar containing 100 µg/ml ampicillin and 1% w/v glucose. The plates were incubated at 37 ^{o}C for overnight.

3.2.3.4 Individual Phage Rescue

The individual phage-infected colony was randomly picked from 2xYT agar containing 100 µg/ml ampicillin and 1% w/v glucose. The cells were grown in wells of a 96-well plate (Nunc, Denmark) containing 100 µl 2xYT supplemented with 100 µg/ml ampicillin and 1% (w/v) glucose. After overnight

incubation at 37 ^{o}C with shaking, small inocula (5 µl) from each well were transferred to a second 96-deep well plate containing 200 µl of 2xYT plus 100 µg/ml ampicillin and 1% (w/v) glucose. The first plate was kept as master stock by adding glycerol to a final concentration of 20% (v/v) and kept at -20 ^{o}C. The second plate was incubated at 37 ^{o}C with shaking (200 rpm) for 2 h, and later phage was rescued by adding 10^{10} KM13 helper phage to each well. Following this they were then incubated at 37 ^{o}C for 1 h with shaking. Then, the supernatant was discarded after centrifugation of the plate at 3,300 x g for 10 min. The pellet was then resuspended in 200-400 µl of 2xYT containing 100 µg/ml ampicillin and 50 µg/ml kanamycin, and cultured at 30 ^{o}C overnight (18-20 h) with shaking (250 rpm). The overnight culture was spun at 3,300 x g for 15 min, and 100 µl of the supernatant-containing phage was used in monoclonal phage ELISA.

3.2.4 Monoclonal phage ELISA

3.2.4.1 Whole cell virus

A single colony from each round of biopanning was randomly picked and cultured in 96-deep well plate (costar, USA) followed by super-infection by KM13 helper phage as described above. Phage supernatants were collected after centrifugation and subjected to ELISA for screening of monoclonal anti-rabies virus phage-scFv. The Immuno 96 microWell^{TM} plate (Nunc, Denmark) was immobilized with 0.1 IU of inactivated rabies at 37 ^{o}C for 3 h following by 4 ^{o}C overnight in 100 mM $NaHCO_3$ and 2% skim milk in 100 µl PBS buffer as a control. The plate was stabilized with 5% w/v sucrose, 0.3% w/v BSA, and 50 mM $NaHCO_3$ for 45 min. The plate was then washed 3 times with PBS and blocked with PBS containing skimmed milk (2%, w/v, MPBS) for 1 h. The phage supernatant was added to Immuno 96

microWell^{TM} plate (Nunc, Denmark). Phage was allowed to bind to the target by incubation for 2 h and the unbound phage was washed off by 3 times PBST and 3 times PBS. The binding phage-scFv was detected with HRP-conjugated anti-M13 antibody (1:5000) (Amersham-Pharmacia Biotech, Sweden). The color of the reaction was developed with ABTS reagent (Emresco, USA). The reaction was quantified by measuring the absorbance at 405 nm.

3.2.4.2 Glycoprotein plate

Rabies G protein pre-coated 8-well strips from BioRad (USA) were used for monoclonal phage ELISA. After the plate was blocked with 100 µl of 3% BSA in PBS, the phage supernatant was added to Immuno 96 microWell^{TM} plate (Nunc, Denmark). Then, phage was allowed to bind the target by incubated for 2 h. The unbound phage was washed off by washing 5 times with buffer from the kit. The binding phage-scFv was detected with HRP-conjugated anti-M13 antibody (1:5000). The resulting absorbance was read at 450 nm for 3, 3′, 5, 5′-Tetramethylbenzidine substeate (TMB, Emresco, USA) or 405 nm for ABTS substeate.

3.2.5 Expression of soluble scFv

3.2.5.1 HB2151

To express soluble scFv antibody without pIII domain, phage scFv was infected into an *E. coli* HB2151 (non-suppressor host). One to ten microliter phage particles of the selected clone were used to infect 500 µl of mid-log phase of *E. coli* HB2151 at an OD_{600} of approximately 0.4, and incubated at 37 ^{o}C for 30 min. Then, the infected bacteria were streaked on 2xYT agar containing 100 µg/ml ampicillin and 1% w/v glucose. After that, the plates were incubated at 37^{o}C overnight. A single colony was placed into 5 ml of 2xYT containing 100 µg/ml

ampicillin and 2% w/v glucose. The culture was incubated overnight with shaking at 250 rpm, 30 ^{o}C. One percent v/v of each overnight culture was used to inoculate into 2xYT containing 100 µg/ml ampicillin and 2% w/v glucose and incubated with shaking at 30 ^{o}C until the OD_{600} reached 0.9. The expression was induced by the addition of 1 mM isopropylthio-galactoside (IPTG, Emresco, USA). After continuing incubation at 30 ^{o}C for 20 h, the secreted antibody could be found in the supernatant. The hexahistidine tag or myc tag was used for detection and purification.

3.2.5.2 Subcloning and expression in *E. coli* BL21 (DE3)

The genes of scFv antibody clones IRA7c, IIIRC2c, IYF5c and IIYG4v were sub cloned into pET27b vectors (Figure 8) between *Nco*I and *Not*I restriction sites. Plasmid were purified from each clone (QIAgen spin Mini-prepkit, USA) and transformed into *E. coli* BL21. The *E. coli* BL21 harboring recombinant plasmid was grown at 37 ^{o}C in 5 ml of M9ZB media containing 50 µg/ml kanamycin and 2% w/v glucose. The culture was incubated for overnight with shaking at 200 rpm, 30 ^{o}C. One percent v/v of each overnight culture was used to inoculate into M9ZB media supplemented with 2% w/v glucose and 50 µg/ml kanamycin. Then, the culture was incubated with shaking at 30 ^{o}C for 4 h. After that, the cells were centrifuged at 16 ^{o}C, 5000 rpm for 15 min followed by resuspension with M9ZB media containing 1% w/v glycerol, 50 µg/ml kanamycin and 1 mM isopropylthio-galactoside (IPTG, Emresco, USA). After continuing incubation at 16 ^{o}C for 20 h with shaking, the cell pellet was collected by centrifugation at 16 ^{o}C, 8,000 rpm for 15 min. The secreted antibody could be found in the supernatant. The hexahistidine tag was used for detection and purification.

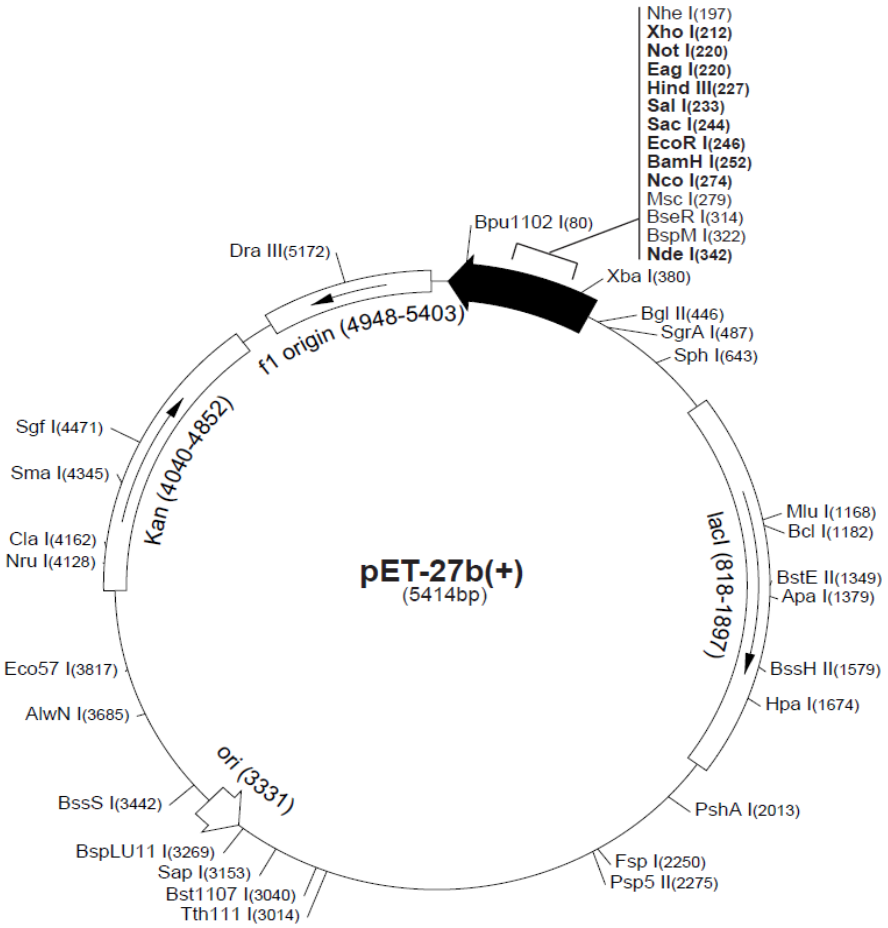


Figure 8. pET27b Vector.

3.2.6 Subcloning and expression of scFv-AP fusions

The scFv gene clone (IRA7c and IYF5c) from the pMOD1 phagemid vector was double digested with the restriction enzymes *Nco*I and *Not* I and subcloned into pMY607AP, an alkaline phosphatase fusion vector (Yamabhai M., unpublished). The integrity of the constructs was confirmed by automated DNA sequencing. To produce soluble scFv-alkaline phosphatase (scFv-AP) fusions, a single colony of *E. coli* DH5αF' harboring recombinant plasmid was inoculated into 5 ml of 2xYT containing 100 µg/ml ampicillin and 2% w/v glucose for overnight at 37 ^{o}C with

shaking (200 rpm). After the culture supernatant was centrifuged at 3,000 x g for 10 minute, cell pellets were resuspended in 2xYT containing 100 µg/ml ampicillin without glucose and incubation continued shaking at 200 rpm, 37 ^{o}C with for 2 h. Expression of soluble scFv-AP was induced by adding 1 mM isopropylthio-galactoside (IPTG), and the cultures were grown overnight at room temperature with shaking. The culture supernatant containing the secreted scFv-AP fusion product was collected by centrifuged and used in direct ELISA of soluble scFv–AP fusion.

3.2.7 Soluble scFv antibody ELISA

3.2.7.1 Monoclonal scFv antibody ELISA

Monoclonal scFv antibody supernatants were collected after centrifugation and subjected to ELISA as described above. The Immuno 96 microWell^{TM} plate (Nunc, Denmark) was immobilized with 0.1 IU of inactivated rabies. After the plate was stabilized for 45 min, the plate was washed 3 times with PBS and blocked with PBS containing skimmed milk (2%, w/v, MPBS) for 1 h. The monoclonal scFv antibody supernatant (100-200 µl) was added into Immuno 96 microWell^{TM} plate (Nunc, Denmark). After incubation for 2 h, the plate was washed off by 3 times with PBST and PBS. The monoclonal scFv antibody was detected with His prob-HRP (1:5000) or Mouse Anti-Myc Tag Monoclonal Antibody (1:5000). The color of the reaction was developed with ABTS reagent (Fluka, Switzerland). The reaction was quantified by measuring the absorbance at 405 nm.

3.2.7.2 Direct ELISA of soluble scFv–AP fusion

The 96-well microtiter plate was coated with inactivated rabies (0.1 IU PVRV, PCEC) at 37 ^{o}C for 3 h following by 4 ^{o}C overnight in 100 mM $NaHCO_3$ and 2% skim milk or 3% BSA in 100 µl PBS buffer as a control. The plate

was stabilized with 5% w/v sucrose, 0.3% w/v BSA, and 50 mM $NaHCO_3$ for 45 min. Then, the wells were washed 3 times with PBS and blocked with PBS containing skimmed milk (2%, w/v, MPBS) for 1 h. Later, 150 µl of scFv-AP supernatant and 50 µl of 4% skim milk were added into each well and incubated at room temperature for 2 h. After five time washings with PBST and PBS, 100 µl p-Nitrophenyl phosphate substrate (pNPP; SIGMA, Switzerland) was added to each well. After incubation for 20 min at room temperature, absorbance was measured at 405 nm.

3.2.8 Purification of soluble scFv antibody

ScFv antibody containing a hexahistidine tag was purified using Ni++ ions immobilized on resin by covalent linkage to nitrilotriacetic acid (NTA). The supernatant was immediately applied to a Ni–NTA agarose affinity column containing 1–2 ml of bed volume (QIAGEN GmbH, Hilden, Germany), and the chromatography was carried out gravitationally at 4 ^{o}C, following the Qiagen's protocol. The column was washed two times with 50 ml of wash buffer (20 mM Tris–HCl buffer, pH 8.0 and 150 mM NaCl) containing 20 mM imidazole. Ni–NTA bound enzyme was eluted with 250 mM imidazole in the same buffer. The eluted fractions were then dialyzed by SnakeSkin dialysis tubing (10K MWCO, Thermoscientific, USA) followed by centrifugal dialysis using Amicon® Ultra-4 Centrifugal Filter Devices (Mr 10,000 cut-off, Millipore, Ireland) to remove imidazole and concentrated soluble scFv antibody. SDS-PAGE electrophoresis was used to monitor antibody purity.

3.2.9 Western blot

For Western blot analysis, samples were run on SDS-PAGE and the scFv were blotted onto PVDF membrane (Biorad, USA). The proteins were transfered to a membrane using a membrane transfer machine and the membrane was blocked

with 3% BSA in PBS for 1 h. Then, the membrane was washed with PBS. After that, the membrane was incubated with His probe-HRP (a dilution of 1:5000 in PBS) for 1 h followed by washing with PBS. The 3, 3', 5-5'-Tetrametrylbenzidine (TMB) substrate (Emresco, USA) was used for developing the blue color on the membrane.

3.2.10 Neutralization assay

The neutralization capacity of the soluble scFv antibody and phage scFv antibody were detected by the Rapid Fluorescent Focus Inhibition Test (RFFIT). HRIG was used as the reference serum. Both forms of scFv monoclonal antibody were incubated with CVS (Challenge virus standard strain) and then the mixture was further incubated on the monolayered NA cell (Neuroblastoma cell). Briefly, serial dilution of soluble scFv antibody (IRA7c, IIYG4v, IYF5c and scFv anti-Alfa toxin 3C1) and phage scFv antibody (IRA7c, IIIRC2c, IRC3c, IYC11c, IYC12c, IYD1c, IYF5c, IIRD5v, IIYB5v, IIYG4v, IIYE5v, IIYG8v, IIYD4v) were mixed with an equal volume of CVS (100 TCID50) diluted with MEM containing 2% FCS and then incubated in a 5% CO_2 incubator at 37 ^oC for 90 min. Next, 50 µl of BHK-21 cells (10^5/well) were added and incubated at 37 ^oC for 21 h. After fixation with 90% acetone and washing 2 times with PBS buffer, cells infected with rabies virus were stained directly with fluorescent isothiocyanate (FITC)-labeled anti-rabies virus conjugate. The infected cells were counted using a fluorescence microscope. The neutralizing potencies (IU) of the established clones against CVS were calculated by comparison with the WHO international standards (equine anti-rabies immunoglobulin, National Institute for Biological Standards & Control) by the Spearman-Karber method. A RFFIT titer of ≥0.5 IU/ml was defined as an adequate concentration of neutralizing ability against RABV (Matsumoto et al, 2010).

3.2.11 DNA and Amino acid sequence analysis

3.2.11.1 Plasmid preparation

The colonies of the bacteria harboring the desired plasmid were picked into 5 ml media containing appropriate antibiotic. After the cultures were incubated for 16-18 h at 37 ^{o}C with shaking, the cultures were centrifuged at 6,800 x g for 3 min to collect the cells for DNA extraction. Then plasmid minipreparations were made from each clone (QIAgen spin Mini-prepkit, USA).

3.2.11.2 Restriction fragment analysis (cut insert check)

Digestion reactions of DNA were performed in a total volume of 10 µl. The reaction mixtures consisted of 1 µl of DNA, 0.2 µg of vector DNA 1x NEB buffer 3, 1 µg/ml BSA, 1 U of *Nco*I and 1 U of *Not*I. The reaction was incubated at 37 ^{o}C for overnight and was run on 1% (w/v) agarose gel (Emresco, USA) in TAE buffer at 100 volts for 45 min.

3.2.11.3 Finger printing analysis

The restriction fragment analysis was performed by using *BstN*I. The digestion reactions of DNA were performed in a total volume of 10 µl. The reaction mixtures consisted of 2 µl of DNA, 0.2 µg of vector DNA 1x NEB buffer 3, 1 µg/ml BSA, 1 U of *BstN*I. The reactions were incubated at 65 ^{o}C for overnight and were run on 1% (w/v) agarose gel in TAE buffer at 100 volts for 45 min.

3.2.11.4 DNA sequence analysis

The positive clones were confirmed by ELISA twice. Each clone of positive phagemid DNA was extracted using DNA miniprep kit (Qiagen) and the restriction fragment analysis was performed by using *BstN*I. The selected positive

clone with variable restriction pattern was confirmed by automated DNA sequencing (Macrogen, Korea) and analyzed with Igblast software (http://www.ncbi.nlm.nih.gov/igblast/) and the sequence alignment of the scFv antibodies was done using CLUSTALW2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

3.2.11.5 Bioinformatic Analysis for 3D structure prediction

For 3D structure modeling, the nucleotide sequences of clone IRA7c, IIIRC2c and IYF5c were translated to an amino acid sequence using Expasy (http://web.expasy.org/translate/). Suitable templates for homology modeling were searched for in the PDB database using the phyre^2 program. The binding sites which are CDR 1, 2, 3 of scFv Human antibodies were indicated by pymol program.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Selection of scFv fragments specific to rabies virus by biopanning

4.1.1 Selection of scFv fragments specific to rabies from non-immunized library (Yamo-I library), using PCEC as a target

The phage clones specific to rabies virus from the 1st round were screened for the positive clone by ELISA method. The mouse anti-M13 antibodies conjugated to HRP was used for detection and the color of the reaction was developed with ABTS substrate. The reaction was quantified by measuring the absorbance at 405 nm. The phage clones that had the absorbance value at least two times higher than binding of phage to the 2% w/v skim milk in PBS (2%, w/v, MPBS) were scored as a positive clones. After confirmation of the binding by phage ELISA, five positive clones (Figure 9) were analyzed by automated DNA sequencing (Macrogen, Korea). The DNA sequencing data revealed that clone D1 and H10 were identical. Clone F5, C11 and C12 of phages displaying anti-rabies virus were unique (Sequence data shown in APPENDIX C). Thus, four unique positive clones were selected from non-immunized library (YAMO-I library), using PCEC as target.

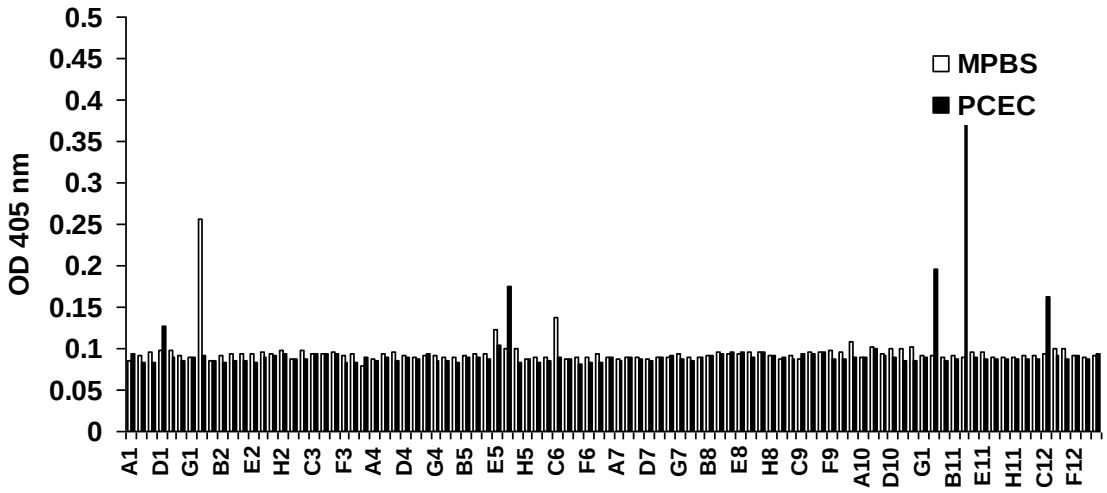


Figure 9. Binding of specific phage to PCEC from 1st round of panning. Clones were selected from Yamo-I library (non-immunized library). Five positive clones (D1, F5, H10, C11 and C12) showed specific binding to PCEC and not to 2 % (w/v) skim milk (MPBS negative control).

4.1.2 Selection of scFv fragments specific to rabies from immunized library (Yamo-Rb library), using PCEC as a target

The phage clones specific to rabies virus from the 1st and 5th rounds were screened for the positive clone by ELISA method. The specific clones were isolated from the immunized library (Yamo-Rb library) by using PCEC as a target. After 5th rounds of bio-panning**,** the enrichment of specific clone was shown. There were 13.54 % and 25 % of the positive clone from the 1st and 5th rounds, separately (figure 10-11). After confirmation of the binding by ELISA, the DNA of the positive clones from the 1st and 5th rounds were extracted and digested by *Bst*NI. The restriction fragment analysis demomstrated that almost of the clones show the same DNA pattern

(Figure 12). To characterize the ELISA positive clones, ten representative clones from a total of twenty-four ELISA positive clones (Figure 12) were analyzed by sequencing. The DNA sequencing data revealed that all eight clones of phages displaying anti-rabies were identical (IRA7c); whereas the anti-Rabies virus clones IIIRC2c and IRC3c were unique (Sequence data shown in APPENDIX C). This indicated that the phages recognizing the target are gradually enriched after several rounds of bio-panning were performed. But the variant of the phages recognizing the target are decreased. In summary, three positive clones were selected from immunized library (Yamo-Rb library) by using PCEC as target.

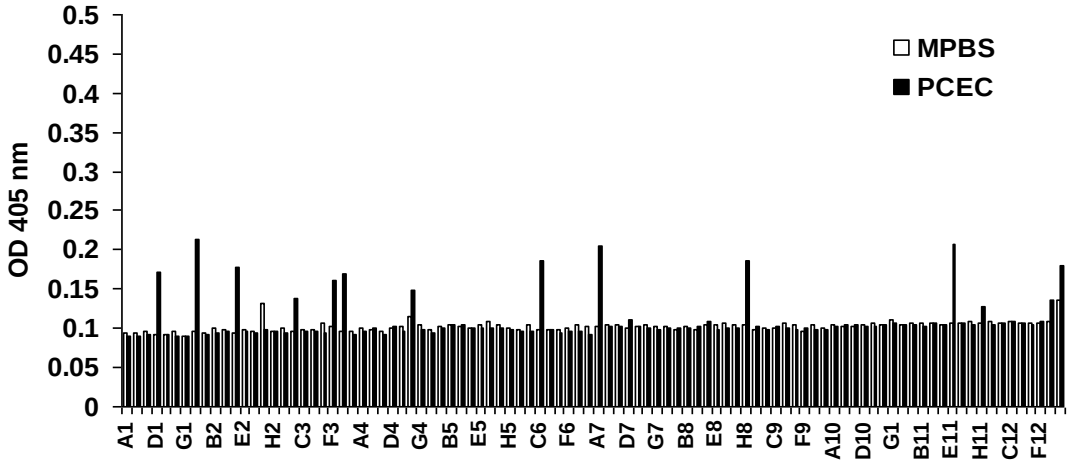


Figure 10. Binding of specific phage to PCEC from 1st round of panning. Clones were selected from Yamo-Rb immunized library. Thirteen positive clones (D1, H1, D2, B3, F3, G3, F4, C6, A7, H8, E11, E12 and H12) showed specific binding to PCEC and not to 2% (w/v) skim milk (MPBS negative control).

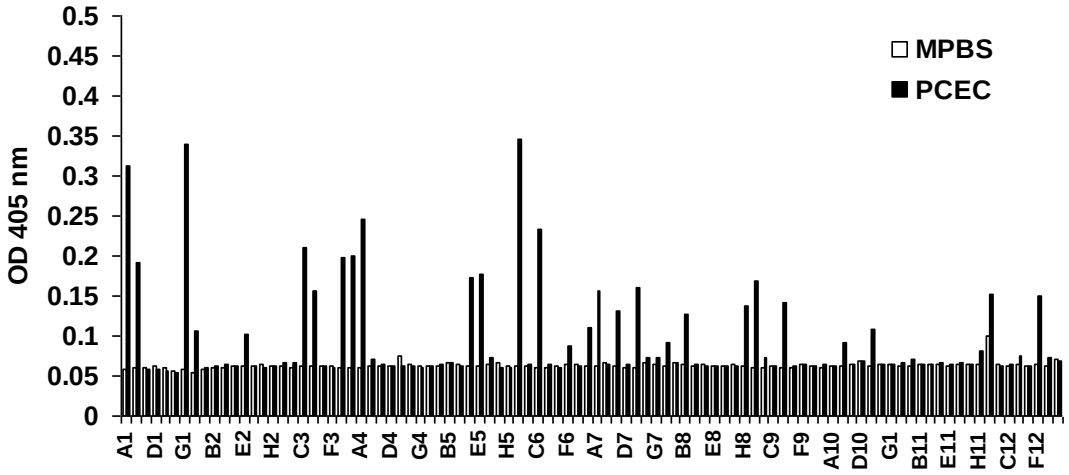


Figure 11. Binding of specific phage to PCEC from 5th round of panning. Clones were selected from Yamo-Rb immunized library. Twenty-four positive clones (A1, B1, G1, H1, E2, C3, D3, G3, H3, A4, D5, E5, A6, C6, H6, A7, C7, E7, B8, H8, A9, D9, A12, and F12) showed specific binding to PCEC and not to 2% (w/v) skim milk (MPBS negative control).

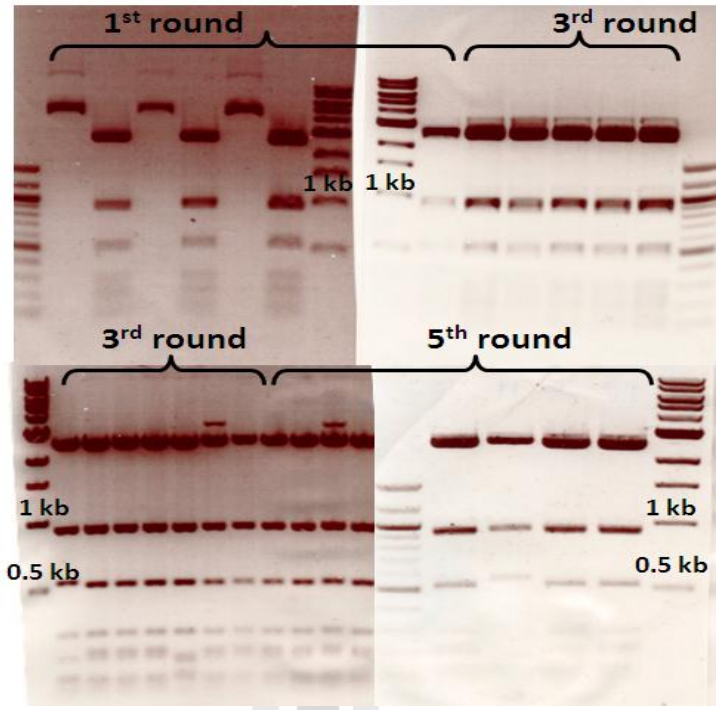


Figure 12. DNA fingerprint analysis. The twenty-four scFv clones were digested with *Bst*N1. The restriction patterns were analyzed on 1.5% agarose gel.

4.1.3 Selection of scFv fragments specific to rabies from non-immunized library (Yamo-I library), using PVRV as a target.

The phage clones specific to rabies virus from the 1st, 2nd and 4th rounds were screened for the positive clone by ELISA method. The specific clones were isolated from the non-immunized library (Yamo-I library) by using PVRV as a target. After 4th rounds of Bio-panning**,** the enrichment of specific clone was shown. There were 3.13 %, 59.38 % and 95.83 % of the positive clone from the 1st, 2rd and 4th rounds, respectively (Figure 14-16). After confirmation of the binding by ELISA, the DNA of the positive clones were extracted and digested by *Bst*NI. Six different

patterns were identified, showing all examined clones to be unique (Figure 13). The variation in patterns indicated the framework diversity in scFv phage. To characterize the ELISA positive clones, ten representative clones (Figure 13; lene 3, 4, 5, 6, 11, 13, 14, 15, 18 and 21) from a total of twenty-three ELISA positive clones (Figure 13) were analyzed by sequencing. The DNA sequencing data revealed that there were five unique clones of phages displaying anti-rabies; which are clones IIYB5v, IIYG4v, IIYE5v, IIYG8v and IIYD4v (Sequence data shown in APPENDIX C).

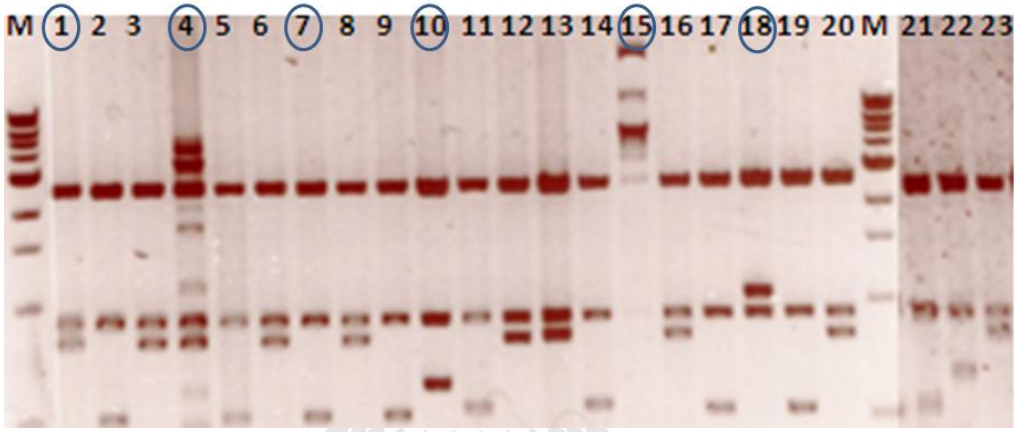


Figure 13. DNA fingerprint analysis. The twenty-three scFv clones were digested with *Bst*N1. The restriction patterns were analyzed on 1.5% agarose gel.

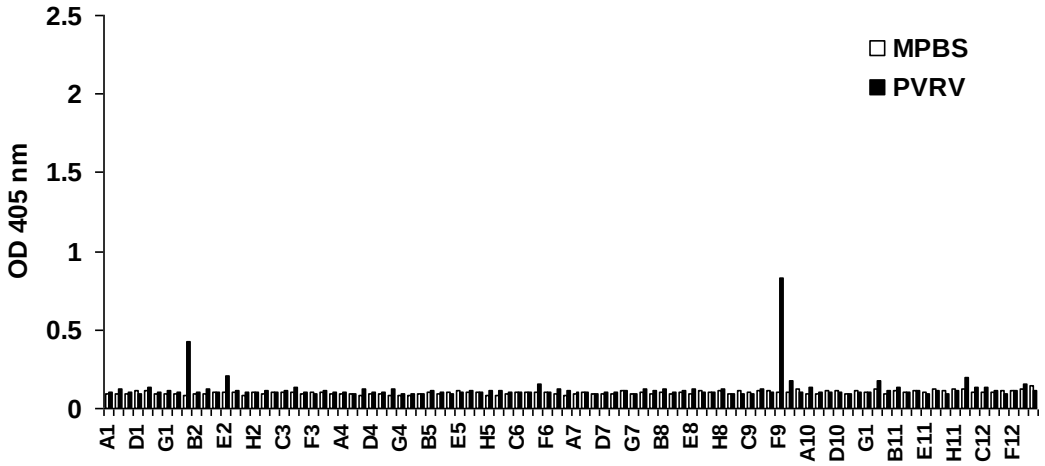


Figure 14. Binding of specific phage to PVRV from 1st round of panning. Clones were selected from Yamo-I library. Three positive clones (A2, E2, and F9) showed specific binding to PVRV and not to 2% (w/v) skim milk (MPBS negative control).

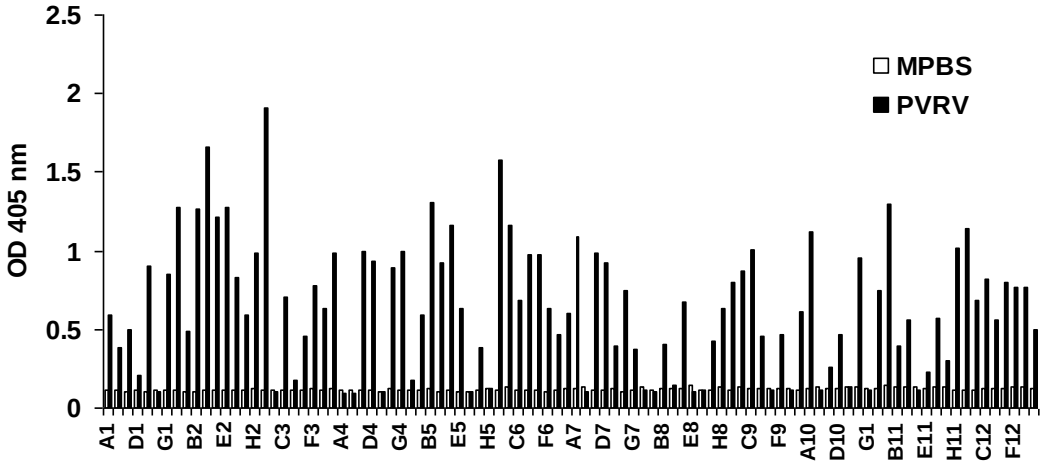


Figure 15. Binding of specific phage to PVRV from 2nd round of panning. Clones were selected from Yamo-I library. Almost all of the selected clones (57/96) showed specific binding to PVRV and not to 2% (w/v) skim milk (MPBS negative control).

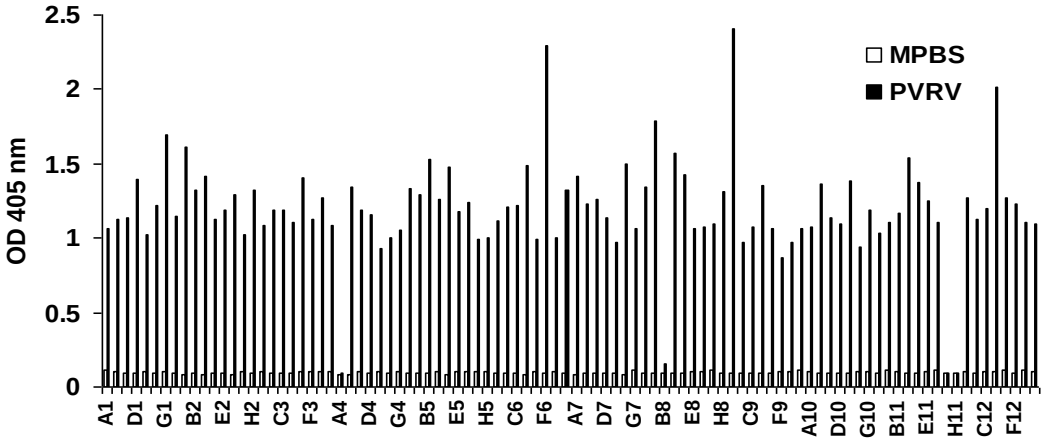


Figure 16. Binding of specific phage to PVRV from the 4th round of panning. Clones were selected from Yamo-I library. Almost of the selected clones (92/96) showed specific binding to PVRV and not to 2% (w/v) skim milk (MPBS negative control). None of them were sequence.

4.1.4 Selection of scFv fragments specific to rabies from immunized library (Yamo-Rb library), using PVRV as a target

The phage clones specific to rabies virus from the 1st, 2nd and 4th rounds were screened for the positive clone by ELISA method (Figure 17-19). The specific clones were screened from the immunized library (Yamo-Rb library) by using PVRV as a target. After 4th rounds of Bio-panning**,** the enrichment of specific clone was shown as same as the performing of selection of scFv fragments specific to rabies virus from non-immunized library (Yamo-I library) using the same target. There are 1.04 %, 73.96 % and 90.63% of the positive clone from the 1st, 2rd and 4th rounds were found (Figure 18-20). At the 1st and the 4th rounds of bio-panning, the specific phages

from Yamo-I library (3.13% and 95.83%) were found more than those of the specific phages from Yamo-Rb library (1.04% and 90.63%). Whereas, the specific phages from Yamo-Rb library (73.96%) were found more than those of the specific phages from Yamo-I library (59.38%) at the second rounds of bio-panning. However, the signal absorbance value indicated that the specific clones from Yamo-I library (OD 405 nm up to 2.5) bound to the PVRV stronger than the specific clones from Yamo-Rb library (OD 405 nm ~0.2-0.7). In order to analyze the diversity of scFv, DNA segments encoding the scFv genes from ten randomly picked clones were examined. The phagemid DNA of these clones was digested with *Bst*NI, and their fingerprint patterns were compared. Two different patterns were identified and sequence (Figure 17; lene 4 and 10). However, only clone IIRD5v (Sequence data shown in APPENDIX C) is the real binder after comfirmation by ELISA (Data not shown).

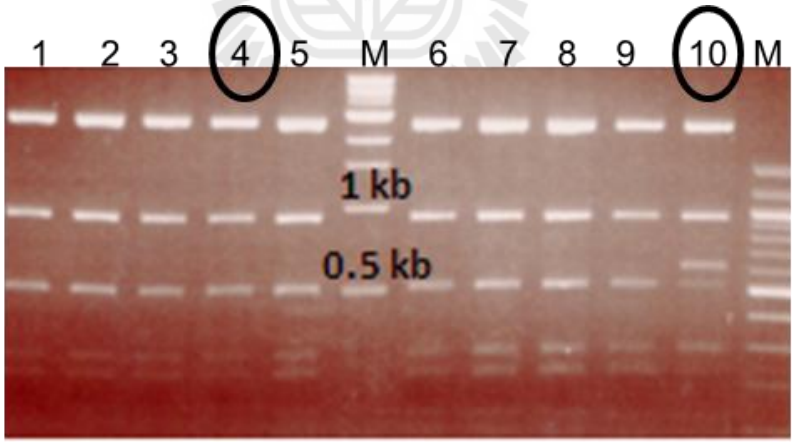


Figure 17. DNA fingerprint analysis. The ten scFv clones was digested with *Bst*N1. The restriction patterns were analyzed on 1.5% agarose gel. Clone 4 and 10 were submitted for automated DNA sequencing analysis.

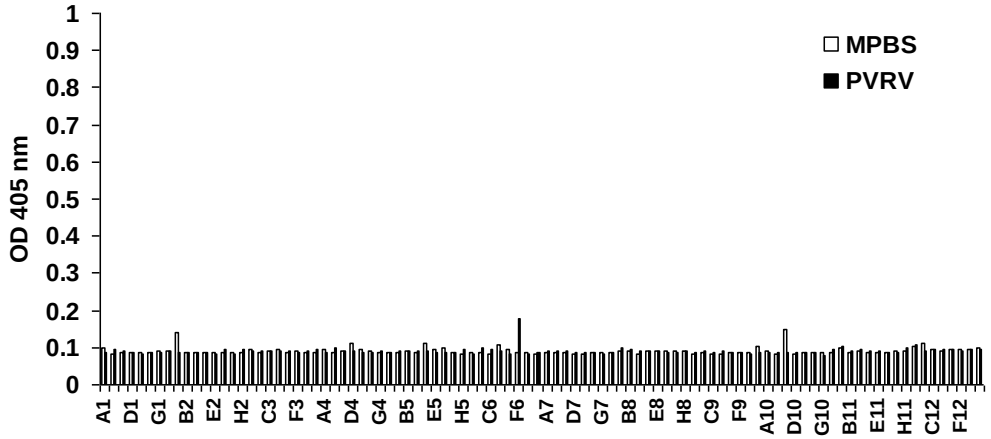


Figure 18. Binding of specific phage to PVRV from 1st round of panning. Clones were selected from Yamo-Rb immunized library. Only one clone (F6) showed specific binding to PVRV and not to 2% (w/v) skim milk (MPBS negative control).

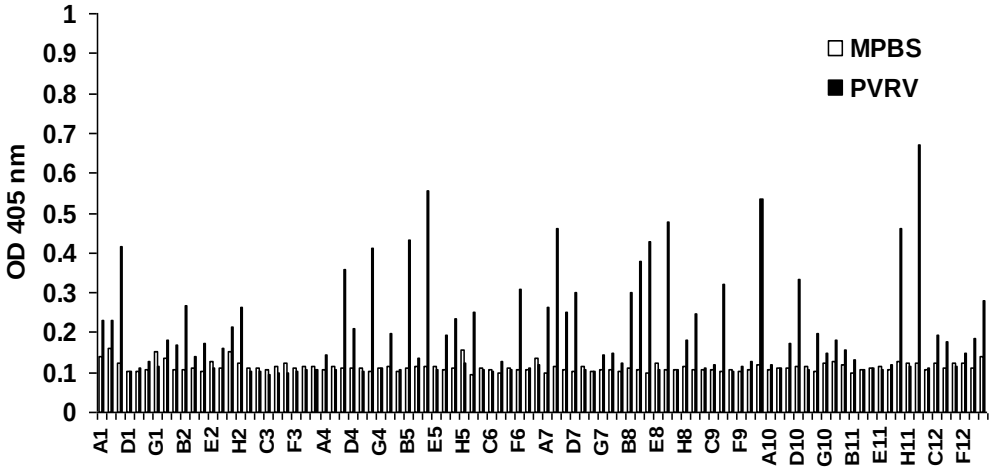


Figure 19. Binding of specific phage to PVRV from 2nd round of panning. Clones were selected from Yamo-Rb library. Almost of the selected clones (29/96) showed specific binding to PVRV and not to 2% (w/v) skim milk (MPBS negative control).

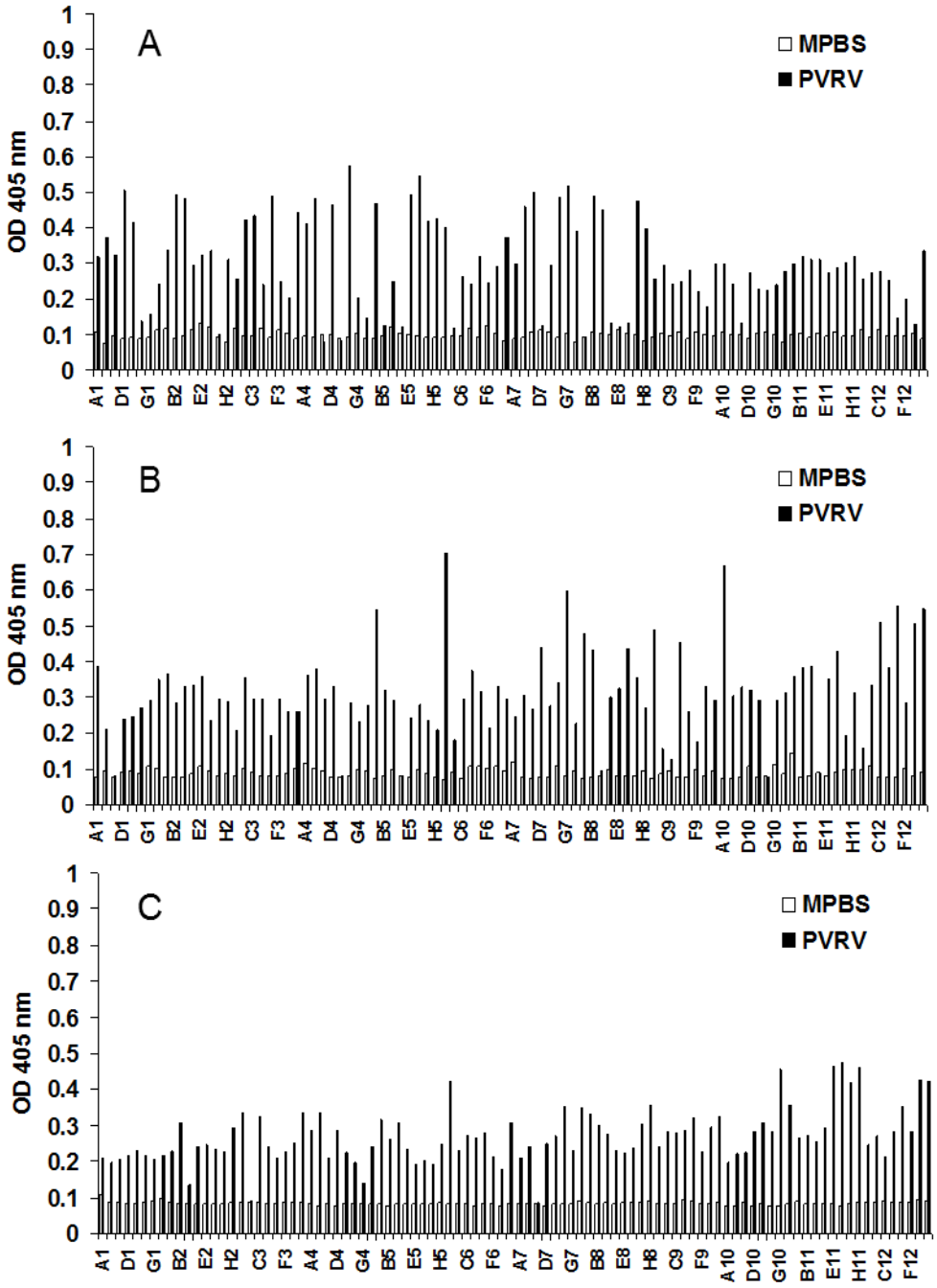


Figure 20. Binding of specific phage to PVRV from 4th round of panning. Clones were selected from Yamo-Rb library. Almost of the selected clones (261/288) showed specific binding to PVRVand not to 2% (w/v) skim milk. A) Clone 1-96; B) Clone 97-192; C) Clone 193-288.

4.1.5 Selection of scFv fragments specific to rabies from immunized library (Yamo-Rb library), using PCEC and PVRV as a target

The specific clones were screened from the immunized library (Yamo-Rb library) by using PVRV or/and PCEC as a target. At the 1st and 4th round of biopanning, both targets were mixed and immobilized for using in biopanning process. In second round, the PVRV was used as a target and the PCEC was used as a target for the third round**.** None of the selected clones showed the binding to the target at the first round (Figure 22) while almost of the selected clones showed specific binding to the both targets after the 4th round of bio-panning (Figure 23). After the positive clone form the 4th round of biopanning were randomly digested with *BstN*I, their fingerprint patterns were compared**.** All of the clones showed the same pattern. The same patterns indicated the same framework diversity in scFv phage clone (Figure 21). Clones IVB4cv (Figure 23; lene 5) were analyzed by sequencing (Sequence data shown in APPENDIX C).

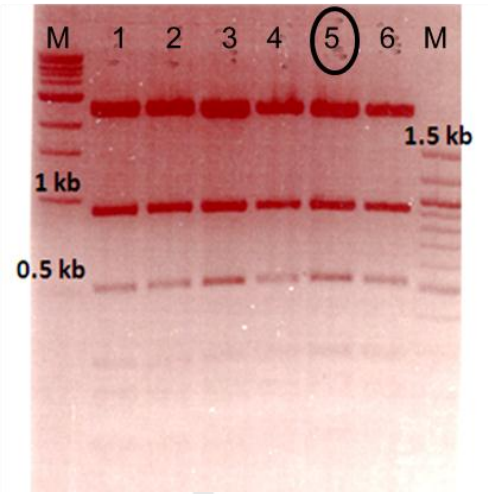


Figure 21. DNA fingerprint analysis. The six scFv clones were digested with *Bst*NI. Clone 5 was submitted for automated DNA sequencing analysis.

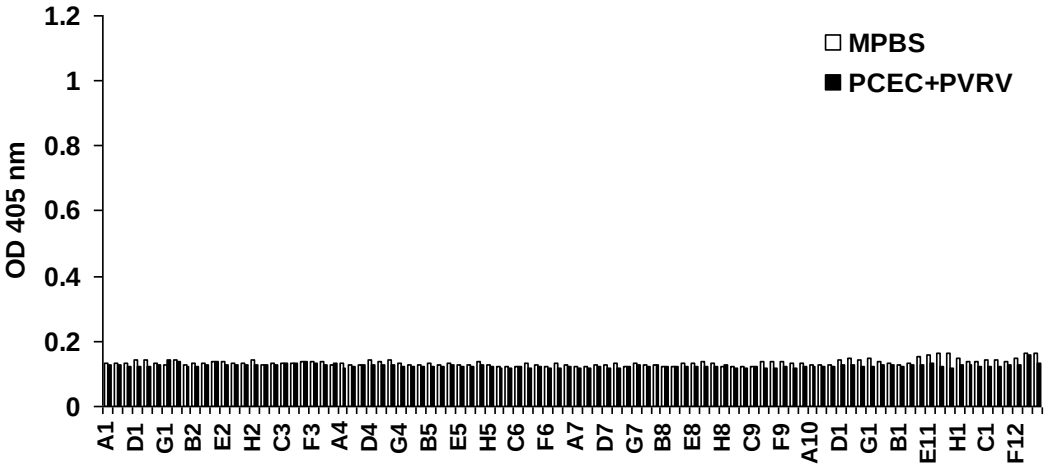


Figure 22. Binding of specific phage to PCEC together with PVRV from 1st round of panning. Clones were selected from Yamo-Rb immunized library. None showed specific binding to PCEC together with PVRV and not to 2% (w/v) skim milk (MPBS negative control).

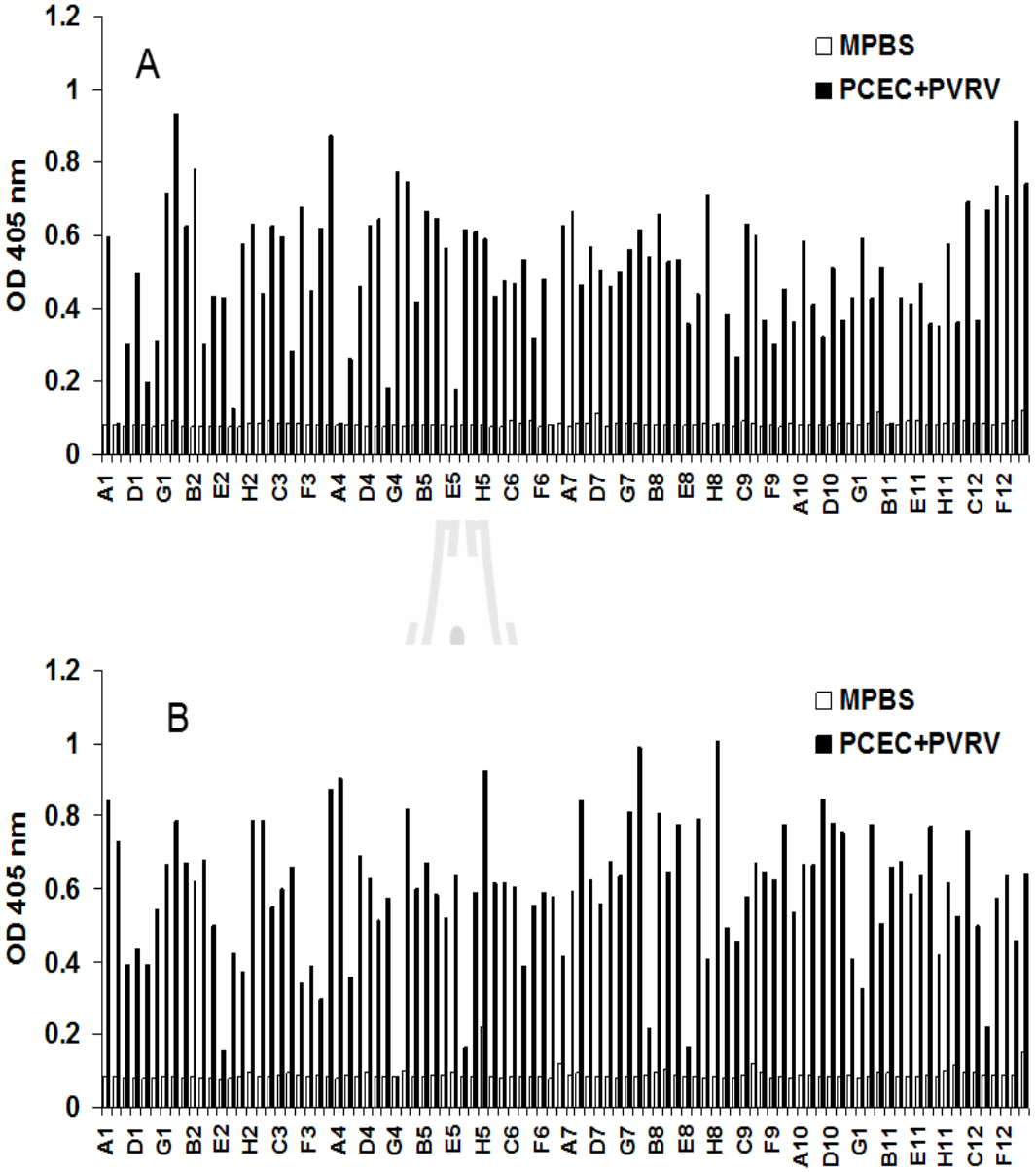


Figure 23. Binding of specific phage to PCEC together with PVRV from 4th round of panning. Clones were selected from Yamo-Rb immunized library. Almost of the selected clones (266/284) showed specific binding to PCEC together with PVRV and not to 2% (w/v) skim milk (MPBS negative control). A) Clone 1-96; B) Clone 97-192.

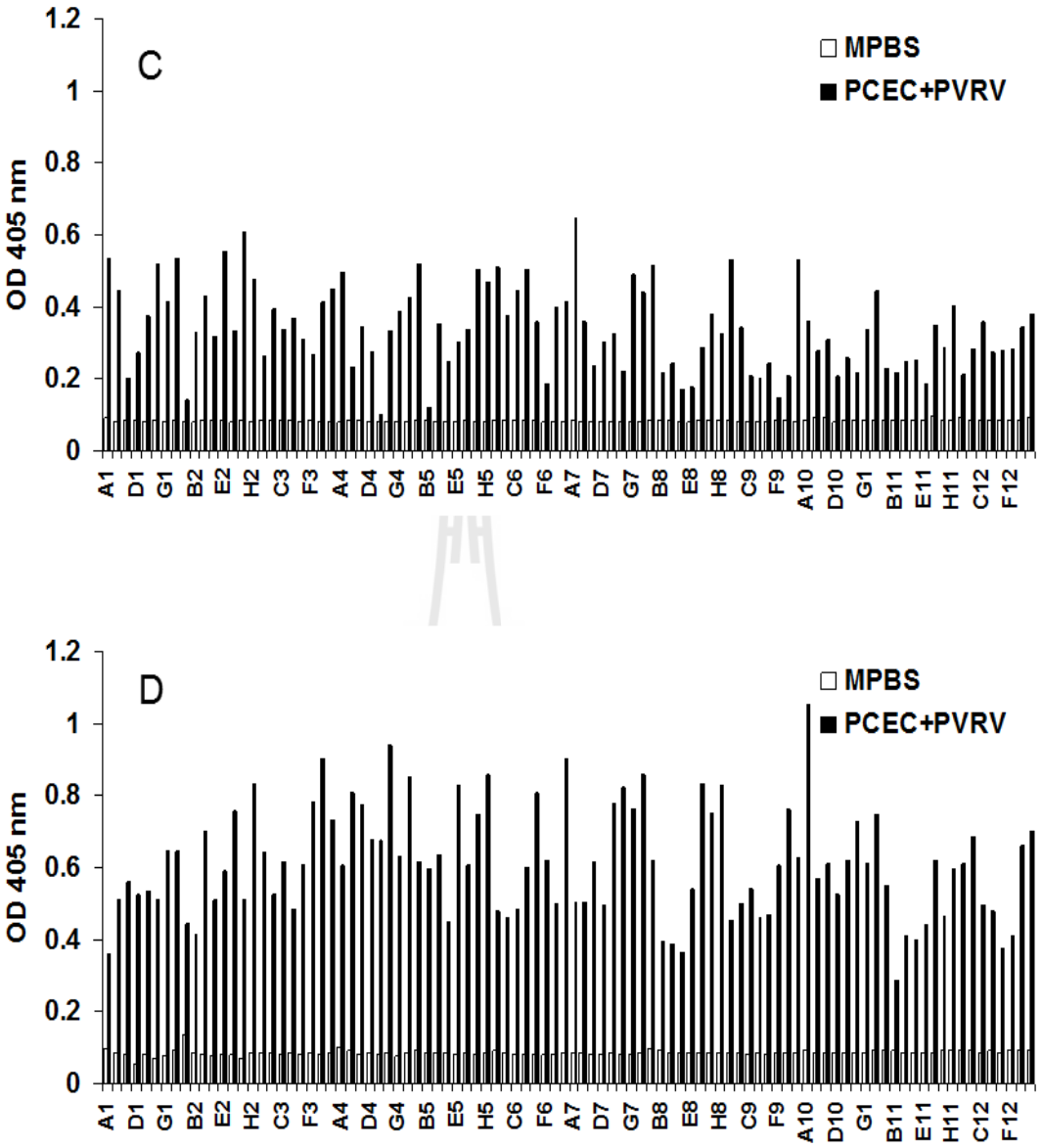


Figure 23 (cont.). Binding of specific phage to PCEC together with PVRV from 4th round of panning. Clones were selected from Yamo-Rb immunized library. C) Clone 192-288; D) Clone 289-384. Almost of the selected clones (266/284) showed specific binding to PCEC together with PVRV and not to 2% (w/v) skim milk (MPBS negative control).

4.1.6 Selection of scFv fragments specific to rabies from non-immunized library (Yamo-I library) and immunized library (Yamo-Rb library), using glycoprotein as a target

Both non-immunized library (Yamo-I library) and immunized library (Yamo-Rb library) were used for selection of the phage clone specific to rabies virus glycoprotein. The phage clones specific to rabies virus glycoprotein from the 3rd round were screened for the positive clone by ELISA method (Figure 24). Phage anti-Ervinia Clone B10 as a control that should showed specific binding to Ervinia and not to rabies virus glycoprotein. Six antigens (PCEC, PVRV, BSA, MPBS, rabies virus glycoprotein and Ervinia) were tested. Clone R1, R2, R4, Y6 and Y8 showed specific binding to glycoprotein and not to other antigens.

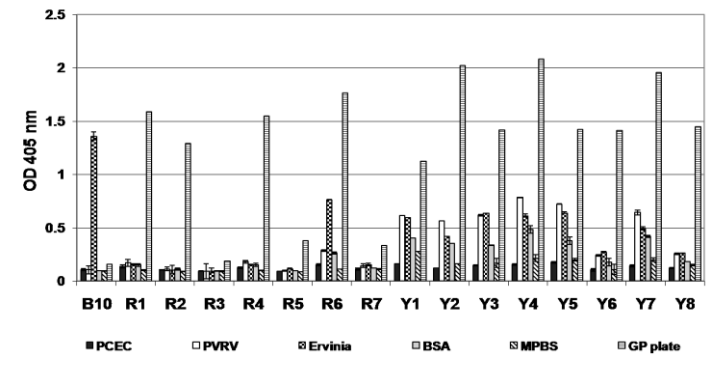


Figure 24. Binding of specific phage to PCEC, PVRV and glycoprotein plate from 3rd round of panning. Clones were selected from Yamo-Rb immunized library (clone R1-R7) and Yamo non-immunized library (Y1-Y8). Clone R1, R2, R4, Y6 and Y8 showed specific binding to glycoprotein plate and not to PCEC, PVRV, MPBS and BSA. Phage anti-Ervinia Clone B10 showed specific binding to Ervinia.

4.2 Summary of the selection of scFv fragments specific to rabies by biopanning

In summary, a total of 14 positive clones from various methods of biopanning using PCEC or/and PVRV as a target were isolated (Table 8). These are IRA7c, IIIRC2c, IRC3c, IYC11c, IYC12c, IYD1c, IYF5c, IIRD5v, IIYB5v, IIYG4v, IIYE5v, IIYG8v, IIYD4v and IVB4cv (Table 7). After DNA sequence analysis, we found that clones IVB4cv and IRA7c were identical (Table 9). These two clones were isolated from the same library, i.e., Yamo-Rb by using different biopanning method. Clone IVB4cv was obtained from the fourth round of biopanning using a combination of both PCEC and PVRV as targets; whereas clone IRA7c was from the first round of panning using PCEC as a target. Clones IRA7c, IIIRC2c, IRC3c, IYC11c, IYC12c, IYD1c and IYF5c were selected by using PCEC as a target; while IIRD5v, IIYB5v, IIYG4v, IIYE5v, IIYG8v, IIYD4v were selected by using PVRV as a target. The ELISA results in Figure 25 showed that the positive clones always bind strongly to the target that was used for biopanning. Nine clones were obtained from naïve library (YAMO-I) and four clones were from immunized library (Yamo-Rb). Five clones which are IRA7c, IIIRC2c, IIYG4v, IIYE5v, and IVB4cv showed cross-reactivity to both PCEC and PVRV targets.

Three unique phage-displayed anti-rabies were selected from non- immunized (YAMO-I) and immunized (Yamo-Rb) using glycoprotein as a target. There are R1, R4 and Y6 (Table 7). Two of them are small peptide fragment. The others clone is consisted of only VL fragment.

Table 7. A total of 16 unique clones from various method of biopanning using PCEC, PVRV and Glycoprotein as a target**.**

	Naïve library (YAMO-I)	**Immunized library (Yamo-Rb)**
PCEC	IYC11c, IYC12c, IYD1c and IYF5c	IRA7c (IVB4cv) ,IIIRC2c and IRC3c
PVRV	IIYB5v, IIYG4v, IIYE5v, IIYG8v and IIYD4v	IIRD5v
Glycoprotein	Y6	R1 and R4

Table 8. Overview of selection of antibodies against rabies virus.

Target	PCEC		PVRV		PCEC+PVRV	Glycoprotein	
Library	YAMO-I	Yamo-Rb	YAMO-I	Yamo-Rb	Yamo-Rb	YAMO-I	Yamo-Rb
Rounds of panning	1	5	4	4	4	3	3
Number of clones after 1st round	$4.3x10^2$	$3.4x10^2$	$1.3x10^3$	$1.3x10^3$	$1.7x10^3$	$2x10^3$	$1.36x10^5$
Number of binding phage after 1st round	4/192	13/96	3/96	1/96	0/96	-	-
Number of clones after 2nd round	-	$3.11x10^5$	$1.7x10^5$	$2.1x10^3$	$1.2x10^3$	$5.95x10^6$	$7.05x10^6$
Number of binding phage after 2nd round	-	-	57/96	29/96	-	-	-
Number of clones after 3rd round	-	$8.7x10^5$	$1.4 x10^6$	$8.0x10^4$	$1.5x10^3$	$5.05x10^6$	$1.93x10^6$
Number of binding phage after 3rd round	-	44/48	-	-	-	2/8	3/7
Number of clones after 4th round	-	$1.47x10^8$	$4.7 x10^6$	$8.2x10^4$	$1.4x10^3$	-	-
Number of binding phage after 4th round	-	-	92/96	261/288	366/384	-	-
Number of clones after 5th round	-	$4.7x10^6$	-	-	-	-	-
Number of binding phage after 5th round	-	24/96	-	-	-	-	-
Number of difference scFv clones	4	3	5	1	1	1	2

Table 9. Amino acids sequence of selected positive clones against rabies.

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
VH									
IRA7c	QVQLVQSGGGLVQPGGSL RLSCAASGFSFS	DYGMHW	VRQIPGKGLEVA	VIYARGINTYYGDSVKGR	FTISRDNSKNILYLEMNRLSSEDTAVYYCAT	DDPPSGTGSYHVNWGQGTL	IGHV3-33*01	18	VH3
IIIRC2c	QVQLQESGPGLVKPFGDPGPHCTVSGGSLS	SVNSYWD	FIRQPPGKGLEWIG	SIYYRGTTYYNPSLKS	RVTLSVDTSQNQISLKLTSLTAADTAVYYCAR	ESVTRGTFDMWGQGTMV	IGHV4-39*07	19	VH4
IRC3c									
IYC11c	QLVESGAEVKKPGASVKVSCKASGYSFT	AYYIHW	VRQAPGQGLEWMG	WINPNSGTTTYAQRFQG	RVTMTRDTSISTAYMELSRLRSDDTAVYYCAR	DFGGYWRWGAFDIWGQGT TV	IGHV1-2*02	7	VH1
IYC12c	ESGGGLVQPGGSLRLSC AASGFTFS	SYAMSW	VRQAPGKGLEWVSS	ITYSGTATSYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	GYSTFDYWGQGTLVTV	IGHV3-23*01	6	VH3
IYD1c	ESGGGLVQPGGSLRLSC AASGFTFS	SYAMSW	VRQAPGKGLEWVSA	ITYNGASTAYADSVKG	RFTISRDNS*NTLYLQMNSLRAEDTAVYYCAK	GYSTFDYWGQGTLVTV	IGHV3-23*01	6	VH3
VL									
IRA7c	QSALTQPASVSGSPGQS ITISC	TGTSSDVGSYNLVS	WYQQHPGKAPKLMIY	EVSKRPSG	VPDRFSGSKSGNTASLTVSGLQAEDEADYYC	SSYTSITAAAVFGTGTKVTVL	IGLV2-14*02	7	VL2
IIIRC2c	QSALTQPASVSGSPGQSITISC	TGTSSDVGGYNYVS	WYQQHPGKAPNLMIY	VSNRPSG	VSNRFSGSKSGNTASLTISRLQAEDEGIYFC	SAYTSSSSLGVFGTGTKLTVL	IGLV2-14*01	8	VL2
IRC3c	QSALTQPASVSGSPGQSITISC	TGTSSDVGGYNYVA	WYQHHPGKAPKLMIY	DVSNRPSG	VSNRFSGSKSGNTASLTISGLQAEDEADYYC	SSYTSSSTLVVFGGGTKVTVL	IGLV2-14*01	3	VL2
IYC11c	SYVLTQPPSVSVAPGKT ARITC	GGNNIGSKSVH	WYQQKPGQAPVLVVY	DDSDRPSG	IPERFSGSNSGNTATLTISRVEAGDEADYYC	QVWDSSSDHYVFGTGTQLTV	IGLV3-21*03	0	VL3
IYC12c	QAVLTQPSSTSGTPGQR VTISC	SGGSSNIGSNTVN	WYRHLPGTAPKLLIYI	DDRRPSDI	PDRFSGSRSGTSASLAISGLQSEDEADYYC	AAWDDSLNGLVFGGGTQLTV	IGLV1-44*01	13	VL1
IYD1c	SSKLTQDPAVSVALGQT VRITC	QGDSLRSYYAS	WYQQKPGQAPVLVIY	GKNNRPSG	IPDRFSGSKSGTSASLDISGLQSEDEADYYC	AAWDDSLNGVLFGGGT	IGLV3-19*01	8	VL3

Table 9. Amino acids sequence of selected positive clones against rabies. (Continued)

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
VH									
IYF5c	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	SYGISW	VRQAPGQGLEWMG	WISAYNGNTNYAQKLQG	RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAD	GGNFDYWGQGTLVTV	IGHV1-18*01	0	VH1
IIRD5v	VQLVESGTEVRKPGDSVKVSCKSGYTFT	DYYLHW	VRQAPGQGLEWVG	WIYPKRGGTHSAQKFQG	RVTMTRDTSINTAYMELTRLRSDDTAVYFCAR	DRDIEDAFDIWGQGTMVTV	IGHV1-2*02	15	VH1
IIYB5v	QV*LVQSGAEVKKPGSSVKVSCKASGGTFS	SYAIS	WVRQAPGQGLEMG	GIIPIFGTANYAQKFQG	RVTTADESTSTAYMELSSLRSEDTAVYYCAR	DRELPGFDYWGQGTLVTVYYCAR	IGHV1-69*01	1	VH1
IIYG4v	QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWLG	RTYYRSKWYNDYAVSVKS	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	ERMGGFDPWGQGTLV	IGHV6-1*01	0	
IIYE5v	QVQLQQSGPGLVKPPQTLSLTCAISGDSVS	SNTAAWN	WIRQSPSRGLEWLG	RTYYRSKWHNDYAVSVNS	RISINPDTSKNQFSLQLDSVTPEDTAVYYCAR	DRYYGSGSYYRGFDYW	IGHV6-1*01	5	VH6
IIYG8v	EVQLVESGGGLVQPGRSLRLSCAASGFTFD	DYAMH	WVRQAPGKGLEWVS	GISWNSGSIGYADSVKG	RFITSRDNAKSSLYLQMNSLRAEDTALYYCAK	GGHRGAFDIWGQGTMVTV	IGHV3-9*01	1	VH3
VL									
IYF5c	QAVLTQPSSASGTPGQRVTISC	SGSSSNIGSNYVY	WYQQLPGTAPKLIY	RNNQRPSG	VPDRFSGSKSGTSASLAISGLRSEDEADYYC	AAWDDSLSGPVFGGGTKLTVL	IGLV1-47*01	2	VL1
IIRD5v	QSALTQPASVSGSPGQSITISC	TGTSSDVGGYNYVS	WYQQHPAKAPNLLIY	DVSNRPSG	VSNRFSGSKSGNTASLTISGLQAEDEADYFC	SSYTTSSTLVFGGGTKVTVL	IGLV2-14*01	5	VL2
IIYB5v	VLTQPPSASGTPGQRVTISC	SGSSSNIGSNTVN	WYQQLPGTAPKLLIY	SNNQRPS	GVPDRFSGSKSGTSASLAISGLQSEDEADYYC	ATWDNRNAEWVFGGGTKLTV	IGLV1-44*01	3	VL1
IIYG4v	NFMLTQPHSVSESPGKTVTISC	TRTSGSIASNYVQ	WYQQRPGSSPTTVIF	EDTARPSG	VPARFSGSIDPFSNSASLTISGLKAEDEADYYC	HSYDVHNQVFGGGTKLTVL	IGLV6-57*01	11	
IIYE5v	NFMLTQPHSVSGSPGKTVTLSC	TRNSGSIASAYVQ	WFRQRPGSAPTTVIY	EDNQRPSG	VPARFSGSIDSSNSASLTISGLQTEDEADYYC	QSYDFTNYVFGTGTQLTV	IGLV6-57*01	11	VL6
IIYG8v	VLTQPPSASGTPGQRVTISC	SGGSSNIGGNTVN	WYQVPPRTAPKLIY	NNNQRPSG	VPDRFSGSKSGTSASLAISGLQSEDEADYYC	ATWDDSLHGVVFGGGTKVTVL	IGLV1-44*01	8	VL1

Table 9. Amino acids sequence of selected positive clones against rabies. (Continued)

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
VH									
IIYD4v	QVNLRESGGVVVQPGGSL RLSCAAS	GFTFNNYN	MNWVRQAPGKGLEWVSS	ISSGSSYI	YYADSVKGRFTISRDNAKNSLFLQMNSLRAEDTAVYYC	ARVGGRHAYGMDV	IGHV3-026	11	VH3
IVB4cv	QV*LVQSGGGLVQPGGSL RLSCAAS	GFTFSSYA	MSWVRQAPGKGLEWVSA	ISGSGGST	YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC	AKNPVTSYYFDY	IGHV3-157	3	VH3
VL									
IIYD4v	QSVLTQPPLVSGTPGQRV TISCTGT	SSDVGGYNY	VSWYQQHPGKAPKLIY	DVS	NRPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYC	SSYTSSSTLV	IGLV2-137	7	VL2
IVB4cv	QAVLTQPSSLSASPGASA RLTCTLR	SGLNVGAYR	MYWYQKKPGSPPQFLLM	YKSESSN	HRASGLSSRFSGSKDSSANEGILSISGLQSADAADYYC	MIWHNSVVEIGGGTQLT	IGLV5-062	22	VL5

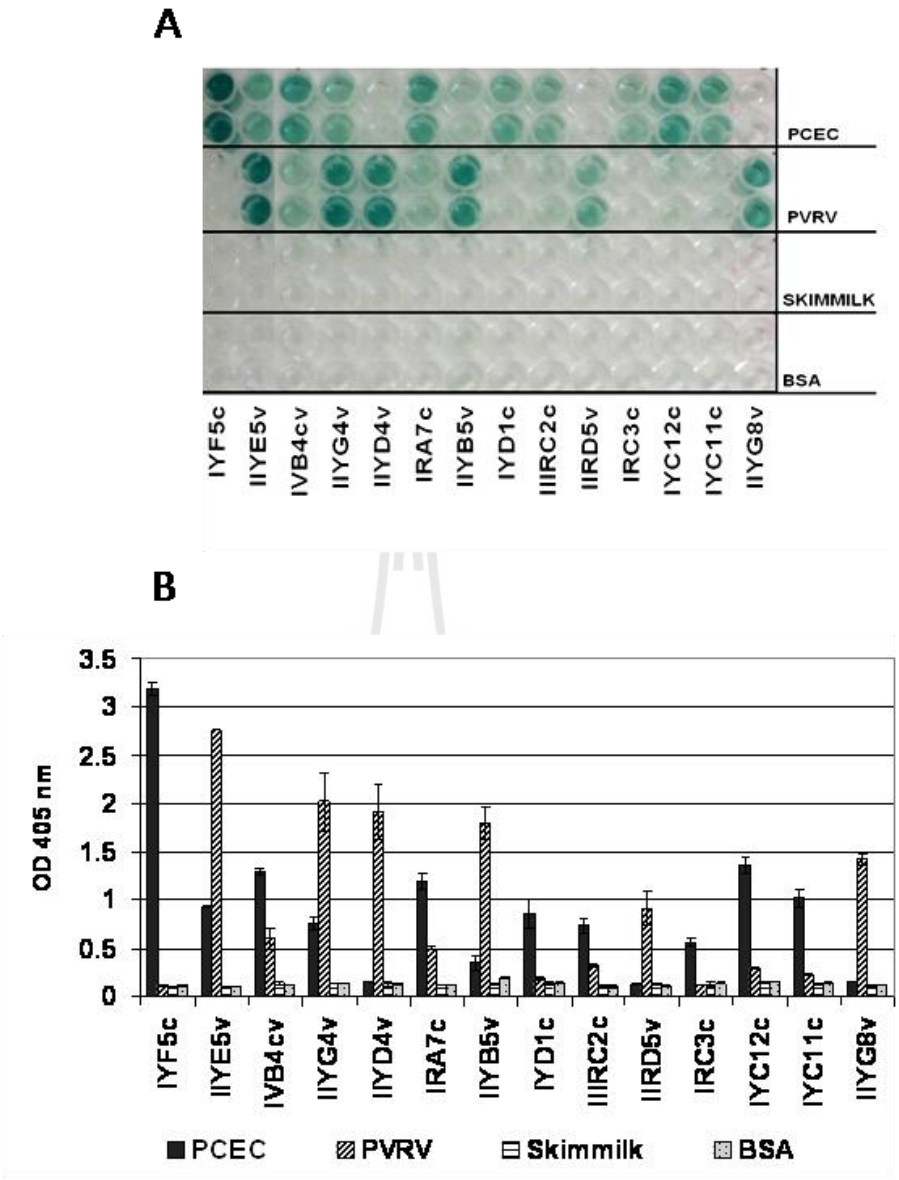


Figure 25. ELISA of isolated 14 clones. A. ELISA plate showing positive reactions (green color) with PCEC or PVRV and negative reaction (clear) with 2% skim milk and 3 % BSA. B. ELISA signal at O.D. 405 nm.

Finally, the individual clone from each selection was tested by ELISA together using PCEC, PVRV or Glycoprotein as a target (Figure 26-27). Clones IRA7c(A7), IIIRC2c(C2), IRC3c(C3), IYC11c(C11), IYC12c(C12), IYD1c(D1) and IYF5c(F5) were selected by using PCEC as a target; while IIRD5v(D5), IIYB5v(B5), IIYG4v(G4), IIYE5v(E5), IIYG8v(G8), IIYD4v(D4) were selected by using PVRV as a target. Specific binding of VL of human IgG to the target has never been report before. A very small antibody fragment might be able to pass through blood brain barrier and can be use to treat the disease. Clones IIIRC2c (C2) and IRC3c (C3) showed the binding to the glycoprotein even if they were selected by using PCEC for biopanning. This indicated that these clones bound to the glycoprotein of the inactivated rabies virus. Clones R1, R2, R4, Y6 and Y8 were selected by using glycoprotein as a target. Every clone bound strongly to the target that was used for selection. All of the clones isolated using viral glycoprotein contained only partial scFv fragments and none of them could bind to whole the inactivated virus (PCEC and PVRV).

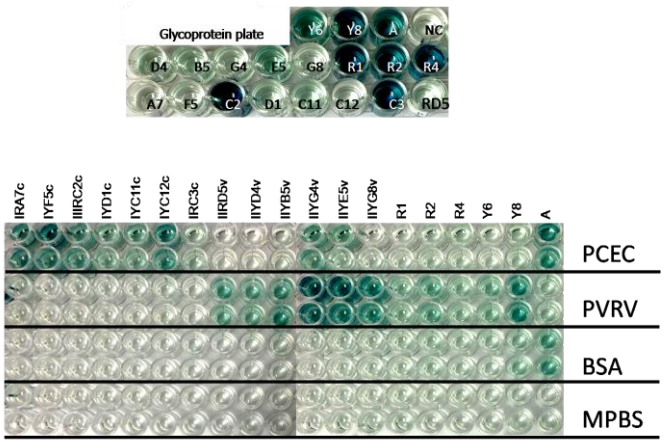


Figure 26. ELISA plate showing positive reactions (green color) with PCEC, PVRV or Glycoprotein and negative reaction (clear) with 2% skim milk and 3% BSA. (A) The immunized human serum is a positive control and NC is a non-immunized human serum (negative control).

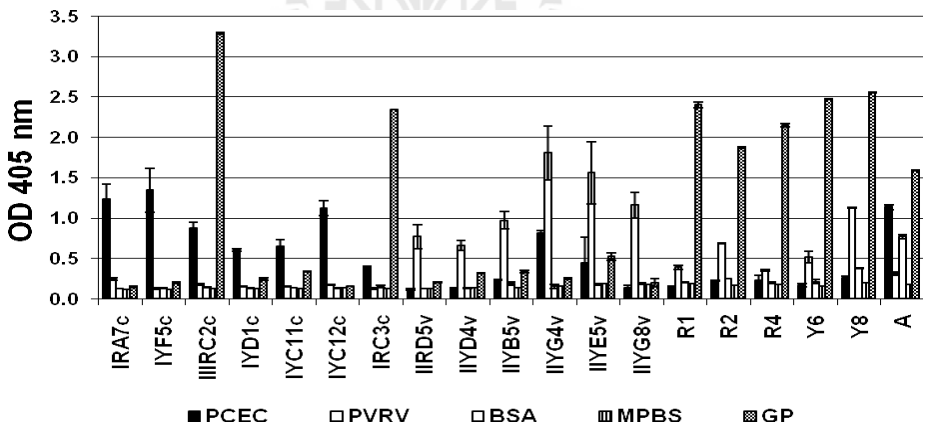


Figure 27. Binding of specific phage to PCEC, PVRV and glycoprotein plate. Positive clones were selected from Yamo-Rb immunized library and Yamo non-immunized library with various method of biopanning.

4.3 DNA and Amino acid sequence analysis

4.3.1. Finger printing analysis

The *BstN*I fingerprinting analysis of 14 positive clones was performed. All the fingerprinting of the positive clones were selected by using PCEC or/and PVRV as a target. The fingerprinting was shown in Figure 28. In this figure pMOD (empty vector) was digested to compare with the positive scFv clones (pMOD+scFv gene).

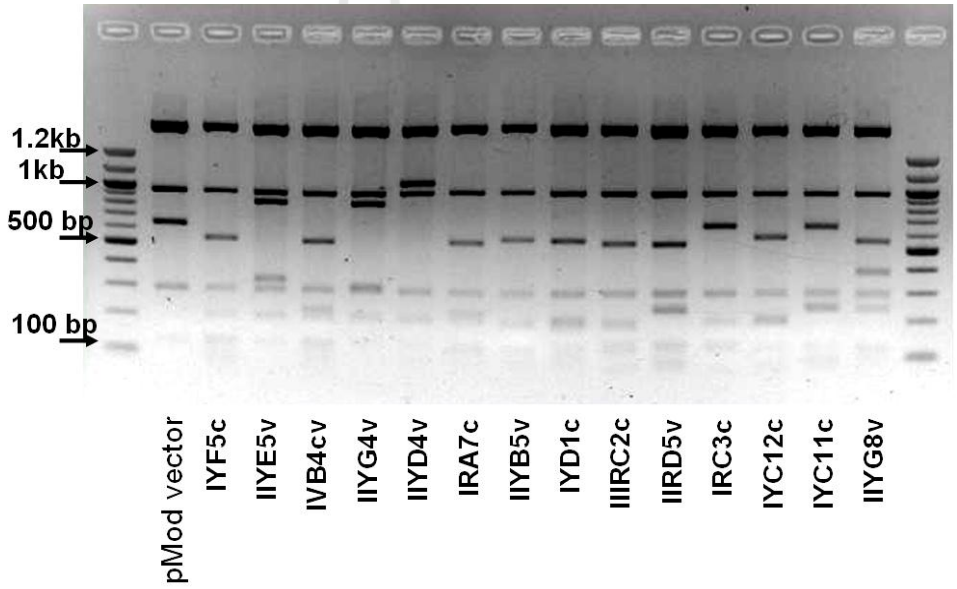


Figure 28. DNA fingerprint patterns of scFv gene from the selection against inactivated rabies vaccines (PVRV and PCEC). The *BstN*I digest of scFv genes identified fourteen positive clones compare to pMOD1 vector. Lene 1 and 17, DNA marker (100 bp ladder).

4.3.2. DNA sequence analysis

4.3.2.1 Selected positive clones from biopanning using PCEC or/and PVRV as a target.

After the positive cloned were confirmed by automated DNA sequencing. The DNA sequence of each scFv clone was analyzed with Igblast and the sequence alignment of the 14 scFv antibodies was done using CLUSTALW software. The origin of germline and family of all the isolated VH and VL segments were summarized in Table 9; whereas, the amino acid sequence alignment of all positive clones were illustrated in Figure 29. It is interesting to note that clone IRC3c, which consist only VL, could still bind to the target, even if the signal is quite low when compared to other positive clones. There have been many reports on the binding of V_{HH}, which is a single-domain antibody (sdAb) that consists only of VH domain from camelidae (Kierny et al., 2012, Hultberg et al., 2011 and Vanlandschoot et al., 2011). Our data is the first report on the binding of single VL domain of human antibody. It is very small antibody fragment and it might be able to pass through blood brain barrier and can be use to treat the disease.

Figure 29 showed the amino acid sequence alignment of all isolated scFv clones. The TAG amber stop codons were found in clone IIYB5v, IIYD4v and IYD1c. These codons are translated as glutamine instead of stop codon in *E. coli* suppressor strain such as DH5αF' or TG1; therefore, the functional antibody can be displayed on filamentous phage. The complementary determining regions (CDRs) of the heavy and light chain of the antibody (VH/CDR1, VH/CDR2, VH/CDR3, VL/CDR1, VL/CDR2 and VL/CDR3) and the linker sequence were indicated. Amino

acid sequence analysis revealed that all isolated positive clones have only the lambda type of the light chain (VL) from family 1, 2, 3, and 6; while variable heavy chains (VH) were from family 1, 3, 4 and 6. No clone containing the kappa type of the light chain (VK) was isolated (Table 9). The previous work shown that anti-rabiesD7 was identical with clone IYF5c which was previously selected from the same library by using the same target (Pansri et al, 2009).

```
IYF5c           MAQVQLVQSGAEVKKPGASVKVSCKASG--YTFTSYGISWVRQAPGQGLEWMGWISAYNG 58
IIYB5v          MAQVQLVQSGAEVKKPGSSVKVSCKASG--GTFSSYAISWVRQAPGQGLEWMGGIIPIFG 58
IYC11c          ---MQLVESGAEVKKPGASVKVSCKASG--YSFTAYYIHWVRQAPGQGLEWMGWINPNSG 55
IVB4cv          MAQVQLVQSGGGLVQPGGSLRLSCAASG--FSFSDYGMHWVRQIPGKGLEWVAVIYARGI 58
IRA7c           MAQVQLVQSGGGLVQPGGSLRLSCAASG--FSFSDYGMHWVRQIPGKGLEWVAVIYARGI 58
IIIRC2c         MAQVQLQESGPGLVKPFGDPGPHCTVSGGSLSSVNSYWDFIRQPPGKGLEWIGSIY-YRG 59
IRC3c           MAQVNLRESG---------------------------------------------------- 10
IIRD5v          MAEVQLVESGTEVRKPGDSVKVSCKASG--YTFTDYYLHWVRQAPGQGLEWVGWIYPKRG 58
IYD1c           MAQVNLRESGGGLVQPGGSLRLSCAASG--FTFSSYAMSWVRQAPGKGLEWVSAITYNGA 58
IYC12c          MAQVNLRESGGGLVQPGGSLRLSCAASG--FTFSSYAMSWVRQAPGKGLEWVSSITYSGT 58
IIYG8v          MAEVQLVESGGGLVQPGRSLRLSCAASG--FTFDDYAMHWVRQAPGKGLEWVSGISWNSG 58
IIYE5v          MAQVQLQQSGPGLVKPPQTLSLTCAISGDSVSSNTAAWNWIRQSPSRGLEWLGRTYYRSK 60
IIYG4v          MAQVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYRSK 60
IIYD4v          MAQVQLVQSGGGVVQPGRSLRLSCAASGFTFSS--YAMHWVRQAPGKGLEWVAVISYDGS 58
                   ::* :**

IYF5c           -NTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCADGG---------NFDYWG 108
IIYB5v          -TANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARDRE------LPGFDYWG 111
IYC11c          -TTTYAQRFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARDFGGY--WRWGAFDIWG 112
IVB4cv          -NTYYGDSVKGRFTISRDNSKNILYLEMNRLSSEDTAVYYCATDDPPS--GTGSYHVNWG 115
IRA7c           -NTYYGDSVKGRFTISRDNSKNILYLEMNRLSSEDTAVYYCATDDPPS--GTGSYHVNWG 115
IIIRC2c         -TTYYNPSLKSRVTLSVDTSQNQISLKLTSLTAADTAVYYCARESVTR--GT---FDMWG 113
IRC3c           ------------------------------------------------------------ 
IIRD5v          -GTHSAQKFQGRVTMTRDTSINTAYMELTRLRSDDTAVYFCARDRDIE--DA---FDIWG 112
IYD1c           -STAYADSVKGRFTISRDNSQNTLYLQMNSLRAEDTAVYYCAKG--------YSTFDYWG 109
IYC12c          -ATSYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKG--------YSTFDYWG 109
IIYG8v          -SIGYADSVKGRFTISRDNAKSSLYLQMNSLRAEDTALYYCAKGGH------RGAFDIWG 111
IIYE5v          WHNDYAVSVNSRISINPDTSKNQFSLQLDSVTPEDTAVYYCARDRYYGSGSYYRGFDYWG 120
IIYG4v          WYNDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARERMG-------GFDPWG 113
IIYD4v          -NKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARLDITMIPEGPDAFDIWG 117


IYF5c           QGTLVTVSSGGGGSGGGGSGGGGS-QAVLTQPSSASGTPGQRVTISCSGSSSNIGSNY-V 166
IIYB5v          QGTLVTVSSGGGGSGGGGSGGDGS-SYVLTQPPSASGTPGQRVTISCSGSSSNIGSNT-V 169
IYC11c          QGTTVTVSSGGGGSGGGGSGGSGS-SYVLTQPPSVSVAPGKTARITCGGN--NIGSKS-V 168
IVB4cv          QGTLVTVSSGGGGSGGGGSGGGGS-QSALTQPASVSGSPGQSITISCTGTSSDVGSYNLV 174
IRA7c           QGTLVTVSSGGGGSGGGGSGGGGS-QSALTQPASVSGSPGQSITISCTGTSSDVGSYNLV 174
IIIRC2c         QGTMVTVSSGGGGSGGGGSGGGGS-QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYV 172
IRC3c           --TTVTVSSGGGGSGGGGSGGGGS-QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYV 67
IIRD5v          QGTMVTVSSGGGGSGGGGSGGGGS-QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYV 171
IYD1c           QGTLVTVSSGGGGSGGGGSGGGGS-SSKLTQDPAVSVALGQTVRITCQGDS--LRSYY-A 165
IYC12c          QGTLVTVSSGGGGSGGGGSGGSGS-QAVLTQPSSTSGTPGQRVTISCSGGSSNIGSNT-V 167
IIYG8v          QGTMVTVSSGGGGSGGGGSGGGGS-SYVLTQPPSASGTPGQRVTISCSGGSSNIGGNT-V 169
IIYE5v          QGTMVTVSSGGGGSGGGGSGGGGS-NFMLTQPHSVSGSPGKTVTLSCTRNSGSIASAY-V 178
IIYG4v          QGTLVTVSSGGGGSGGGGSSGGGS-NFMLTQPHSVSESPGKTVTISCTRTSGSIASNY-V 171
IIYD4v          QGTMVTVSSGGGGSGGGGSGGGGSSNFMLTQPHSVSESPGKTVTISCTRSSGSIASNY-V 176
                  * ***************.*.** .  ***  :.* : *:   ::*      : .   .
```

```
IYF5c           YWYQQLPGTAPKLLIYRNNQRPSGVPDRFSGSK--SGTSASLAISGLRSEDEADYYCAAW 224
IIYB5v          NWYQQLPGTAPKLLIYSNNQRPSGVPDRFSGSK--SGTSASLAISGLQSEDEADYYCATW 227
IYC11c          HWYQQKPGQAPVLVVYDDSDRPSGIPERFSGSN--SGNTATLTISRVEAGDEADYYCQVW 226
IVB4cv          SWYQQHPGKAPKLMIYEVSKRPSGVPDRFSGSK--SGNTASLTVSGLQAEDEADYYCSSY 232
IRA7c           SWYQQHPGKAPKLMIYEVSKRPSGVPDRFSGSK--SGNTASLTVSGLQAEDEADYYCSSY 232
IIIRC2c         SWYQQHPGKAPNLMIYDVSNRPSGVSNRFSGSK--SGNTASLTISRLQAEDEGIYFCSAY 230
IRC3c           AWYQHHPGKAPKLMIYDVSNRPSGVSNRFSGSK--SGNTASLTISGLQAEDEADYYCSSY 125
IIRD5v          SWYQQHPAKAPNLLIYDVSNRPSGVSNRFSGSK--SGNTASLTISGLQAEDEADYFCSSY 229
IYD1c           SWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSK--SGTSASLDISGLQSEDEADYYCAAW 223
IYC12c          NWYRHLPGTAPKLLIYIDDRRPSDIPDRFSGSR--SGTSASLAISGLQSEDEADYYCAAW 225
IIYG8v          NWYQVPPRTAPKLLIYNNNQRPSGVPDRFSGSK--SGTSASLAISGLQSEDEADYYCATW 227
IIYE5v          QWFRQRPGSAPTTVIYEDNQRPSGVPARFSGSIDSSSNSASLTISGLQTEDEADYYCQSY 238
IIYG4v          QWYQQRPGSSPTTVIFEDTARPSGVPARFSGSIDPFSNSASLTISGLKAEDEADYYCHSY 231
IIYD4v          QWYQQRPGSAPTTVIYEDNQRPSGVPDRFSGSIDSSSNSASLTISGLKTEDEADYYCQSY 236
                 *::  *  :*  :::    ***.:. *****    ..:*:* :* :.: **. *:*  :

IYF5c           DDSLSGP-VFGGGTKLTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA 271
IIYB5v          DNRLNAEWVFGGGTKLTVLRAAAHHHHHHGAAGPEQKLISEEDLNGTA 275
IYC11c          D-SSSDHYVFGTGTQLTVLRAAAHHHHHHGAAGPEQKLISEEDLNGTA 273
IVB4cv          T-SITAAAVFGTGTKVTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA 279
IRA7c           T-SITAAAVFGTGTKVTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA 279
IIIRC2c         T-SSSSLGVFGTGTKLTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA 277
IRC3c           T-SSSTLVVFGGGTKVTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA 172
IIRD5v          T-TSSTL-VFGGGTKVTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA 275
IYD1c           D-DSLNGVLFGGGTKVTALRAAAHHHHHHGAAGPEQKLISEEDLNGTA 270
IYC12c          D-DSLNGLVFGGGTQLTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA 272
IIYG8v          D-DSLHGVVFGGGTKVTVLRAAAHHHHHHGAAGPEQKLISEEDLNGTA 274
IIYE5v          D---FTNYVFGTGTQLTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA 283
IIYG4v          D---VHNQVFGGGTKLTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA 276
IIYD4v          D---SSNVVFGGGTKVTVLGAAAHHHHHHGAAGPEQKLISEEDLNGTA 281
                  :** **::*.* **************************
```

Figure 29. Amino acid sequence alignments of all positive clones are shown. The GC-rich sequence that links V_H and V_L segments is indicated (*Italic*). The three complementary determining regions (CDRs) are marked with box. These scFv antibodies are linked with 6xHis and Myc epitope at the C-terminal. Under line Q is glutamine that is translated from an amber stop *codon* in suppressor *E. coli* strains.

4.3.2.2 Selected positive clones from biopanning using glycoprotein as a target

The positive clones selected from biopanning using glycoprotein as a target were confirmed by automated DNA sequencing. The DNA sequence of each clone was analyzed with Igblast and the sequence alignment of the 5

positive clones were done using CLUSTALW software (Figure 30). Clone R1 and R2 are identical. Both clones contain just 77 amino acids whereas the normal scFv usually contain approximately ~250 amino acids. Clone R4 is similar to these two clones (R1 and R2). This clone contains 76 amino acids. Clone Y6 and Y8 are also identical. They are consist only VL. All of the selected clone could still bind to the target (Figure 26-27), even if none of the clones contains full length of scFv. All clones contain concensus motif DIVMTQSP.

```
R1              MAQVNLRESGTTVTVSSGGGGSGGGGSGGGGSDIVMTQSPS------------------- 41
R2              MAQVNLRESGTTVTVSSGGGGSGGGGSGGGGSDIVMTQSPS------------------- 41
R4              MAQVQLQESGTMVTVSSGGGGSGGGGSGGGGSDIVMTQSP-------------------- 40
Y6              MAQVNLRESGNPGHRLLRWWWQRGGGSGGGGSDIQMTQSPSSLSASVGDRVTVTCRASQS 60
Y8              MAQVNLRESGNPGHRLLRWWWQRGGGSGGGGSDIQMTQSPSSLSASVGDRVTVTCRASQS 60
                ****:*:***.          . *********** *****

R1              ------------------------------------------------------------
R2              ------------------------------------------------------------
R4              ------------------------------------------------------------
Y6              ISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC 120
Y8              ISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC 120


R1              -----------GPRLEIKRAAAHHHHHHGAAGPEQKLISEEDLNGTA 77
R2              -----------GPRLEIKRAAAHHHHHHGAAGPEQKLISEEDLNGTA 77
R4              -----------GTKLEIKRAAAHHHHHHGAAGPEQKLISEEDLNGTA 76
Y6              QQSYSTPTFGQGTRLEIKRAAAHHHHHHGAAGPEQKLISEEDLNGTA 167
Y8              QQSYSTPTFGQGTRLEIKRAAAHHHHHHGAAGPEQKLISEEDLNGTA 167
```

Figure 30. Amino acid sequence alignments of all positive clones selected from biopanning using glycoprotein as a target. Amino acid sequence alignments are shown. These clone sequence are linked with 6xHis and Myc epitope at the C-terminal.

4.4 Production of soluble antibody fragment

4.4.1 Expression and purification of soluble antibody fragment in *E. coli* HB2151

The pMOD-I phagemid vector harboring human scFv gene of the positive clones were expressed in *E. coli* HB2151, which is non-suppressor strain. This strain of *E. coli* reads the amber stop codon (TAG) as a stop signal, thus the soluble scFv antibody is expressed. Thirteen anti-rabies clones were selected for expression. These are IRA7c, IIIRC2c, IRC3c, IYC11c, IYC12c, IYD1c, IYF5c, IIRD5v, IIYB5v, IIYG4v, IIYE5v, IIYG8v and IIYD4v. Anti-alfatoxin clone 3C1 (Rangnoi et al., 2011) and used as a control. After overnight of expression, the secreted antibody could be found in the supernatant. The supernatant were testd by ELISA technique. Since these scFv antibodies are linked with 6xHis and Myc epitope, functional monoclonal scFv antibody could be detected with His prob-HRP or mouse anti-Myc tag monoclonal Antibody. Only one out of thirteen clones produced functional antibody fragment against rabies virus (Figure 32). The result showed that His prob-HRP could detect the expressed antibodies better than mouse anti-Myc tag monoclonal antibody (Figure 31-32).

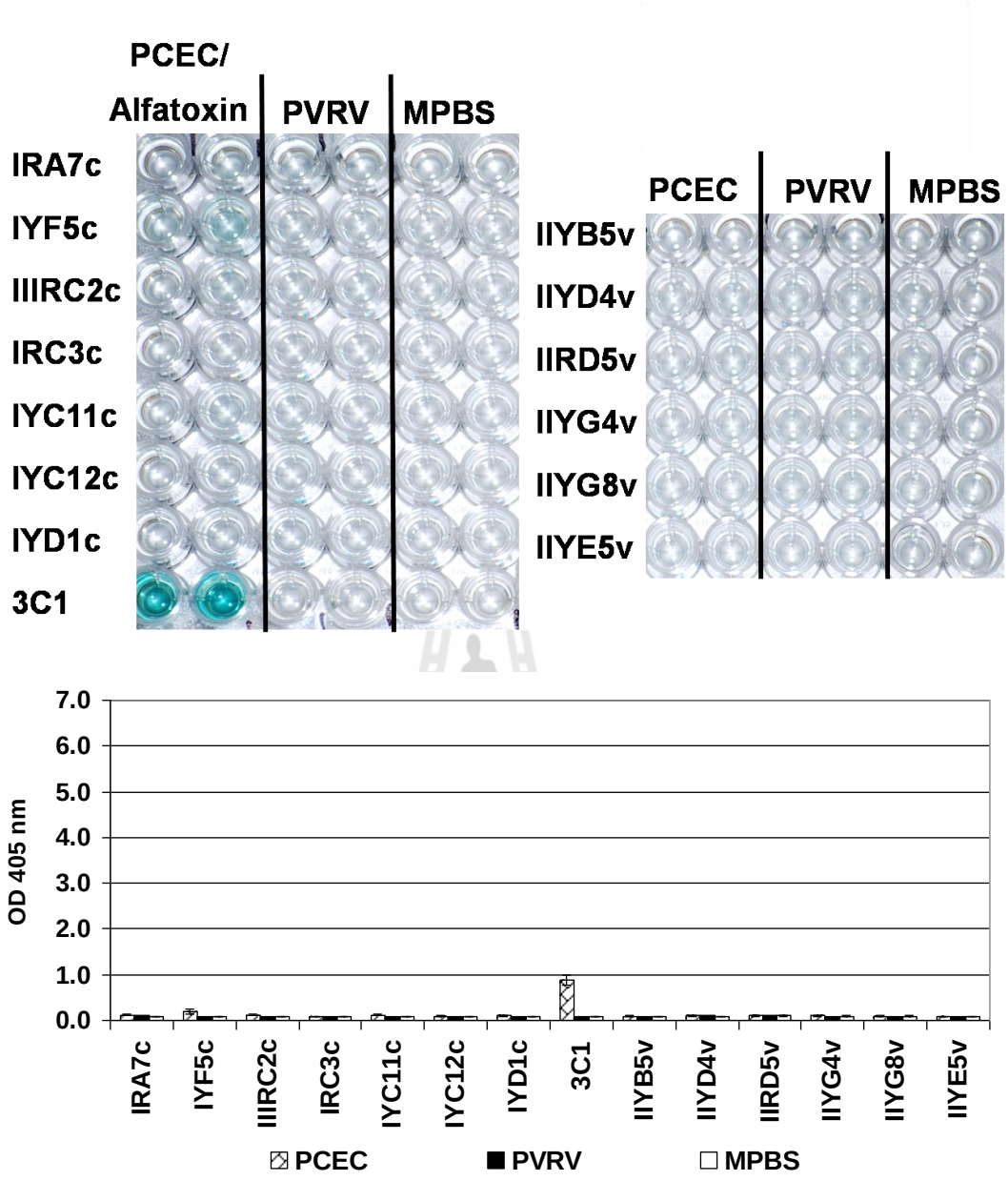


Figure 31. ELISA for testing of the binding of soluble scFv antibody fragment to inactivated rabies vaccines (PVRV and PCEC) compare to MPBS and BSA. The monoclonal scFv antibody was detected with Mouse Anti-Myc Tag Horseradish peroxidase (HRP)

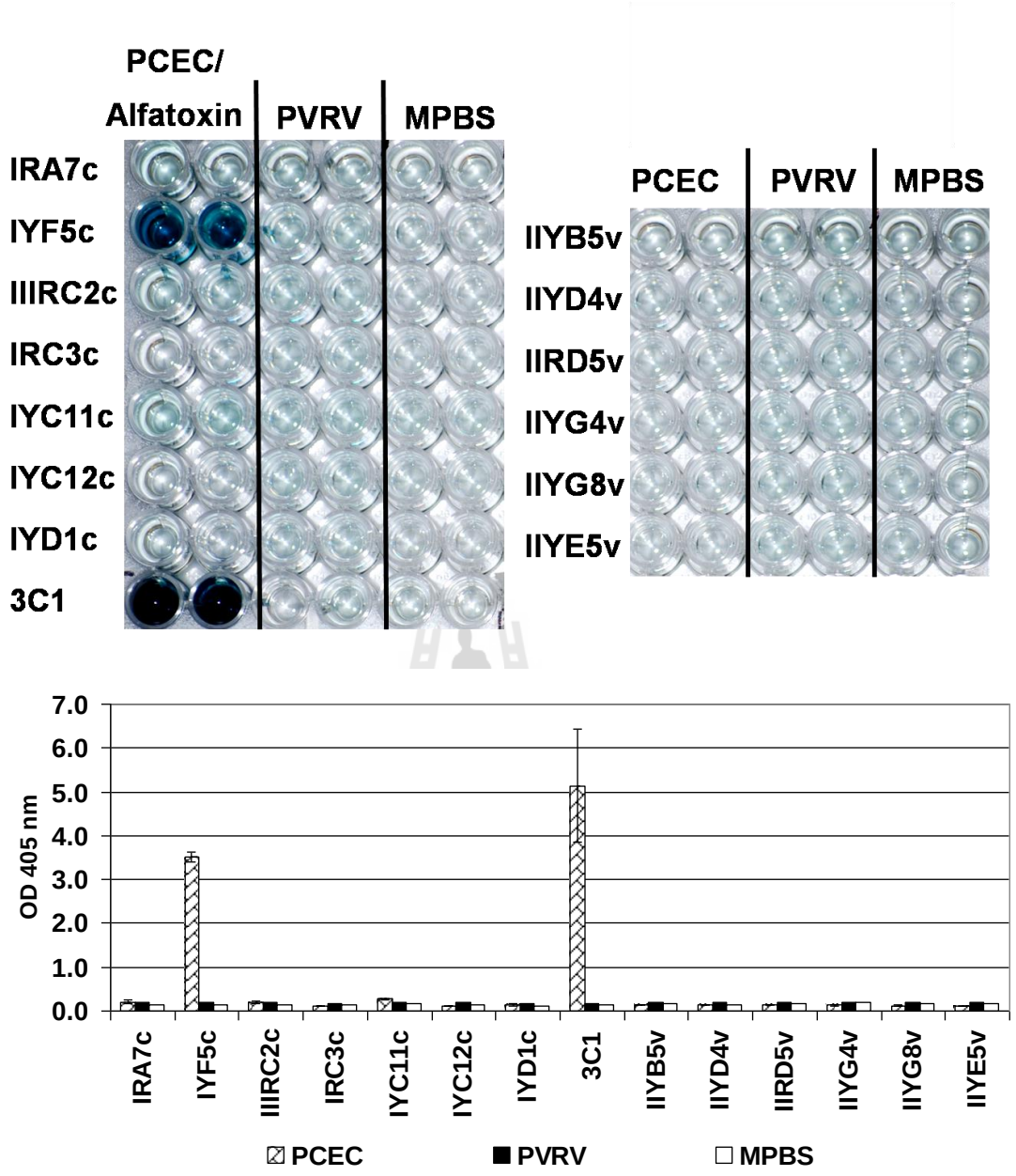


Figure 32. ELISA for testing of the binding of soluble scFv antibody fragment to inactivated rabies vaccines (PVRV and PCEC) compare to MPBS and BSA. The monoclonal scFv antibody was detected with His probe Horseradish peroxidase (HRP).

Soluble anti-Aflatoxin clone 3C1 and anti-Rabies virus clone IYF5c were successfully purified by IMAC (Figure 33). The recombinant antibody scFv proteins were purified from the supernatant. These scFv antibodies encode a carboxy-terminal to hexa histidine tag. Thus, the Ni–NTA nickel-charged resin was added to the supernatant for purification of scFv.

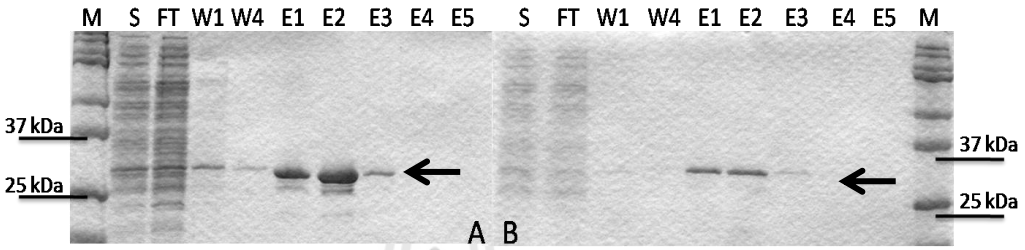


Figure 33. SDS PAGE of purified scFv antibody. SDS-PAGE stained with CBB; (A) Anti-Aflatoxin clone 3C1 and (B) Anti-Rabies virus clone IYF5c; Lene M, protein molecular weight marker; lene S, culture supernatant fraction; lene FT, flow-through fraction; lene W1 and W4, wash fraction; lene P, purified scFv antibody by IMAC.

Purified anti-rabies virus clone IYF5c was tested the binding affinity by ELISA. Ten fold dilution of PCEC target were coated into the well. The binding of purified anti-rabies virus was detected by HIS-prob HRP. The signal indicated that IYF5c could not bind to the target at $0.5x10^3$ IU (Figure 34). The various amount of purified anti-rabies virus clone IYF5c (5 µg, 2.5 µg, 1.25 µg, 0.5 µg, 0.25 µg and 0 µg) were also tested the binding ability by ELISA (Figure 35).

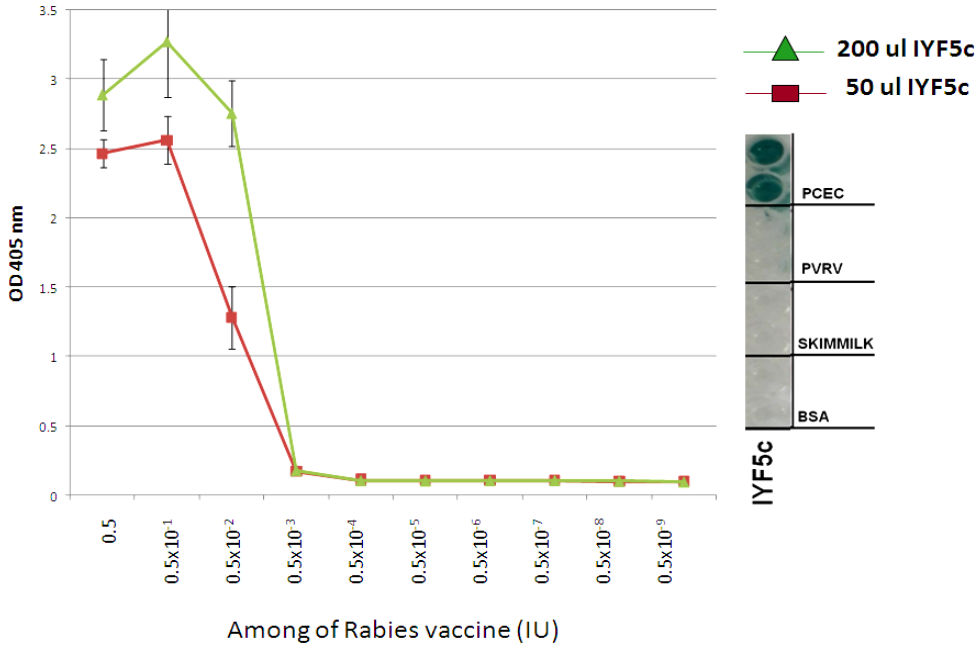


Figure 34. Binding affinity of soluble anti-rabies IYF5c (I).

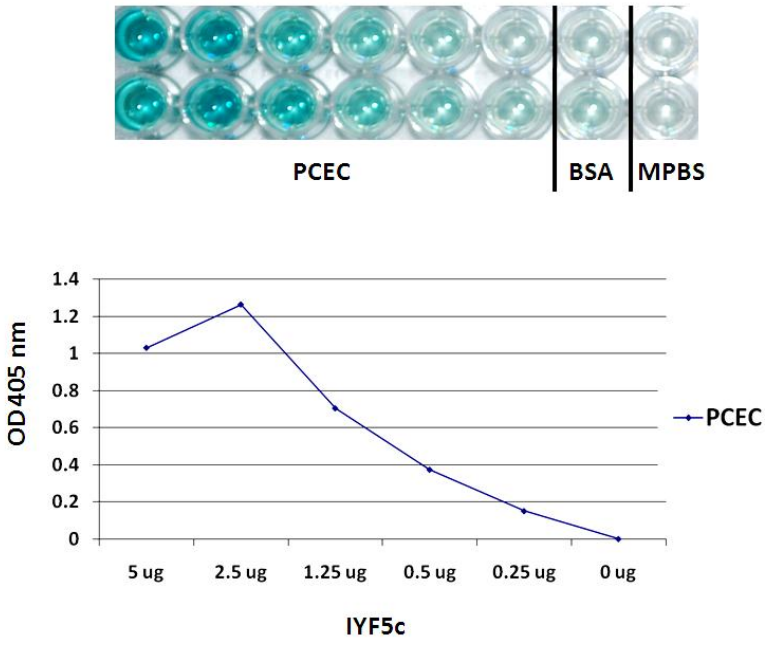


Figure 35. Binding ability of soluble anti-rabies IYF5c (II).

4.4.2 Expression and purification of soluble antibody fragment in *E. coli* BL21

Since only 1 scFv clones could be produced in *E. coli* HB2151, the selected scFv genes (IIYG4v, IRA7c, IYF5c and IIIRC2c) were cloned into pET21b between *Nco*I and *Not*I site for expression in *E. coli* BL21 (Figure 37). This expression vector allows the expression of 6xHistidine-tag-fusion-proteins. Restriction fragment analysis was performed in order to confirm the successful of cloning. The agarose gel in figure 36 showed the positive clones were digested by restriction endonuclease (*Nco*I and *Not*I) and generated 2 fragments with the approximately size ~5000 bp of vector and ~800 bp of scFv genes.

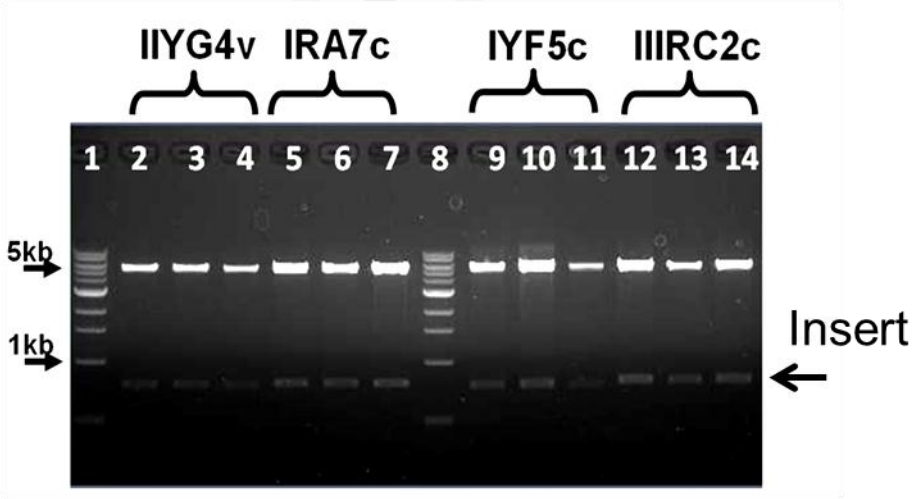


Figure 36. Agarose gel analysis of *Nco*I and *Not*I digestion product to check positive clone. Lene 1 and 8, 1 kb DNA ladder; lene 2-4, clone IIYG4v were digested with enzymes; lene 5-7, clone IRA7c were digested with enzymes; lene 9-11, clone IYF5c were digested with enzymes; lene 12-14, clone IIIRC2c were digested with enzymes.

Recombinant expression plasmids carrying the IIYG4v, IRA7c, IYF5c and IIIRC2c genes were transformed into *E. coli* BL21 (DE3). The expression was performed overnight at 16 ^{o}C with shaking. The secreted antibody could be found in the supernatant. The supernatants were used to test by Western blot and ELISA techniques. The recombinant scFv fused with 6xHistidine could be detected by HIS-prob HRP as shown in figure 37 and figure 38. The results of Western blot analysis (Figure 37) showed that the scFv-hexahistidine fusion antibodies with a size of about 30 kDa were successfully expressed in *E. coli* BL21 (DE3). The ELISA confirms the binding of the soluble scFv to the target. Only clone IYF5c showed high signal by ELISA. Whereas, the signal of the others clone are relatively low.

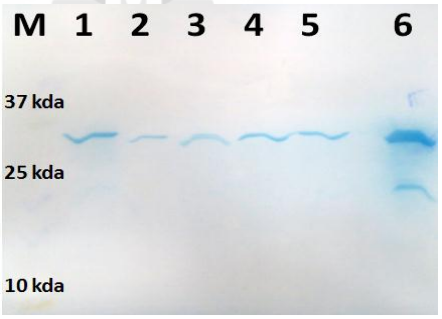


Figure 37. Western blot analysis. Western blot probed with His prob-HRP. Color was developed by using TMB substrate. Lane (M): Protein Molecular weight Markers (Sigma Colour burst prestain); Lane1: pMOD1+ IYF5c (Positive control). Lane 2: pET21b+ IIYG4v. Lane 3: pET21b+ IRA7c. Lane 4: pET21b+ IYF5c. Lane 5: pET21b+ IIIRC2c and Lane 6: pMOD1+ 3C1 (Positive control)

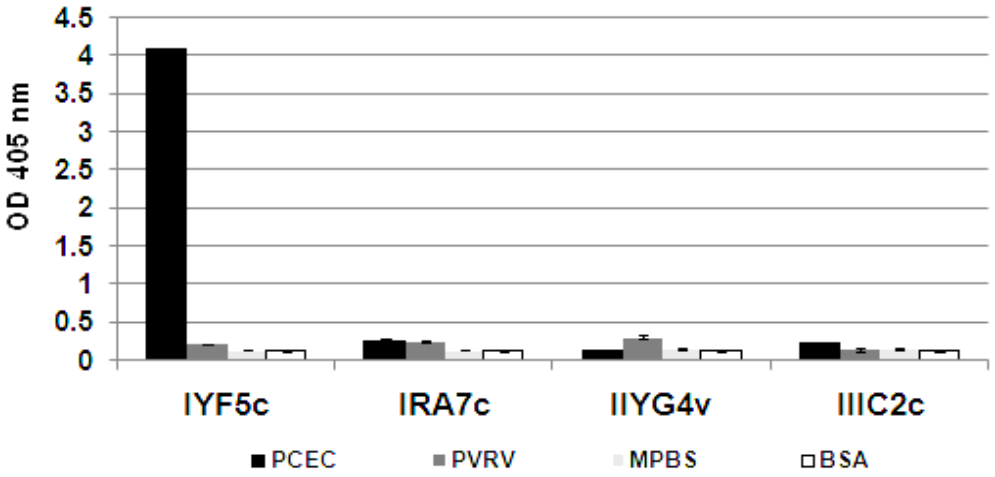


Figure 38. ELISA for testing of the binding of soluble scFv antibody fragment to inactivated rabies vaccines (PVRV and PCEC) compare to MPBS and BSA.

The recombinant antibody scFv proteins were purified from the supernatant. After purification by IMAC, the purified fraction was examined on SDS-PAGE gel (Figure 39). The results of SDS-PAGE showed that the scFv-hexahistidine fusion antibodies with a size of about 30 kDa were successfully purified from the supernatant using Ni-NTA resin.

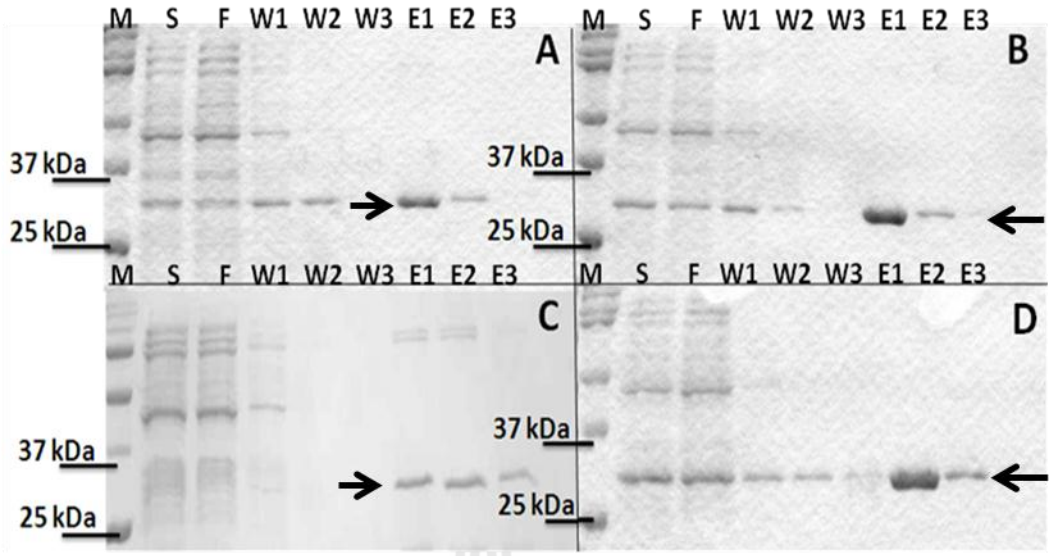


Figure 39. SDS-PAGE of purified scFv antibody. SDS-PAGE stained with CBB; (A) Anti-Rabies virus clone IRA7c, (B) Anti-Rabies virus clone IYF5c, (C) Anti-Rabies virus clone IIIC2c, (D) Anti-Rabies virus clone IIYG4v; Lene M, protein molecular weight marker; lene S, culture supernatant fraction; lene FT, flow-through fraction; lene W1-W3, wash fraction; lene P, purified scFv antibody by IMAC.

4.4.3 Expression and purification of soluble antibody fragment in *E. coli* DH5f'

The selected scFv genes (IRA7c and IYF5c) were cloned into pMY607AP. The two scFv-AP clones; scFv-IRA7c-AP and scFv-IYF5c-AP were expressed by using DH5αF'. Crude cell lysate and supernatant were used to test the specific to bind inactivated rabies by ELISA using PNPP as substrate. The yellow color was developed and measured absorbance at 405 nm. The supernatant of clone

IRA7c-AP showed the signal bound to PCEC. scFv-AP fusion result indicated that this format can be used for diagnostic purpose. Moreover, scFv-GFP format could be further developed to be used as diagnostic probe in the future.

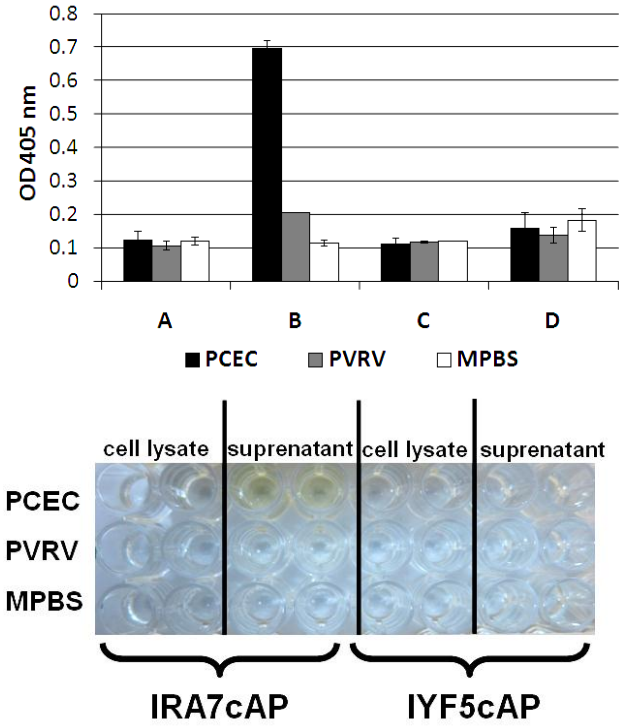


Figure 40. The binding of scFv-IRA7c-AP and scFv-IYF5c-AP; (A) scFv-IRA7c-AP from crude cell lysate, (B) scFv-IRA7c-AP from crude supernatant , (C) scFv- IYF5c -AP from crude cell lysate, (D) scFv- IYF5c AP from crude supernatant, PNPP was used to be substrate for AP. Absorbance signals were present with standard deviation of duplicate wells as depicted above.

4.5 Neutralization assay

The neutralization capacity of the scFv antibody was detected by the Rapid Fluorescent Focus Inhibition Test (RFFIT). By using this method, the field where virus has been neutralized (Figure 41A) or the field where virus was not neutralized (Figure 41B) were counted under a fluorescence microscope and then calculated as IU/ml. The neutralizing potencies (IU) of the established clones against RABV were calculated by comparison with theWHO international standards (equine anti-rabies immunoglobulin, National Institute for Biological Standards & Control) by the Spearman-Karber method.

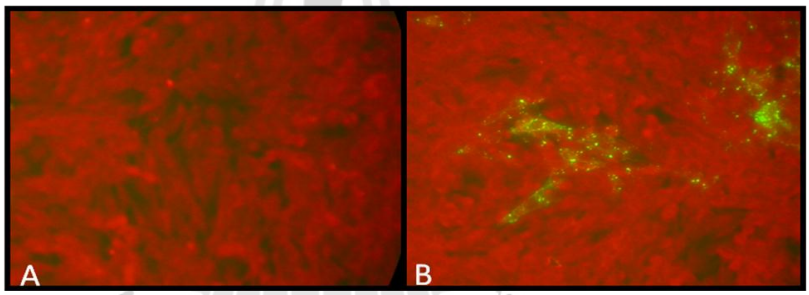


Figure 41. An example of a microscopic field from neutralization assay. (A) An example of a microscopic field where virus has been neutralized. (B) An example of a microscopic field where virus was not neutralized.

Phage displayed antibodies (Crude supernatant or PEG precipitate) and soluble scFv antibodies were used for RFFIT. The neutralizing efficiency was shown in table 10-12. The neutralizing efficiency of soluble scFv antibodies were calculated by comparison with WHO international standards and expressed as IU per mg. The

neutralization abilities of IRA7c and IIIRC2c against CVS were 4.54 IU/mg and 0.2 IU/mg as shown in Table 12. Among the different methods for preparation of antibodies, the soluble scFv antibodies performed the best neutralize activity to the rabies virus. IRA7c can neutralize the virus better than IIIRC2c as shown in Table 10-12. Phage displayed antibody showed the lower neutralizing activity than soluble scFv when testing by RFFIT method. It is interesting to note that the ELISA results prove that IRA7c was not bind to the glycoprotein whereas the IIIRC2c could bound to the glycoprotein. While clone IYF5c interacted specifically and strongly to the PCEC target, it did not neutralize the virus. Only clones IRA7c and IIIRC2c, which were derived from immunized library showed neutralization of the rabies virus *in vitro* at 4.54 IU/mg and 0.20 IU/mg, respectively. The result proved that the ELISA signal is not corresponded with the neutralizing activity. Finally, the soluble scFv antibody IRA7c was concentrated using Amicon® Ultra-4 Centrifugal Filter Devices and tested by RFFIT. The neutralizing efficiency was increased about 10 times after the concentration of soluble scFv antibody IRA7c was concentrated 10 times (Table 12). Nevertheless, the neutralization assay would be better to performed with other RABV strains (such as ERA, HEP-Flury, and Nishigahara) to analyze the activity of the recombinant antibody against a broad spectrum of RABV (Masumoto et al., 2010). There are many reports worldwild about recombinant monoclonal antibody against Rabies virus by phage display technology (Ray et al., 2001; Kramer et al., 2005; Zhao et al., 2008; Kaku et al., 2011; Aavula et al., 2011; Ando et al., 2005; Houimel et al., 2009; Liu et al., 2011; San et al., 2012; Hultberg et al., 2011; Boruah et al., 2013). Almost of them showed the neutralizing activity higher than this work. However, it is

important to note that this antibody is 100% Thai human. Nevertheless, the neutralizing potency and stability of these antibodies can be further improved by various methods of affinity maturation (Dean et al., 2012). These two human MAbs against rabies are expected to be utilized as a tool for future post-exposure prophylaxis. For therapeutic use, clone IRA7c should be converted to full-length IgG.

Table 10. *In vivo* neutralizing potency determined by RFFIT using phage scFv antibody (PEG precipitate)

Phage scFv antibody (PEG precipitate)	**IU/ml**
IRA7c	0.06
IYF5c	Negative
IIIC2c	0.03
IRC3c	Negative
IYC11c	Negative
IYC12c	Negative
IYD1c	Negative
IIRD5v	Negative
IIYB5v	Negative
IIYG4v	Negative
IIYE5v	Negative
IIYG8v	Negative
IIYD4v	Negative

Table 11. *In vivo* neutralizing potency determined by RFFIT using phage scFv antibody (crude supernatant)

Phage scFv antibody (crude supernatant)	**IU/ml**
IRA7c	0.12
IYF5c	Negative
IIIC2c	0.08

Table 12. *In vivo* neutralizing potency determined by RFFIT using soluble scFv

Soluble scFv antibody (concentration)	**IU/ml**	**IU/mg**
IRA7c (500 ug/ml)	2.27	4.54
IRA7c (4900 ug/ml)	20.13	4.10
IYF5c (284 ug/ml)	Negative	Negative
IIIC2c (500 ug/ml)	0.10	0.20
IIYG4v (125.21 ug/ml)	Negative	Negative
3C1 (368 ug/ml)	Negative	Negative

4.6 Bioinformatic Analysis for 3D structure prediction

The nucleotide sequences of clone IRA7c, IIIRC2c and IYF5c were translated to an amino acid sequence and three dimention structures were predicted by homology. The binding sites which are CDR 1, 2, 3 of scFv Human antibodies were indicated by pymol program. Surface cartoon representation of binding site of the single chain variable fragment (scFv) of clone IRA7c and IIIRC2c are shown in Figure 42. The three complementary determining regions (CDR1, CDR2 and CDR3) are indicated. As shown in Figure 42, the three complementary determining regions of clone IRA7c and IIIRC2c form flat structure, in contrast to deep binding pocket of Anti-alfatoxin the previous report of 3D structure prediction showed the binding pocket, a deep cavity, is highly complementary to the hapten AFB1 (Li et al., 2012). The schematic ribbon cartoon of IRA7c, IIIRC2c and IYF5c was depicted in Figure 43, 44 and 45.

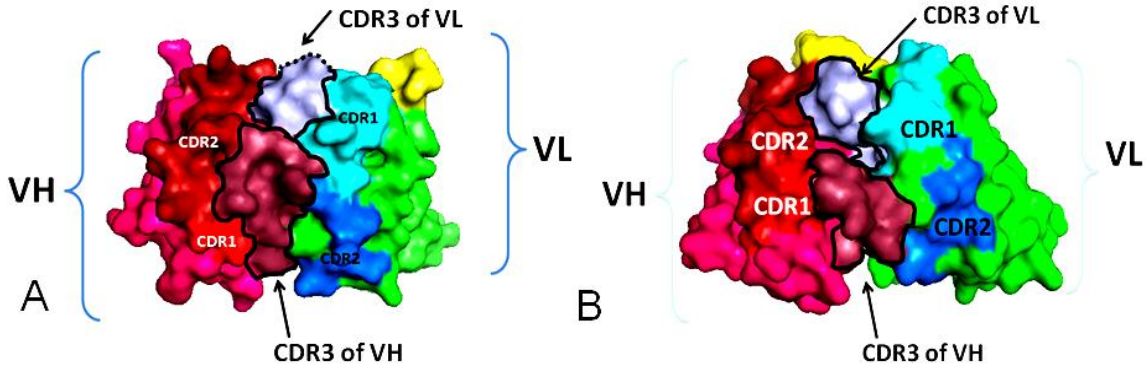


Figure 42. Three-dimensional structure prediction. A) IRA7c. B) IIIRC2c.

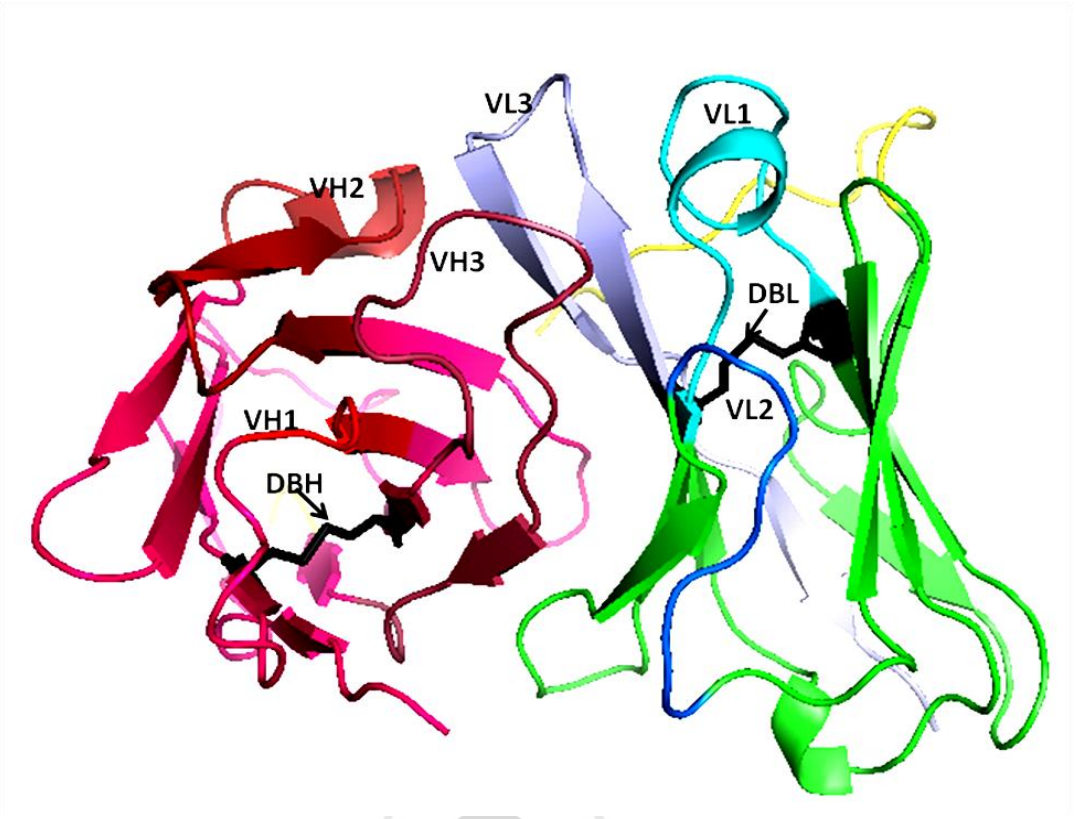


Figure 43. Three-dimensional structure prediction of neutralized single chain variable fragment (scFv) against rabies; IRA7c. The disulfide bond of heavy chain (DBH) and the disulfide bond of the light chain (DBL) are located.

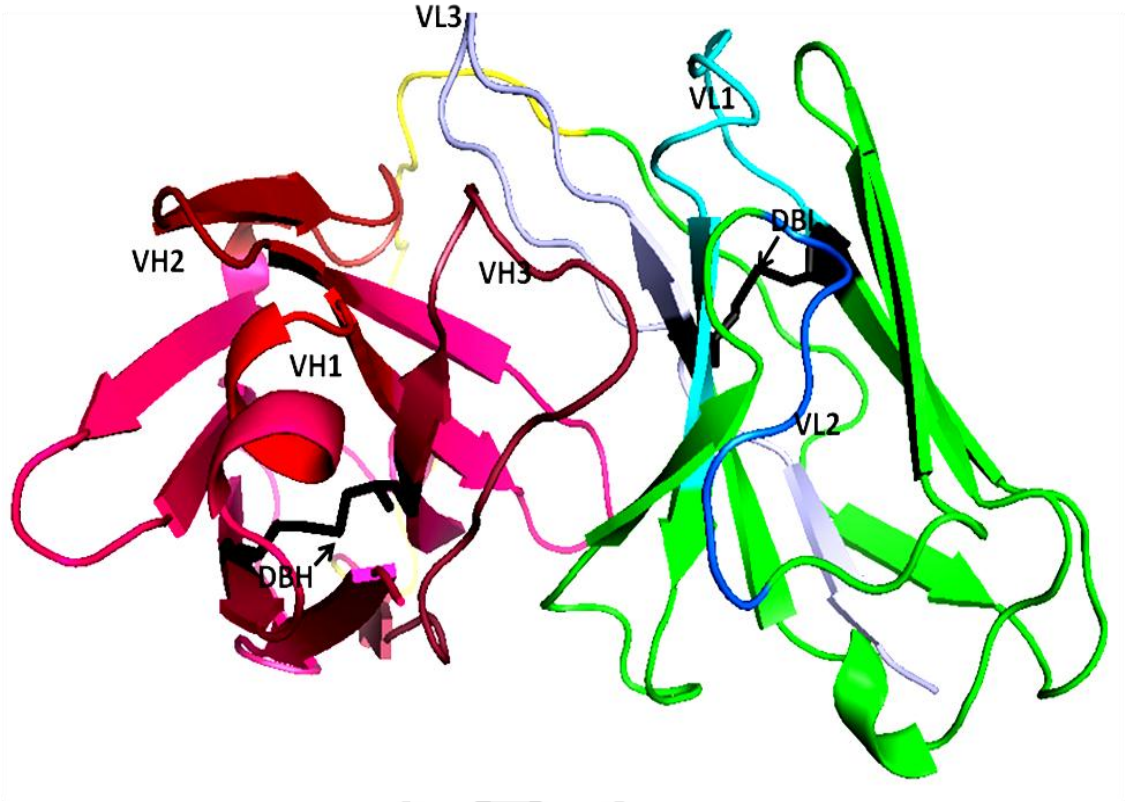


Figure 44. Three-dimensional structure prediction of neutralized single chain variable fragment (scFv) against rabies; IIIRC2c. The disulfide bond of heavy chain (DBH) and the disulfide bond of the light chain (DBL) are located.

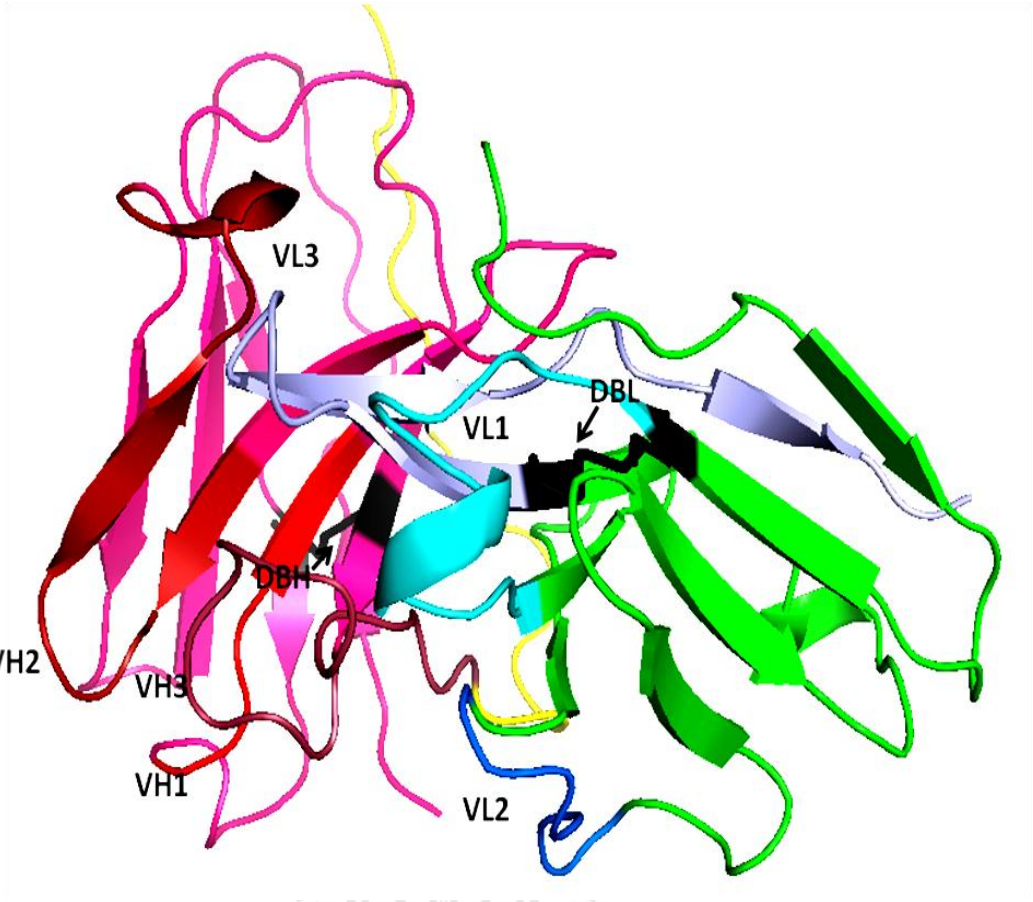


Figure 45. Three-dimensional structure prediction of non-neutralized single chain variable fragment (scFv) against rabies; IYF5c. The disulfide bond of heavy chain (DBH) and the disulfide bond of the light chain (DBL) are located.

CHAPTER V

CONCLUSION

1. Thirteen unique phage-displayed anti-rabies scFv antibodies were successfully selected from non-immunized (YAMO-I) and immunized (Yamo-Rb) libraries using PVRV or/and PCEC as a target. These are clones IRA7c, IIIRC2c, IRC3c, IYC11c, IYC12c, IYD1c and IYF5c (selected by using PCEC as a target) IIRD5v, IIYB5v, IIYG4v, IIYE5v, IIYG8v, IIYD4v (selected by using PVRV as a target).

2. Three unique phage-displayed anti-rabies were selected from non-immunized (YAMO-I) and immunized (Yamo-Rb) using glycoprotein as a target. There are R1, R4 and Y6. Two of them are small peptide fragment. The others clone is consisted of only VL fragment. All clones contain concensus motif DIVMTQSP.

3. All scFv antibodies possessed variable heavy chains (VH) from family 1, 3, 4 and 6 and only λ light chains (VL) from family 1, 2, 3, and 6. No κ light chain (Vκ) was isolated.

4. Two clones consisted of only VL fragments. One was from immunized (Yamo-Rb) library using PCEC as a target. The others one is from non-immunized (YamoI) library using glycoprotein as a target.

5. Two out of 13 selected phage scFv antibodies can neutralize the virus using RFFIT assay. There are clone IRA7c and IIIRC2c.

6. Soluble scFv clone IRA7c, IIIRC2c, IYF5c and IIYG4v were expressed and tested by RFFIT assay. Only clones IRA7c and IIIRC2c showed neutralizing activity. Clone IRA7c neutralized rabies virus better than clone IIIRC2c.

7. This result indicated that both non-immunized library from exposed population and immunized library can be used to generate scFv nanobodies for detection of the virus, but only antibody from immunized library can neutralize the virus.

Table 13. Characteristics of all selected positive anti-rabies clones.

Clone	ELISA result (Phage)			ELISA result (Soluble scFv)			ELISA result (scFv AP)	Neutralization			Remark
	GP	PCEC	PVRV	GP	PCEC	PVRV	PCEC	Crude Phage	PEG/Nacl Phage	Soluble scFv	
IYF5c	-	+++	-	N/A	+++	-	-	No	No	No	-
IYC11c	-	++	-	N/A	N/A	N/A	N/A	N/A	No	N/A	-
IYC12c	-	++	-	N/A	N/A	N/A	N/A	N/A	No	N/A	-
IYD1c	-	++	-	N/A	N/A	N/A	N/A	N/A	No	N/A	-
IIYG4v	-	++	+++	N/A	+	++	N/A	N/A	No	No	-
IIYG8v	-	-	+	N/A	N/A	N/A	N/A	N/A	No	N/A	-
IIYD4v	-	-	+	N/A	N/A	N/A	N/A	N/A	No	N/A	-
IIYE5v	-	+	+	N/A	N/A	N/A	N/A	N/A	No	N/A	-
IIYB5v	-	-	+++	N/A	N/A	N/A	N/A	N/A	No	N/A	-
Y6(Y8)	+++	-	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Consist only VL

Table 13. Characteristics of all selected positive anti-rabies clones. (Continued)

Clone	ELISA result (Phage)			ELISA result (Soluble scFv)			ELISA result (scFv AP)	Neutralization			Remark
	GP	PCEC	PVRV	GP	PCEC	PVRV	PCEC	Crude Phage	PEG/Nacl Phage	Soluble scFv	
IRA7c	-	++	+	N/A	+	-	++	Yes	Yes	Yes (4.54 IU/mg)	-
IIIC2c	+++	++	+	N/A	+	-	N/A	Yes	Yes	Yes (0.20 IU/mg)	-
IRC3c	+++	+	-	N/A	N/A	N/A	N/A	N/A	No	N/A	Consist only VL
IIRD5v	-	-	++	N/A	N/A	N/A	N/A	N/A	No	N/A	-
R1(R2)	+++	-	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Short peptide
R4	+++	-	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Short peptide

Note: Signal of OD 405 nm

- No signal compared with negative control

+ Low signal compared with negative control

++ Medium signal compared with negative control

+++ High signal compared with negative control

No haven't got neutralizing activity

Yes have neutralizing activity

N/A is an abbreviation for not applicable

มหาวิทยาลัยเทคโนโลยีสุรนารี

REFERENCES

Aavula, S. M., Nimmagadda, S. V., Biradhar, N., Sula, S., Chandran, D., Lingala, R., Villuppanoor, S.A. (2011): Generation and characterization of an scFv directed against site II of rabies glycoprotein**. Biotechnology Research International**, 1-11.

Ahmad, Z.A., Yeap, S.K., Ali, A.M., Ho, W.Y., Alitheen, N.B.M., Hamid, M. (2012): scFv Antibody: principles and clinical application. **Clinical and Developmental Immunology,** 1-15.

Almagro, J. C., and Fransson, J. (2008): Humanization of antibodies**. Frontiers in Bioscience** 13, 1619-1633.

Ando T, Yamashiro T, Takita-Sonoda Y, Mannen K, Nishizono A. (2005): Construction of human Fab library and isolation of monoclonal Fabs with rabies virus-neutralizing ability. **Microbiology Immunology** 49, 311-22.

Barbas, C. F., 3rd, Bain, J.D., Hoekstra D.M., Lerner R.A. (1992): Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. **Proceeding of the National Academic of Science of the United State of America** 89, 4457-4461.

Batra, S., Srinivasan, T., Rastogi, S.K., Kundu, B. (2002): Identification of enzyme inhibitors using combinatorial libraries. **Current Medicinal Chemistry** 9, 307-319.

Bakker, A.B.H., Marissen, W.E., Kramer R.A., Rice, A.B., Weldon, W.C., Niezgoda, M., Hanlon, C.A., Thijsse, S., Backus, H.H., de Kruif, J., et al. (2005):

Novel human monoclonal antibody combination effectively neutralizing natural rabies virus variants and individual *in vitro* escape mutants. **Journal of Virology** 79, 9062-9068.

Benhar, I. (2007): Design of synthetic antibody libraries. **Expert Opinion Biological Therapy** 7, 763-779.

Boruah, B. M., Liu, D., Ye, D., Gu, T.J., Jiang, C.L., Qu, M., Wright, E., Wang, W., He, W., Liu, C., Gao, B. (2013): Single domain antibody multimers confer protection against rabies infection. **PLoS ONE** 8, 1–10.

Booy, E.P., Johar, D., Maddika, S., Pirzada, H., Sahib, M.M., Gehrke, I., Loewen, S., Louis, S.F., Kadkhoda, K., Mowat, M., Los, M. (2006): Monoclonal and bispecific antibodies as novel therapeutics. **Archivum Immunologiae et Therapiae Experimentalis** 54, 85-101.

Both, L., Banyard, A. C., Dolleweerd, C. V., Horton, D.L., Ma J. K-C., Fooks A. R. (2012): Passive immunity in the prevention of rabies. **The Lancet Infectious Diseases** 12, 397–407.

Briggs, D.J., Benzhoff, A., Nicolay, U., Silikwin, S., Dumavibhat, B., Tongswas, S., Wasi, C. (2000): Antibodies response of patients aster postexpoture rabies vaccination intradermal doses of purified chick embryo cell vaccine or purified Vero cell rabies vaccine. **Bulletin of the World Health Organization** 78, 693–698.

Bugli, F., Graffeo, R., Sterbini, F.P., Torelli, R., Masucci, L., Sali, M., Grasso, A., Rufini, S., Ricci, E., Fadda, G., Pescatori, M. (2008): Monoclonal antibody fragment from combinatorial phage display library neutralizes alpha-latrotoxin activity and abolishes black widow spider venom lethality in mice. **Toxicon** 51, 547-554.

Burton, D.R., Barbas, C.F., 3rd, Persson, M.A., Koenig, S., Chanock, R.M., Lerner, R.A. (1991): A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. **Proceeding of the National Academic of Science of the United State of America** 88, 10134-10137.

Burtrum, D., Zhu, Z., Lu, D., Anderson, D.M., Prewett, M., Pereira, D.S., Bassi, R., Abdullah, R., Hooper AT, Koo H, et al. (2003): A fully human monoclonal antibody to the insulin-like growth factor I receptor blocks ligand-dependent signaling and inhibits human tumor growth *in vivo*. **Cancer Reserch** 63, 8912-8921.

Buscombe, J.R. (2006): The future of infection imaging. Q J. **Nuclear Medicine and Molecular Imaging** 50, 99-103.

Cai, X., Garen, A. (1995): Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. **Proceeding of the National Academic of Science of the United State of America** 92, 6537-6541.

Carter, P., Merchant, A.M. (1997): Engineering antibodies for imaging and therapy. **Current Opinion of Biotechnology** 8, 449-454.

Chang, C.N., Landolfi, N.F., Queen, C. (1991): Expression of antibody Fab domains on bacteriophage surfaces. Potential use for antibody selection. **Journal of Immunology** 147, 3610-3614.

Clackson, T., Hoogenboom, H.R., Griffiths, A.D., Winter, G. (1991): Making antibody fragments using phage display libraries. **Nature**, 352:624-628.

Crameri, A., Cwirla, S., Stemmer, W.P. (1996): Construction and evolution of antibody-phage libraries by DNA shuffling. **Natature Medicine** 2, 100-102.

Dauvillier, S., Merida, P., Visintin, M., Cattaneo, A., Bonnerot, C., Dariavach, P. (2002): Intracellular single-chain variable fragments directed to the Src homology 2 domains of Syk partially inhibit Fc epsilon RI signaling in the RBL-2H3 cell line. **Journal of Immunology** 169, 2274-2283.

De Kruif, J., Bakker, A.B., Marissen, W.E., Kramer, R.A., Throsby, M., Rupprecht, C.E., Goudsmit, J. (2007): A human monoclonal antibody cocktail as a novel component of rabies postexposure prophylaxis. **Annual Review of Medicine** 58, 359-368.

Dodet, B., Goswamib, A., Gunasekerac, A., Guzmand, F.D., Jamalie, S., Montalbanf, C., Purbag, W., Quiambaoh, B., Salahuddini, N., Sampathj, G., Tang, Q., Tantawichien, T., Wimalaratne, O., Ziauddin, A. (2008): Rabies awareness in eight Asian countries. **Vaccine** 26, 6344–6348.

Donna, J., Fisher, M.D. (1995): Epidermiology and prevention of rabies. **Current Problems in Padiatrics**, 304-313.

Duan, Y., Gua, T.J., Jianga, C.L., Yuana, R.S., Zhanga, H.F., Houa, H.J.,Yua, X.H., Chena, Y., Zhanga, Y., Wua, Y.G., Kong, W. (2012): A novel disulfide-stabilized single-chain variable antibody fragment against rabies virus G protein with enhanced in vivo neutralizing potency. **Molecular Immunology** 51, 188– 196.

Fermer,C., Andersson, I., Nilsson, K., Nilsson, O. (2004): Specificity rescue and affinity maturation of a low-affinity IgM antibody against pro-gastrin-releasing peptide using phage display and DNA shuffling. **Tumour Biology**, 25:7-13.

Filpula, D. (2007): Antibody engineering and modification technologies. **Biomolecular Engineering** 24, 201-215.

Fromant, M., Blanquet, S., Plateau, P. (1995): Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction. **Analytical Biochemistry** 224, 347-353.

Gejima, R., Tanaka, K., Nakashima, T., Hashiguchi, S., Ito, Y., Yoshizaki, K., Sugimura, K. (2002): Human single-chain Fv (scFv) antibody specific to human IL-6 with the inhibitory activity on IL-6-signaling. **Human Antibodies** 11, 121-129.

Goudsmit, J., Marissen, W.E., Weldon, W.C., Niezgoda, M., Hanlon, C.A., Rice, A.B., Kruif, J., Dietzschold, B., Bakker, A.B., Rupprecht, C.E. (2006): Comparison of an anti-rabies human monoclonal antibody combination with human polyclonal anti-rabies immune globulin. **Journal of Infectious Diseases** 193, 796-801.

Graus, Y.F., de Baets, M.H., Parren, P.W., Berrih-Aknin, S., Wokke, J., van Breda Vriesman, P.J., Burton, D.R. (1997): Human anti-nicotinic acetylcholine receptor recombinant Fab fragments isolated from thymus-derived phage display libraries from myasthenia gravis patients reflect predominant specificities in serum and block the action of pathogenic serum antibodies. **Journal of Immunology** 158, 1919-1929.

Hale, G. (2006): Therapeutic antibodies-delivering the promise? **Advanced Drug Delivery Reviews** 58, 633-639.

Hancock, R.E., Sahl, H.G. (2006): Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. **Nature Biotechnology** 24, 1551-1557.

Hemachudha T., Ugolini G., Wacharapluesadee S., Sungkarat W., Shuangshoti S., Laothamatas J.(2013): Human rabies: neuropathogenesis, diagnosis, and management. **The Lancet Neurology** 12, 498–513.

Hof ,D., Hoeke, M.O., Raats, J.M. (2008): Multiple-antigen immunization of chickens facilitates the generation of recombinant antibodies to autoantigens. **Clinical & Experimental Immunology** 151, 367-377.

Houimel M., Dellagi K. (2009a): Isolation and characterization of human neutralizing antibodies to rabies virus derived from a recombinant immune antibody library. **Journal of Virological Methods** 161, 205–215.

Houimel M., Dellagi K. (2009b): Peptide mimotopes of rabies virus glycoprotein with immunogenic activity. **Vaccine** 27, 4648–4655.

Hoogenboom, H.R., Griffiths, A.D., Johnson, K.S., Chiswell, D.J., Hudson, P., Winter, G. (1991): Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. **Nucleic Acids Research** 19, 4133-4137.

Hoogenboom, H.R., Winter, G. (1992): By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. **Journal of Molecular Biology** 227, 381-388.

Hoogenboom, H.R. (1997): Designing and optimizing library selection strategies for generating high-affinity antibodies. **Trends in Biotechnology** 15, 62-70.

Howard, G.C., Kaser, M.R (Ed). (2006): Making and Using Antibodies: A Practical handbook: CRC, 408p.

Hultberg, A., Temperton, N.J., Rosseels, V.R., Koenders, M., Gonzalez-Pajuelo, M., Schepens, B., Iban˜ez, L.I., Vanlandschoot, P., Schillemans, J., Saunders, M., Weiss, R.A., Saelens, X., Melero, J.A., Verrips C.T., Gucht, S.V., de Haard H.J., (2011): Llama-Derived Single Domain Antibodies to Build Multivalent, Superpotent and Broadened Neutralizing Anti-Viral Molecules. **PLoS One** 6, e17665.

Jolliffe, L.K. (1993): Humanized antibodies enhancing therapeutic utility through antibody engineering. **International Review of Immunology** 10, 241-250.

Kaku, Y., Noguchi, A., Hotta, K., Yamada, A., Inoue S. (2011): Inhibition of rabies virus propagation in mouse neuroblastoma cells by an intrabody against the viral phosphoprotein. **Antiviral Research** 91, 64-71.

Kay, B.K., Winter, J., McCafferty, J. (Ed). (1996): Phage Display of Peptides and Proteins: A laboratory manual. London: Academic Press: San Diego, CA, 344p.

Kay, B.K., Kasanov, J., Yamabhai, M. (2001a): Screening phage-displayed combinatorial peptide libraries. **Methods** 24, 240-246.

Kay BK, Hamilton, P.T. (2001b): Identification of enzyme inhibitors from phage-displayed combinatorial peptide libraries. **Combinatorial Chemistry & High Throughput Screening** 4, 535-543.

Kehoe¸ J, W., Kay, B.K. (2005): Filamentous phage display in the new the promise of pretargeting strategies using bispecific antibodies and radiolabeled haptens millennium. **Chemical Review** 105, 4056-4072.

Kierny, M. R., Cunningham, T. D., Kay, B. K. (2012): Detection of biomarkers using recombinant antibodies coupled to nanostructured platforms. **Nano reviews**, 1-24.

Korpimaki, T., Rosenberg, J., Virtanen, P., Lamminmaki, U., Tuomola, M., Saviranta, P. (2003): Further improvement of broad specificity hapten recognition with protein engineering. **Protein Engineering** 16, 37-46.

Kovaleva, M., Bussmeyer, I., Rabe, B., Grotzinger, J., Sudarman, E., Eichler, J., Conrad, U. Rose-John, S., Scheller, J. (2006): Abrogation of viral interleukin-6 (vIL-6)-induced signaling by intracellular retention and neutralization of vIL-6 with

an anti-vIL-6 single-chain antibody selected by phage display. **Journal of Virology** 80, 8510-8520.

Kramer, R.A., Marissen, W.E., Goudsmit J., Visser T.J., Clijsters-Van der Horst M, Bakker AQ, de Jong M, Jongeneelen M, Thijsse S, Backus H.H.J., Rice, A.B., Weldon, W.C., Rupprecht, C.E., Dietzschold, B., Bakker A.B.H., de Kruif, J. (2005): The human antibody repertoire specific for rabies virus glycoprotein as selected from immune libraries. **European Journal of Immunology** 35, 2131-2145.

Kristensen, P., Winter, G. (1998): Proteolytic selection for protein folding using filamentous bacteriophages. **Folding and Design** 3, 321-328.

Kruif, J.D., Bakker, A.B.H., Marissen, W.E., Kramer, R. A., Throsby, M., Rupprecht, C.E., Goudsmit. J. (2007): A human monoclonal antibody cocktail as a novel component of rabies postexposure prophylaxis. **Annual Review of Medicine** 58, 359–68

Ladner RC, Sato AK, Gorzelany J, de Souza M. (2004): Phage display-derived peptides as therapeutic alternatives to antibodies. **Drug Discovery Today** 9, 525-529.

Lagerwerf, F., Worsta, S., Van Corvena, E., Kostensea, S., et al. (2008): First administration to humans of a monoclonal antibody cocktail against rabies virus: Safety, tolerability, and neutralizing activity. **Vaccine** 26, 5922–5927.

Latham, P. W. (1999): Therapeutic peptides revisited. **Nature Biotechnology** 17, 755-757.

Lee, M.S., Lee, J.C., Choi, C.Y., Chung, J. (2008): Production and characterization of monoclonal antibody to botulinum neurotoxin type B light chain by phage display. **Hybridoma (Larchmt)** 27, 18-24.

Li, X., Li, P., Zhang, Q., Li, Y., Zhang, W., Ding, Z. (2012): Molecular Characterization of Monoclonal Antibodies against Aflatoxins: A Possible Explanation for the Highest Sensitivity. **Analytical chemistry** 84, 5229−5235

Li, Y., Li, H., Wang, M.N., Lu, D., Bassi, R., Wu, Y., Zhang, H., Balderes, P., Ludwig, D.L., Pytowski, B., Kussie, P., Piloto, O., Small, D., Bohlen, P., Witte, L., Zhu, Z., Hicklin, D.J. (2004): Suppression of leukemia expressing wild-type or ITD-mutant FLT3 receptor by a fully human anti-FLT3 neutralizing antibody. **Blood** 104, 1137-1144.

Liu W., Liu Y., Liu J., Zhai J., Xie Y. (2011): Evidence for inter- and intra-clade recombinations in rabies virus. **Infection, Genetics and Evolution** 11, 1906–1912

Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D., Winter, G. (1991): By-passing immunization. Human antibodies from V-gene libraries displayed on phage. **Journal of Molecular Biology** 222, 581-597.

Martineau, P. (2002): Error-prone polymerase chain reaction for modification of scFvs. **Methods in Molecular Biology** 178, 287-294.

Matsumoto, T.,Yamada, K., Noguchi, K., Nakajima, K., Takada, K., Khawplod, P., Nishizono, A. (2010): Isolation and characterization of novel human monoclonal antibodies possessing neutralizing ability against rabies virus. **Microbiology and Immunology** 54(11), 673-83.

McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J. (1990): Phage antibodies: filamentous phage displaying antibody variable domains. **Nature** 348, 552-554.

McCarron, P.A., Olwill, S.A., Marouf, W.M., Buick, R.J., Walker, B., Scott, C.J. (2005): Antibody conjugates and therapeutic strategies. **Molecular Interventions** 5, 368-380.

Mitra, A., Nan, A., Line, B.R., Ghandehari, H. (2006): Nanocarriers for nuclear imaging and radiotherapy of cancer. **Current Pharmaceutical Design** 12, 4729-4749.

Nissim, A., Hoogenboom, H.R., Tomlinson, I.M., Flynn, G., Midgley, C., Lane, D., Winter, G. (1994): Antibody fragments from a 'single pot' phage display library as immunochemical reagents. **EMBO Journal** 13, 692-698.

Mitmoonpitak, C., Tepsumethanon, V., Wilde, H. (1998): Rabies in Thailand. **Epidemiology Infection**. 120, 165-169.

O'Brien, P.M., Aitken, R (Ed). (2002): Antibody Phage Display. **Methods and protocols New Jersey: Humana Press.**

Okumura A. and Harty R. N. (2011): Rabies virus assembly and budding. **Advance in Virus Research** 79, 23-32

Paz, K., Brennan, L.A., Iacolina, M., Doody, J., Hadari, Y.R., Zhu, Z. (2005): Human single-domain neutralizing intrabodies directed against Etk kinase: a novel approach to impair cellular transformation. **Molecular Cancer Therapeutics** 4, 1801-1809.

Pansri, P., Jaruseranee, N., Rangnoi, K., Kristensen, P., Yamabhai, M. (2009): A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. **BMC Biotechnology** 9, 6-22.

Paschke M. (2006): Phage display systems and their applications. **Apply Microbiology Biotechnology** 70, 2–11

Piloto, O., Levis, M., Huso, D., Li, Y., Li, H., Wang, M.N., Bassi, R., Balderes, P., Ludwig, D.L., Witte, L., et al. (2005): Inhibitory anti-FLT3 antibodies are capable of mediating antibody-dependent cell-mediated cytotoxicity and reducing engraftment of acute myelogenous leukemia blasts in nonobese diabetic/severe combined immunodeficient mice. **Cancer Research** 65, 1514-1522.

Pedersen, J.S., Otzen, D.E., Kristensen, P. (2002): Directed evolution of barnase stability using proteolytic selection. **Journal of Molecular Biology** 323, 115-123.

Persson, M.A., Caothien, R.H., Burton, D.R. (1991): Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. **Proceeding of the National Academic of Science of the United State of America** 88, 2432-2436.

Proba, K., Worn, A., Honegger, A., Pluckthun, A. (1998): Antibody scFv fragments without disulfide bonds made by molecular evolution. **Journal of Molecular Biology** 275, 245-253.

Qi, H., Lu, H., Qiu, H.J., Petrenko, V., Liu, A. (2012): Phagemid vectors for phage display: Properties, characteristics and construction. **Journal of Molecular Biology** 417, 129–143.

Quiambao, B. P., Dimaano, E. M., Ambas, C., Davis, R., Banzhoff, A., Malerczyk, C. (2005): Reducing the cost of post-exposure rabies prophylaxis: efficacy of 0.1 ml PCEC rabies vaccine administered intradermally using the Thai Red Cross post-exposure regimen in patients severely exposed to laboratory-confirmed rabid animals. **Vaccine** 23, 1709–1714.

Rangnoi, K., Jaruseranee, N., O'Kennedy, R., Pansri, P., Yamabhai, M. (2011): One-Step Detection of Aflatoxin-B1 Using scFv-Alkaline Phosphatase-Fusion

Selected from Human Phage Display Antibody Library. **Molecular Biotecnology** 49, 240-249.

Ray, K., Embleton, M.J., Jailkhani, B.L., Bhan, M.K., Kumar, R. (2001): Selection of single chain variable fragments (scFv) against the glycoprotein antigen of the rabies virus from a human synthetic scFv phage display library and their fusion with the Fc region of human IgG1. **Clinical and Experimental Immunology** 125(1), 94-101.

Reilly, R.M. (2006): Radioimmunotherapy of solid tumors. **Journal of Nuclear Medicine** 47, 196-199.

Rodi, D.J., Makowski, L., Kay, B.K. (2002): One from column A and two from column B: the benefits of phage display in molecular-recognition studies. **Current Opinion in Chemical Biology** 6, 92-96.

Rowley, M.J., O'Connor, K., Wijeyewickrema, L. (2004): Phage display for epitope determination: a paradigm for identifying receptor-ligand interactions. **Biotechnology Annual Review** 10, 151-188.

Santos, A.D., Padlan, E.A. (1998): Development of more efficacious antibodies for medical therapy and diagnosis. **Progress in Nucleic Acid Research & Molecular Biology** 60, 169-194.

Sato, A.K., Viswanathan, M., Kent, R.B., Wood, C.R. (2006): Therapeutic peptides: technological advances driving peptides into development. **Current Opinion in Biotechnology**, 17:638-642.

Schier, R., Bye, J., Apell, G., McCall, A., Adams, G.P., Malmqvist, M., Weiner, L.M., Marks, J.D. (1996a): Isolation of high-affinity monomeric human anti-

c-erbB-2 single chain Fv using affinity-driven selection. **Journal of Molecular Biology** 255, 28-43.

Schier. R, McCall, A., Adams, G.P., Marshall, K.W., Merritt, H., Yim, M., Crawford, R.S., Weiner, L.M., Marks, C., Marks, J.D. (1996b): Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. **Journal of Molecular Biology** 263, 551-567.

Schirrmann, T., Meyer, T., Schütte, M., Frenzel, A., Hust M. (2011): Phage display for the generation of antibodies for proteome research, Diagnostics and therapy. **Molecules** 16, 412-426.

Schumacher, T.N., Mayr, L.M., Minor, D.L. Jr., Milhollen, M.A., Burgess, M.W., Kim, P.S. (1996): Identification of D-peptide ligands through mirror-image phage display. **Science** 271, 1854-1857.

Shaw, I., O'Reilly A, Charleton M, Kane M. (2008): Development of a high-affinity anti-domoic acid sheep scFv and its use in detection of the toxin in shellfish. **Analytical Chemistry**, 3205-3212.

Sheets, M.D., Amersdorfer, P., Finnern, R., Sargent, P., Lindquist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J.C., Marks, J.D. (1998): Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. **Proceeding of the National Academic of Science of the United State of America** 95, 6157-6162.

Schnell, M. J., McGettigan, J. P., Wirblich, C., Papaneri A (2010): The cell biology of rabies virus: using stealth to reach the brain. **Nature Reviews Microbiology** 8, 51-61.

Smith, G. P. (1985): Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. **Science** 228, 1315.

Smith, T.G., Wu, X., Franka, R., Rupprecht, C.E. (2011): Design of Future Rabies Biologics and antiviral Drugs. **Advance in Virus Research** 79:346-362

Smothers, J.F., Henikoff, S., Carter, P. (2002): Tech.Sight. Phage display. Affinity selection from biological libraries. **Science** 298, 621-622.

Stowell, C.P. (2006): Therapy with immunoglobulin. applications for monoclonal antibodies. **Journal of Infusion Nursing** 29, S29-44.

Sun, L., Chen, Z., Yu, L., Wei, J., Li, C., Jin, J., Shen, X., Lv, X., Tang, Q., Li, D., Liang, M. (2012): Generation and characterization of neutralizing human recombinant antibodies against antigenic site II of rabies virus glycoprotein. **Applied Microbiology and Biotechnology** 96(2), 357-366.

Teillaud, J.L. (2005): Engineering of monoclonal antibodies and antibody-based fusion proteins: successes and challenges. **Expert Opinion on Biological Therapy** 5 Suppl 1, S15-27.

Ullas, P. T., Desai, A., Madhusudana, S. N. (2012): Rabies DNA vaccines: current status and future. **World Journal of Vaccines** 2, 36-45

Van de Wiele, C., Revets, H., Mertens, N. (2004): Radioimmunoimaging advances and prospects. **Quarterly Journal of Nuclear Medicine and Molecular Imaging** 48, 317-325.

Vanlandschoot, P., Stortelers, C., Beirnaert, E., Itati Ibnez, L., Schepens, B., Depla, E., Saelens, X. (2011): Nanobodies: New ammunition to battle viruses. **Antiviral Research**, 389-407.

Wang, D.D., Su, M.M., Suna, Y., Huang, S.L., Wang, J., Yan, W.Q. (2012): Expression, purification and characterization of a human single-chain Fv antibody fragment fused with the Fc of an IgG1 targeting a rabies antigen in *Pichia pastoris*. **Protein Expression and Purification** 86, 75–81.

Webster, R. E. (1996). Biology of the filamentous bacteriophage. In: Kay BK (ed) **Phage display of peptides and proteins: A laboratory manual**. Academic Press, California, 1-20.

Willats, W.G.T. (2002): Phage display: practicalities and prospects. **Plant Molecular Biology** 50, 837-854

Willemsen, R.A., Ronteltap, C., Chames, P., Debets, R., Bolhuis, R.L. (2005): T cell retargeting with MHC class I-restricted antibodies: the CD28 costimulatory domain enhances antigen-specific cytotoxicity and cytokine production. **Journal of Immunology** 174, 7853-7858.

Winter, G., Griffiths, A.D., Hawkins, R.E., Hoogenboom, H.R. (1994): Making antibodies by phage display technology. **Annual Review of Immunology** 12, 433-455.

Xie, M.H., Yuan, J., Adams, C., Gurney, A. (1997): Direct demonstration of MuSK involvement in acetylcholine receptor clustering through identification of agonist ScFv. **Nature Biotechnology** 15, 768-771.

Yamabhai, M., Hoffman, N.G., Hardison, N.L., McPherson, P.S., Castagnoli, L., Cesareni, G., Kay, B.K. (1998): Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. **Journal of Biolgical Chemistry** 273, 31401-31407.

Yang, L., Cen, J., Xue, Q., Li, J., Bi, Y., Sun, L., Liu, W. (2013: Identification of rabies virus mimotopes screened from a phage display peptide library with purified dog anti-rabies virus serum IgG. **Virus Research** 174, 47–51.

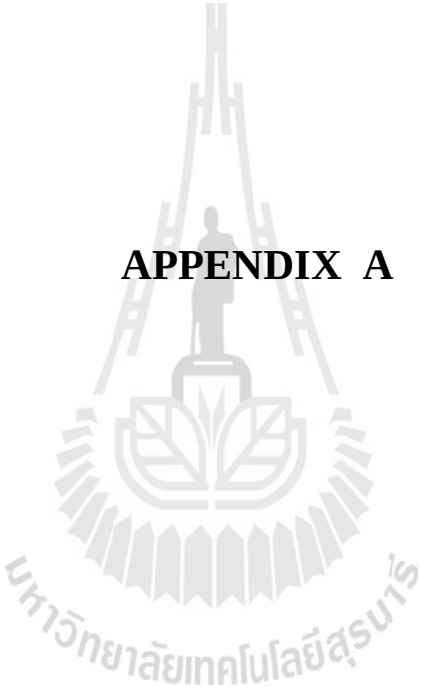
Yang, W.P., Green, K., Pinz-Sweeney, S., Briones, A.T., Burton, D.R., Barbas, C.F., 3rd. (1995): CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. **Journal of Medical Virology** 254, 392-403.

Zhao, X.L., Yin J., Chen, W.Q., Jiang, M., Yang, G., Yang, Z.H. (2008): Generation and characterization of human monoclonal antibodies to G5, a linear neutralization epitope on glycoprotein of rabies virus, by phage display technology. **Microbiology Immunology** 52, 89–93

Zhao, X.L., Chen, W.Q., Chena, W.Q., Yanga, Z.H., Li, J.H., Zhang, S.J., Tian, L.F. (2009): Selection and affinity maturation of human antibodies against rabies virus from a scFv gene library using ribosome display. **Journal of Biotechnology** 144, 253–258

Zhang, X.X., Deng, Q., Zhang, S.Y., Liu, J., Cai, Q., Lu, Z.M., Wang, Y. (2003): Broadly cross-reactive mimotope of hypervariable region 1 of hepatitis C virus derived from DNA shuffling and screened by phage display library. **Journal of Medical Virology** 71, 511-517.

APPENDIX A



APPENDIX A

ADDITIONAL REAGENTS

A.1	**100 mM $NaHCO_3$ pH 8.5**	100 ml
-	$NaHCO_3$	0.84 g
-	DI H_2O	70 ml
-	Adjust pH 8.5 and add DI H_2O to	100 ml
-	Autoclave	
A.2	**20% Glucose**	40 ml
-	Glucose	8 g
-	DI H_2O to	40 ml
-	Filter-sterilize	
A.3	**20% Sucrose**	40 ml
-	Sucrose	8 g
-	DI H_2O to	40 ml
-	Filter-sterilize	
A.4	**2xYT broth**	100 ml
-	Tryptone	1 g
-	Yeast extract	1 g
-	NaCl	0.5 g
-	DI H_2O to	100 ml

- Autoclave

A.5 2xYT with Amp + 1% glucose 400 ml

- Tryptone 1 g
- Yeast extract 1 g
- NaCl 0.5 g
- Bacto agar 3.2 g
- DI H_2O to 400 ml
- Autoclave

A.6 50 mM Glycine-HCl pH 2.0 30 ml

- Glycine 0.11 g
- DI H_2O 20 ml
- Adjust pH to 2.0 by using conc.HCl
- DI H_2O to 30 ml
- Autoclave

A.7 50 mM citric acid monohydrate pH 4.0, 300 ml (Solvent of ABTS substrate used 1 mg ABTS to 1mL of 50 mM citric acid monohydrate pH4.0)

- Citric acid 3.15 g
- DI H_2O 200 ml
- Adjust pH 4.0, add DI H_2O to 300 ml
- Autoclave

A.8 Neutralization solution

8.1 0.2 M $NaH_2PO_42H_2O$ 20 ml

- $NaH_2PO_42H_2O$ 0.62 g

- Add DI H_2O 20 ml
- Autoclave

8.2 0.2 M Na_2HPO_4 60 ml

- Na_2HPO_4 1.70 g
- Add DI H_2O 60 ml
- Autoclave

A.9 2% skimmilk 10 ml

- skim milk 0.2 g
- 1x PBS 10 ml

A.10 5x PEG/NaCl 500 ml

- Polyethylene glycol 8000 65 g
- NaCl 46.8 g
- DI H_2O to 500 ml
- Filter-sterilize 0.2 µm

A.11 10xPBS 1 L

- NaCl 80 g
- KCl 2 g
- Na_2HPO_4 17 g
- KH_2PO_4 1.63 g
- DI water 950 ml,
- adjust pH to 7.4 with 50 ml DI water
- Autoclave

A.12 M9ZB Medium:

12.1 Solution A.

- BactoTryptone 10 g
- Yeast extract 5 g
- NaCl 5 g
- NH_4Cl 1 g
- Dissolve in 800 ml RO water, autoclave.

12.2 Solution B.

- KH_2PO_4 3 g
- Na_2HPO_4 6 g
- Dissolve in 100 ml RO water, autoclave.

12.3 Solution C.

- 20% glucose OR 10% glycerine
- Autoclave.

12.4 Solution D. (1M $MgSO_4$ Mw 120),

- $MgSO_4$ 1.2 g
- Dissolve in 10 ml RO water, autoclave.

For complete medium, combine

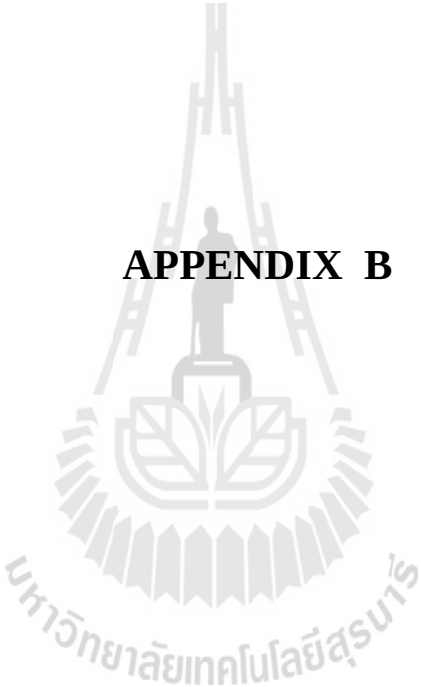
Solution A 400 ml

Solution B 50 ml

Solution C 50 ml

Solution D 0.5 ml

APPENDIX B



APPENDIX B

STEP-BY-STEP METHODS

B.1 Biopanning with PCEC or PVRV target

1. A Maxisorp Immuno tube (Nunc, Denmark) was pre-coated with 0.35-1.4 IU of inactivated rabies virus (PCEC or PVRV), at 37 ^{o}C for 3 h followed by 4 ^{o}C overnight,

Example

Prepare 1 IU of PCEC for immobilized into immuno tube

(Bio-Panning)

PCEC (LEP-Flury strain, Rabipur, Chiron, India)

Potency 7.97 IU/dose/1 ml

7.97 IU	dissolved in	1000	µl
1 IU	”	125.47	µl

- Add 125.47 ul of PCEC into the immuno tube
- Add 100 mM NaHCO3, pH 8.5 into the immuno tube to 200 ul
- Incubate at 37 ^{o}C for 3 h.
- Incubate at 4 ^{o}C for overnight.

Example

Prepare 1 IU of PVRV for immobilized into immuno tube

PVRV (VeroRab, Pitman-more/ W138-153-3M strain, Sanofi-Pasteur, Lyon, France) Potency 9.2 IU/ dose/0.5 ml

9.2 IU	dissolved in	500	µl
1 IU	”	54.35	µl

- Add 54.35 ul of PCEC into the immuno tube
- Add 145.65 ul of 100 mM $NaHCO_3$, pH 8.5 into The Immuno 96 microWellTM plate (Nunc, Denmark)
- Incubate at 37 ^oC for 3 h.
- Incubate at 4 ^oC for overnight.

2. Next day, stabilized with 200 µl of immobilized target by 5 % w/v sucrose, 0.3 % w/v BSA, and 50 mM $NaHCO_3$

Prepare 5 % w/v sucrose, 0.3 % w/v BSA, and 50 mM $NaHCO_3$

Sucrose	0.25	g
BSA	0.015	g
100 mM $NaHCO_3$	2.5	ml
PBS	2.5	ml
Total	10	ml

- Incubate at room temperature for 45 min

3. Wash tube 3 times with PBS (pour into the tube and then pour it immediately out again).

4. Block; Fill tube to brim with PBS containing skimmed milk (2 %, w/v, MPBS) then incubated at room temperature by standing on the bench for 1 h.

5. Prepare phage library

Add phage library (titer 10^{11}) 10 µl in 190 µl of 4%, w/v, MPBS

Mix well and keep it on ice

6. Add phage display scFv antibody library.

- Pour out PBS containing skimmed milk.
- Add 200 µl of the mixture of phage display scFv antibody library.
- Incubate at room temperature for 2 h.

7. Prepare log phase TG1.

- Add 50 µl of overnight *E. coli* TG1 in in 2 ml of 2xYT broth
- Incubate at 37 ^{o}C with shaking for 2 h.

8. Wash tube 10 times with PBST and 10 times PBS.

9. Elute.

- Add 100 µl Trypsin
- Incubate at room temperature for 15 min
- Take trypsin into 1.5 ml tube.
- Acid elution by adding 100 µl of 0.1M glycine HCl pH 2.0
- Incubate at room temperature for 10 min
- Prepare neutralization sol.

0.2 M $NaH_2PO_42H_2O$	16	µl
0.2 M Na_2HPO_4	84	µl
Total	100	µl

- Take glycine into 1.5 ml tube and neutralize with 100 µl neutralization sol.

- ** Elute phage = Eluted phage Trypsin + Eluted phage Glycine with Neutralization sol.

10. Infect; Add Elute phage 150 µl into TG1 log phase 2 ml (keep the rest phage in 4 ^{o}C). Incubate 37 ^{o}C for 30 min (Infected *E. coli* by eluted phage).

11. Make a serial dilution of infected *E. coli* TG1 with 2xYT

12. Spread plate on 2xYT with Amplicillin + 1% glucose

- 1:10 (10^{-1}) spread 100 µl
- 1:100 (10^{-2}) spread 100 µl
- 1:1000 (10^{-3}) spread 100 µl
- All (centrifuged 2ml TG1 log phase) spread all
- All (centrifuged 2ml infected *E.coli* TG1) spread all

13. Phage titre (Output)

Example

	Colonies	Titre (pfu/ul)
10^{-1}	19	$1.9x10^{2}$
10-^{2}	1	$1x10^{2}$
10^{-3}	0	-
All	2,520	-
Negative control	0	-

B.2 Biopanning with Glycoprotein

1. Rabies G protein pre-coated 8-well strips kit from BioRad was block with 200 µl with PBS containing skimmed milk (2 %, w/v, MPBS).

2. Prepare phage library;

- Add phage library (titer 10^{11}) 10 µl in 190 µl of 4 %, w/v, MPBS
- Mix well and keep it on ice

3. Add phage;

- Pour out PBS containing skimmed milk.
- Add 200 µl of phage
- Incubate at room temperature for 2 h.

4. Prepare log phase TG1;

- Add 50 µl of overnight *E. coli* TG1 in 2 ml of 2xYT broth
- Incubate at 37 ^{o}C with shaking for 2 h.

5. Wash 5 times with buffer from kit

6. Elute;

- Add 100 µl Trypsin
- Incubate at room temperature for 15 min
- Take trypsin into 1.5 ml tube.
- Acid elution by adding 100µl of 0.1M glycine HCl pH 2.0 and neutralize with 100 µl solution.
- Incubate at room temperature for 10 min
- Take glycine into 1.5 ml tube with neutralization sol.
- ** Elute phage = Eluted phage Trypsin + Eluted phage Glycine with Neutralization sol.

7. Infect;

- Add Elute phage 150 µl into TG1 log phase 2 ml (keep the rest phage in 4 ^{o}C).

- Incubate 37 ^{o}C for 30 min (Infected *E. coli* by eluted phage).

8. Phage titre (Output)

Make a serial dilution of infected *E. coli* TG1 with 2xYT

Spread plate on 2xYT with Amplicillin + 1 % glucose

- 1:10 (10^{-1}) spread 100 µl
- 1:100 (10^{-2}) spread 100 µl
- 1:1000 (10-3) spread 100 µl
- All (centrifuged 2 ml TG1 log phase) spread all
- All (centrifuged 2 ml infected *E.coli* TG1) spread all

<u>Example</u>

	Colonies	Titre (pfu/ul)
10^{-1}	Too many to count	-
10^{-2}	280	$2.8x10^{3}$
10^{-3}	19	$1.9x10^{3}$
All	4	$4x10^{3}$
Negative control	0	-

B.3 Amplified phage for the next round

1. One milliliter of 2xYT with 1% (w/v) glucose and 100 µg/ml ampillicin media was added to a plate that contained all colonies from first round of selection and loosens *E. coli* with spreader.

2. Scraped cell 5 µl was added to 5 ml of 2xYT with 1 % glucose and 100 µg/ml amplicillin and incubated at 37 °C 200 rpm for 2-3 h.

3. Helper phage 0.5 µl (titre 10^{11}) was added and incubated at 37 °C for 30 min.

4. Spin at 3,000 x g for 10 min. Resuspend in 5 ml of 2xTY containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and 0.1% (w/v) glucose.

5. Incubate shaking at 30 °C overnight (18-20 h)

6. Next day, spin the overnight culture at 3,300 g for 15 min.

7. Add 1 ml PEG/NaCl (20 % Polyethylene glycol 6000, 2.5 M NaCl) to 40 ml supernatant. Mix well and leave for 1 hr on ice.

8. Spin 3,300 x g for 30 min. Pour away PEG/NaCl. Respin briefly for 5 min to remove all remaining supernatant.

9. The phage precipitate pellet was resuspended in 200 µl of 1xPBS and phage particle can be store at 4 °C for several weeks.

10. Repeat selection. For round two-five of selection 100 µl of amplified phage was mixed with 100 µl of 4 % MBPS to perform the selection.

11. The same selection protocol as described in round one of selection was done.

B.4 ELISA

1. Coat a Immuno 96 microWellTM plate with 100 µl (0.1 IU) of inactivated rabies virus (PCEC or PVRV), at 37 °C for 3 h followed by 4 ^{o}C overnight.

2. Stabilized with 100 µl of 5 % w/v sucrose, 0.3 % w/v BSA, and 50 mM $NaHCO_3$ for 45 min.

3. Wash wells 3 times with PBS.

4. Add 200 µl per well of PBS containing skimmed milk (2 %, w/v, MPBS) to block and incubate for 1 hr at room temperature.

5. Pour out PBS containing skimmed milk and add 50 µl of PBS containing skimmed milk (4%, w/v, MPBS)

6. Add 100-150 µl of phage scFv or soluble scFv or 100 µl of 1:100 IgG (serum) in PBS.

7. Incubate for 1-2 h at 37 ^{o}C or room temperature.

8. Wash wells 3 times with PBST and 3 times PBS.

9. Add 100 µl/well of secondary antibody.

Sample	Secondary antibody	Antibody/PBS (Ratio)
Phage scFv (M13)	AntiM13- HRP	4 µl/20ml (1:5000)
Soluble scFv (Myc-Tag)	Mouse Anti-Myc HRP	4 µl/20ml (1:5000)
Soluble scFv (His-Tag)	His probe-HRP	4 µl/20ml (1:5000)
IgG (serum)	Protein A-HRP	2.5 µl/20ml (1:8000)

10. Incubate for 1 h at room temperature.

11. Wash wells 3 times with PBST and 3 times PBS.

12. Add 100 µl of TMB or 200 µl of ABTS.

13. For TMB substrate; incubate for 20 min and add 50 µl of 1 M sulphuric acid to stop the reaction. For ABTS; the reaction was quantified by measuring the absorbance at 405 nm. And read the OD at 450 nm for TMB.

B.5 Expression using *E.coli* BL21 (DE3)

1. Pick single colony of *E.coli* BL21 (DE3) harboring pET27b+scFv into M9ZB (C=20% glucose) medium with 50 µg/ml kanamycin and incubate overnight, shaking at 30 ^{o}C.

2. Inoculate 1:100 into fresh M9ZB (C=20% glucose), shaking 200 rpm for 4 h at 30 °C.

3. Spin at 5,000 rpm, 15 min, 16 °C.

4. Replace medium for M9ZB (C = 10 % glycerine) with 50 µg/ml kanamycin and 1 mM IPTG (final concentration).

5. Incubate with shaking 200 rpm overnight, 16 °C.

6. Spin at 8,000 rpm, 15 min, and 4 °C. The secrete antibody fragment can be found in the supernatant.

B.6 Expression using DH5αF'

1. Pick single colony of *E.coli* DH5αF' harboring pMY607AP+scFv into 5 ml 2xYT medium with 100 µg/ml ampicillin and 1% glucose and incubate overnight, shaking at 37 ^{o}C.

2. Spin at 3,000 rpm for 15 min.

3. Replace medium with 2xYT medium containing 100 µg/ml ampicillin and 1 mM IPTG (final concentration).

4. Incubate with shaking 200 rpm at room temperature for 20 h.

5. Spin at 8,000 rpm, 15 min, 4 °C. The secrete antibody fragment can be found in the supernatant

B.7 Expression using HB2151

1. Pick single colony of *E.coli* HB2151 harboring pMOD1+scFv into 5 ml 2xYT medium with 100 µg/ml ampicillin and 2%glucose and incubate overnight, shaking at 30 ^{o}C.

2. Next day, inoculate 50 µl of overnight culture (1 % inoculum) in 5 ml 2xYT broth containing 100 µg/ml ampicillin and 2 % glucose incubate shaking at 30 ^{o}C until OD600 is 0.9 (~ 4 h).

3. Add IPTG to a final concentration of 1mM and continue shaking at 30 ^{o}C for 20 h.

4. Spin at 3,000 rpm for 10 min to remove bacteria and debris. The secrete antibody fragment can be found in the supernatant.

B.8 Neutralization assay (Rapid Fluorescent Focus Inhibition test; RFFIT)

1. Dilute sample with desired dilution 50 µl/well (2-fold interval) in 96-well tissue culture plates with multichannel pipette by using maintenance medium (2 % DMEM)

2. Add 50 µl of rabies virus suspension (100 $TCID_{50}$), Incubate 37 ^{o}C for 90 min in CO_2 incubater

3. Trypsinize a bottle (75 cm^3) of BHK-cells and prepare cell suspension of 10^6 cell/ml

4. Add 50 ul of BHK-21 (10^5 cell/well),

5. Incubate 37 ^{o}C for 21 h in CO_2 incubater

6. Remove medium after incubation

7. Wash cell monolayer with PBS, Fix with 90 % acetone and dry in the incubator

8. Stain with FITC labeled anti-rabies for 30 min at 37 ^{o}C

9. Wash with PBS and dry in the incubator

10. Observe the wells and count (8 fields per well). Field is judged as positive if it includes at least one attained cell.

11. Calculate the $TCID_{50}$ using the Spearman-Karber method.

$$\text{Log TCID 50} = L - D(s - 0.5)$$

L = Log of the strongest dilution

D = Difference between log dilution

S = sum of proportion of negative filed

Count infected cell using a fluorescence microscope (negative field/8 fields)

Example

Dilution	1:16	1:32	1:64	1:128	1:256
Negative field / 8 fileds	8/8	7/8	4/8	1/8	0/8
Proportion	1	0.88	0.5	0.12	0

Sum of proportions = 1.5 (from 1:32 to 1:256 dilutions)

The starting dilution is 1:32; log = -1.5

The dilution are twofold log 2 = +0.3

Log $TCID_{50}$ = -1.5.0.3 (1.5-0.5) = -1.8

Antilog = dilution giving 50 % reduction in negative fields = 1: 63

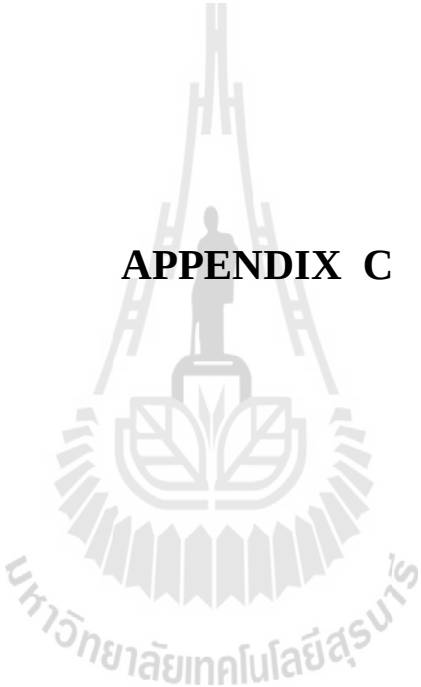
Convert to international units using the WHO standard serum

One tissue culture ID50 (TCID50) is the infectious dose of virus that will result in 50 % of the observed fields having one or more infected cells.

Test Validity: for each test

- Reproducibility of standard $TCID_{50}$
- Reproducibility of challenging virus (working dilution)
- Reproducibility of internal control serum titers
- Good homogenicity of result between two difference wells for each dilution.

APPENDIX C



APPENDIX C

DNA AND AMINO ACID SEQUENCE

C.1 DNA sequence.

C.1.1 IRA7c_pMOD1 (IVB4cv_pMOD1)

>IRA7c_pMOD1 (IVB4cv_pMOD1)

ATGGCCCAGGTGCAGCTGGTGCAGTCTGGGGGAGGCTTGGTCCAGCCTGGGGGGTCCCTGAGACTCTCC
TGTGCAGCGTCTGGATTCAGTTTCAGTGACTATGGCATGCACTGGGTCCGCCAGATTCCAGGCAAGGGA
CTGGAGTGGGTGGCAGTTATATATGCTCGTGGAATCAATACATATTATGGAGACTCCGTGAAGGGCCGA
TTCACCATCTCCAGAGATAATTCCAAGAACATTCTGTATTTGGAAATGAACAGGCTGAGCTCCGAGGAC
ACGGCTGTCTATTACTGTGCGACAGACGATCCCCCGAGTGGTACGGGGTCTTATCACGTCAACTGGGGC
CAGGGAACCCTGGTCACCGTCTCCTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTGGTGGA
TCCCAGTCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCGATCACCATCTCCTGC
ACTGGAACCAGCAGTGATGTTGGGAGTTATAACCTTGTCTCCTGGTACCAACAGCACCCAGGCAAAGCC
CCCAAACTCATGATTTATGAGGTCAGTAAGCGGCCCTCAGGGGTCCCTGATCGCTTCTCTGGCTCCAAG
TCTGGCAACACGGCCTCCCTGACCGTCTCTGGGCTCCAGGCTGAGGATGAGGCTGATTATTACTGCAGC
TCATATACAAGCATCACCGCTGCCGCTGTCTTCGGAACTGGGACCAAGGTCACCGTCCTAGGTGCGGCC
GCACATCATCATCACCATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGAAGAAGACCTGAAC
GGTACCGCATAG

C.1.2 IYF5c_pMOD1

>IYF5c_pMOD1

ATGGCCCAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTCTCC
TGCAAGGCTTCTGGTTACACCTTTACCAGCTATGGTATCAGCTGGGTGCGACAGGCCCCTGGACAAGGG
CTTGAGTGGATGGGATGGATCAGCGCTTACAATGGTAACACAAACTATGCACAGAAGCTCCAGGGCAGA
GTCACCATGACCACAGACACATCCACGAGCACAGCCTACATGGAGCTGAGGAGCCTGAGATCTGACGAC

ACGGCCGTGTATTACTGTGCGGACGGTGGTAACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC

TCCTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCCAGGCTGTGCTCACTCAG

CCGTCCTCAGCGTCTGGGACCCCCGGGCAGAGGGTCACCATCTCTTGTTCTGGAAGCAGCTCCAACATC

GGAAGTAACTATGTATACTGGTACCAGCAGCTCCCAGGAACGGCCCCCAAACTCCTCATCTATAGGAAT

AATCAGCGGCCCTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCTGGCACCTCAGCCTCCCTGGCC

ATCAGTGGGCTCCGGTCCGAGGATGAGGCTGATTATTACTGTGCAGCATGGGATGACAGCCTGAGTGGT

CCGGTATTCGGCGGAGGGACCAAGCTGACCGTCCTAGGTGCGGCCGCACATCATCATCACCATCACGGG

GCCGCAGGGCCCGAACAGAAACTGATCTCTGAAGAAGACCTGAACGGTACCGCATAG

C.1.3 IIIRC2c_pMOD1

>IIIRC2c_pMOD1

ATGGCCCAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTTCGGAGACCCTGGCCCTCAC

TGCACTGTCTCTGGTGGCTCCCTCAGCAGTGTTAATAGTTACTGGGACTTCATCCGCCAGCCCCCAGGG

AAGGGACTGGAGTGGATTGGGAGTATCTATTATAGGGGGACCACTTACTACAACCCGTCCCTCAAGAGT

CGAGTCACCTTATCAGTGGACACGTCCCAGAATCAAATCTCCCTGAAGCTGACCTCTCTGACCGCCGCG

GACACGGCCGTATATTACTGTGCGAGAGAATCAGTCACCAGGGGAACTTTTGATATGTGGGGCCAAGGG

ACAATGGTCACCGTCTCTTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCCAG

TCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCGATCACCATCTCCTGCACTGGC

ACCAGCAGTGACGTTGGTGGTTATAACTATGTCTCCTGGTACCAACAACACCCAGGCAAAGCCCCCAAC

CTCATGATTTATGATGTCAGTAATCGGCCCTCAGGGGTTTCTAATCGCTTCTCTGGCTCCAAGTCTGGC

AACACGGCCTCCCTGACCATCTCTAGGCTCCAGGCTGAGGACGAGGGTATCTATTTCTGCAGCGCATAT

ACAAGCAGCAGCTCTCTCGGGGTCTTCGGCACTGGGACCAAGCTGACCGTCCTAGGTGCGGCCGCACAT

CATCATCACCATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGAAGAAGACCTGAACGGTACC

GCATAG

C.1.4 IYC11c_pMOD1

> IYC11c_pMOD1

ATGCAGCTGGTGGAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTCTCCTGCAAGGCT

TCTGGATACAGCTTCACCGCCTACTATATACACTGGGTGCGACAGGCCCCTGGACAAGGGCTTGAGTGG

ATGGGGTGGATCAATCCTAACAGTGGTACCACAACCTATGCACAGAGGTTTCAGGGCAGGGTCACCATG

ACCAGGGACACGTCCATCAGCACAGCCTACATGGAGCTGAGCAGGCTGAGATCTGACGACACGGCCGTG

TATTACTGTGCGAGAGATTTCGGGGGCTACTGGAGGTGGGGTGCTTTTGATATCTGGGGCCAAGGGACC

ACGGTCACCGTCTCCTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTAGTGGATCCTCCTAT

GTGCTGACTCAGCCACCCTCGGTGTCAGTGGCCCCAGGAAAGACGGCCAGGATTACCTGTGGGGGAAAC

AACATTGGAAGTAAAAGTGTGCACTGGTACCAGCAGAAGCCAGGCCAGGCCCCTGTGCTGGTCGTCTAT

GATGATAGCGACCGGCCCTCAGGGATCCCTGAGCGATTCTCTGGCTCCAACTCTGGGAACACGGCCACC

CTGACCATCAGCAGGGTCGAAGCCGGGGATGAGGCCGACTATTACTGTCAGGTGTGGGATAGTAGTAGT

GATCATTATGTCTTCGGAACTGGGACCCAGCTCACCGTTTTACGTGCGGCCGCAGTCGAC

C.1.5 IYC12c_pMOD1

> IYC12c_pMOD1

ATGGCCCAGGTCAACTTAAGGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCC

TGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGG

CTGGAGTGGGTCTCATCTATTACTTATTCTGGTACTGCTACATCTTACGCAGACTCCGTGAAGGGCCGG

TTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGAC

ACGGCCGTATATTACTGTGCGAAAGGTTATTCTACTTTTGACTACTGGGGCCAGGGAACCCTGGTCACC

GTCTCCTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTAGTGGATCCCAGGCTGTGCTCACT

CAGCCGTCCTCAACGTCTGGGACCCCCGGGCAGAGGGTCACCATCTCTTGTTCTGGAGGCAGCTCCAAC

ATCGGAAGCAATACTGTCAATTGGTACCGGCATCTCCCGGGGACGGCCCCCAAACTCCTCATCTACATT

GATGATCGGCGGCCCTCAGATATCCCTGACCGATTCTCTGGCTCCAGGTCTGGCACCTCAGCCTCCCTT

GCCATCAGTGGGCTCCAGTCTGAGGATGAGGCTGATTATTATTGCGCAGCATGGGATGACAGCCTGAAT

GGTCTCGTTTTTGGCGGAGGGACCCAGCTCACCGTTTTAGGTGCGGCCGCAGTCGAC

C.1.6 IRC3c_pMOD1

> IRC3c_pMOD1 (Consist only VL)

ATGGCCCAGGTCAACTTAAGGGAGTCTGGGACCACGGTCACCGTCTCTTCAGGTGGTGGTGGTAGCGGC

GGCGGCGGCTCTGGTGGTGGTGGATCCCAGTCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCT

GGACAGTCGATCACCATCTCCTGCACTGGAACCAGCAGTGACGTTGGTGGTTATAACTATGTCGCCTGG

TACCAACATCACCCAGGCAAAGCCCCCAAACTCATGATTTATGATGTCAGTAATCGGCCCTCAGGGGTT

TCTAATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGACCATCTCTGGCCTGCAGGCTGAG

GACGAGGCTGATTATTACTGCAGCTCATATACAAGCAGCAGCACTCTCGTGGTATTCGGCGGAGGGACC

AAGGTCACCGTCCTAGGTGCGGCCGCAGTCGAC

C.1.7 IYD1c_pMOD1

> IYD1c _pMOD1

ATGGCCCAGGTCAACTTAAGGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCC

TGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGG

CTGGAGTGGGTCTCAGCTATTACTTATAATGGTGCTTCTACAGCTTACGCAGACTCCGTGAAGGGCCGG

TTCACCATCTCCAGAGACAATTCCTAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGAC

ACGGCCGTATATTACTGTGCGAAAGGTTACTCTACTTTTGACTACTGGGGCCAGGGAACCCTGGTCACC

GTCTCCTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCTCTTCTAAGCTGACT

CAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCAGGATCACATGCCAAGGAGACAGCCTCAGA

AGCTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCCCCTGTACTTGTCATCTATGGTAAAAAC

AACCGGCCCTCAGGGATCCCAGACCGATTCTCTGGCTCCAAGTCTGGCACCTCAGCCTCCCTGGACATC

AGTGGGCTCCAGTCTGAGGATGAGGCTGATTATTACTGTGCAGCATGGGATGACAGCCTGAATGGTGTG

TTATTCGGCGGAGGGACCAAAGTCACCGCCCTACGTGCGGCCGCAGTCGAC

C.1.8 IIRD5v_pMOD1

> IIRD5v_pMOD1

ATGGCCGAGGTGCAGCTGGTGGAGTCTGGGACTGAGGTGAGGAAGCCTGGGGACTCAGTGAAGGTCTCC

TGCAAGGCTTCTGGATACACATTTACCGACTACTATCTGCACTGGGTGCGACAGGCCCCTGGACAAGGG

CTTGAGTGGGTGGGATGGATCTACCCTAAGAGGGGTGGCACACACTCTGCACAGAAGTTTCAGGGCAGG

GTCACCATGACCAGGGACACGTCCATCAACACAGCCTACATGGAGCTGACCAGACTGAGATCTGACGAC

ACGGCCGTATATTTCTGTGCGAGAGATCGCGACATTGAAGATGCTTTTGATATCTGGGGCCAAGGGACA

ATGGTCACCGTCTCTTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCCAGTCT

GCCCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCGATCACCATCTCCTGCACTGGCACC

AGCAGTGACGTTGGTGGTTATAACTATGTCTCCTGGTACCAACAACACCCAGCCAAAGCCCCCAACCTC

CTGATTTATGATGTCAGTAATCGGCCCTCAGGGGTTTCTAATCGCTTCTCTGGCTCCAAGTCTGGCAAC

ACGGCCTCCCTGACCATCTCTGGGCTCCAGGCTGAGGACGAGGCTGATTATTTCTGCAGCTCATATACA

ACCAGCAGCACTCTGGTGTTCGGCGGAGGGACCAAGGTCACCGTCCTAGGTGCGGCCGCACATCATCAT

CACCATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGAAGAAGACCTGAACGGTACCGCATAG

CATCATCATCACCATCACGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGAAGAAGACCTGAACGGTA

CCGCA

C.1.9 IIYB5v_pMOD1

> IIYB5v_pMOD1

AAGGGCTTGAGTGGATGGGAGGGATCATCCCTATCTTTGGTACAGCAAACTACGCACAGAAGTTCCAGG

GCAGAGTCACGATTACCGCGGACGAATCCACGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTG

AGGACACGGCCGTGTATTACTGTGCGAGAGATCGGGAGCTACCAGGATTTGACTACTGGGGCCAGGGAA

CCCTGGTCACCGTCTCCTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTGATGGATCCTCCT

ATGTGCTGACTCAGCCACCCTCAGCGTCTGGGACCCCCGGGCAGAGGGTCACCATCTCTTGTTCTGGAA

GCAGCTCCAACATCGGAAGTAATACTGTAAACTGGTACCAGCAGCTCCCAGGAACGGCCCCCAAACTCC

TCATCTATAGTAATAATCAGCGGCCCTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCTGGCACCT

CAGCCTCCCTGGCCATCAGTGGGCTCCAGTCTGAGGATGAGGCTGATTATTACTGTGCAACATGGGATA

ACAGGCTGAATGCCGAATGGGTGTTCGGCGGAGGGACCAAGCTGACCGTCCTACGTGCGGCCGCACATC

ATCATCACCATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGAAGAAGACCTGAACGGTACCG

CATAG

C.1.10 IIYD4v_pMOD1

> IIYD4v_pMOD1

ATGGCCCAGGTGCAGCTGGTGCAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCC

TGTGCAGCCTCTGGATTCACCTTCAGTAGCTATGCTATGCACTGGGTCCGCTAGGCTCCAGGCAAGGGG

CTGGAGTGGGTGGCAGTTATATCATATGATGGAAGCAATAAATACTACGCAGACTCCGTGAAGGGCCGA

TTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAGATGAACAGCCTGAGAGCTGAGGAC

ACGGCTGTGTATTACTGTGCGAGGTTAGATATTACTATGATACCAGAGGGTCCAGATGCTTTTGATATC

TGGGGCCAAGGGACAATGGTCACCGTCTCTTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGT

GGTGGATCCTCCAATTTTATGCTGACTCAGCCCCACTCTGTGTCGGAGTCTCCGGGGAAGACGGTAACC

ATCTCCTGCACCCGCAGCAGTGGCAGCATTGCCAGCAACTATGTGCAGTGGTACCAGCAGCGCCCGGGC

AGTGCCCCCACCACTGTGATCTATGAGGATAACCAAAGACCCTCTGGGGTCCCTGATCGGTTCTCTGGC

TCCATCGACAGCTCCTCCAACTCTGCCTCCCTCACCATCTCTGGACTGAAGACTGAGGACGAGGCTGAC

TACTACTGTCAGTCTTATGATAGCAGCAATGTGGTATTCGGCGGAGGGACCAAGGTCACCGTCCTAGGT

GCGGCCGCACATCATCATCACCATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGAAGAAGAC

CTGAACGGTACCGCATAG

C.1.11 IIYG4v_pMOD1

> IIYG4v_pMOD1

ATGGCCCAGGTACAGCTGCAGCAGTCAGGTCCAGGACTGGTGAAGCCCTCGCAGACCCTCTCACTCACC

TGTGCCATCTCCGGGGACAGTGTCTCTAGCAACAGTGCTGCTTGGAACTGGATCAGGCAGTCCCCATCG

AGAGGCCTTGAGTGGCTGGGAAGGACATACTACAGGTCCAAGTGGTATAATGATTATGCAGTATCTGTG

AAAAGTCGAATAACCATCAACCCAGACACATCCAAGAACCAGTTCTCCCTGCAGCTGAACTCTGTGACT

CCCGAGGACACGGCTGTGTATTACTGTGCAAGAGAAAGGATGGGGGGGTTCGACCCCTGGGGCCAGGGA

ACCCTGGTCACCGTCTCCTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTAGTGGTGGTGGATCCAAT

TTTATGCTGACTCAGCCCCACTCTGTGTCGGAGTCTCCGGGGAAGACGGTTACCATTTCCTGCACCCGC

ACCAGTGGCAGCATAGCCAGCAACTATGTGCAGTGGTACCAGCAGCGCCCGGGCAGTTCCCCCACCACT

GTGATCTTTGAGGATACCGCAAGACCCTCTGGGGTCCCTGCTCGCTTCTCTGGCTCCATCGACCCCTTC

TCCAACTCTGCCTCCCTCACCATCTCTGGACTGAAGGCTGAGGACGAGGCTGACTACTACTGTCACTCT

TATGATGTCCACAATCAAGTGTTCGGCGGAGGGACCAAGCTGACCGTCCTAGGTGCGGCCGCACATCAT

CATCACCATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGAAGAAGACCTGAACGGTACCGCA

TAG

C.1.12 IIYE5v_pMOD1

> IIYE5v_pMOD1

ATGGCCCAGGTACAGCTACAGCAGTCAGGTCCAGGACTGGTGAAGCCCCCGCAGACCCTCTCACTCACC

TGTGCCATCTCCGGGGACAGTGTCTCTAGCAACACTGCTGCTTGGAACTGGATCAGGCAGTCCCCATCG

AGAGGCCTTGAGTGGCTGGGAAGGACATACTACAGGTCCAAGTGGCATAATGATTATGCAGTATCTGTG

AACAGTCGAATAAGCATCAACCCAGACACATCCAAGAACCAGTTCTCCCTGCAGCTGGACTCTGTGACT

CCCGAGGACACGGCTGTGTATTACTGTGCAAGAGATCGTTACTATGGTTCAGGGAGCTATTATAGGGGG

TTTGACTACTGGGGCCAGGGGACAATGGTCACCGTCTCTTCAGGTGGTGGTGGTAGCGGCGGCGGCGGC

TCTGGTGGTGGTGGATCCAATTTTATGCTGACTCAGCCCCACTCTGTGTCGGGGTCTCCGGGCAAGACG

GTGACCCTCTCCTGCACCCGCAACAGTGGCAGCATTGCCAGCGCCTATGTGCAGTGGTTCCGGCAGCGC

CCGGGCTCTGCCCCCACTACAGTGATCTATGAAGATAATCAAAGACCTTCTGGGGTCCCTGCTCGGTTC

TCTGGCTCCATCGACAGCTCCTCCAACTCTGCCTCCCTCACCATCTCTGGACTGCAGACTGAGGACGAG

GCTGACTACTACTGTCAGTCTTATGATTTCACCAACTATGTCTTCGGAACTGGGACCCAGCTCACCGTT

TTAGGTGCGGCCGCACATCATCATCACCATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGAA

GAAGACCTGAACGGTACCGCATAG

C.1.13 IIYG8v_pMOD1

> IIYG8v_pMOD1

ATGGCCGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGCAGGTCCCTGAGACTCTCC

TGTGCAGCCTCTGGATTCACCTTTGATGATTATGCCATGCACTGGGTCCGGCAAGCTCCAGGGAAGGGC

CTGGAGTGGGTCTCAGGTATTAGTTGGAATAGTGGTAGCATAGGCTATGCGGACTCTGTGAAGGGCCGA

TTCACCATCTCCAGAGACAACGCCAAGAGCTCCCTGTATCTGCAAATGAACAGTCTGAGAGCTGAGGAC

ACGGCCTTGTATTACTGTGCAAAAGGGGGCCACCGGGGTGCTTTTGATATCTGGGGCCAAGGGACAATG

GTTACCGTCTCTTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCTCCTATGTG

CTGACTCAGCCACCCTCAGCGTCTGGGACCCCCGGGCAGAGGGTCACCATCTCTTGTTCTGGAGGCAGC

TCCAACATCGGAGGTAACACTGTAAATTGGTACCAGGTCCCCCCAAGAACGGCCCCCAAACTCCTCATC

TATAATAATAATCAGCGGCCCTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCGGGCACCTCAGCC

TCCCTGGCCATCAGTGGGCTCCAGTCCGAGGATGAGGCTGATTATTACTGTGCAACATGGGATGACAGC

CTGCATGGTGTGGTATTCGGCGGAGGGACCAAGGTCACCGTCCTACGTGCGGCCGCACATCATCATCAC

CATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGAAGAAGACCTGAACGGTACCGCATAG

C.1.14 R1_pMOD1 (R2_pMOD1)

> R1_pMOD1 (R2_pMOD1)

ATGGCCCAGGTCAACTTAAGGGAGTCTGGGACCACGGTCACCGTCTCCTCAGGTGGTGGTGGTAGCGGC

GGCGGCGGCTCTGGTGGTGGTGGATCCGACATCGTGATGACCCAGTCTCCCAGTGGGCCAAGACTGGAG

ATTAAACGTGCGGCCGCACATCATCATCACCATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCT

GAAGAAGACCTGAACGGTACCGCATAG

C.1.15 R4_pMOD1

> R4_pMOD1

CATGGCCCAGGTGCAGCTGCAGGAGTCGGGGACAATGGTCACCGTCTCTTCAGGTGGTGGTGGTAGCGG
CGGCGGCGGCTCTGGTGGTGGTGGATCCGACATCGTGATGACCCAGTCTCCAGGGACCAAGCTGGAGAT
CAAACGTGCGGCCGCACATCATCATCACCATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGA
AGAAGACCTGAACGGTACCGCATAG

C.1.16 R6_pMOD1 (R8_pMOD1)

> Y6_pMOD1 (Y8_pMOD1)

ATGGCCCAGGTCAACTTAAGGGAGTCTGGGAACCCTGGTCACCGTCTCCTCAGGTGGTGGTGGTAGCGC
GGCGGCGGCTCTGGTGGTGGTGGATCCGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCT
GTAGGAGACAGAGTCACCGTCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAG
CAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAGATTTT
GCAACTTACTACTGTCAACAGAGTTACAGTACCCCTACCTTCGGCCAAGGGACACGACTGGAGATTAAA
CGTGCGGCCGCACATCATCATCACCATCACGGGGCCGCAGGGCCCGAACAGAAACTGATCTCTGAAGAA
GACCTGAACGGTACCGCATAG

C.2 Amino acid sequence

C.2.1 IRA7c_pMOD1 (IVB4cv_pMOD1)

>IRA7c_pMOD1 (IVB4cv_pMOD1)

M A Q V Q L V Q S G G G L V Q P G G S L R L S C A A S G F S F S D Y G
M H W V R Q I P G K G L E W V A V I Y A R G I N T Y Y G D S V K G R F
T I S R D N S K N I L Y L E M N R L S S E D T A V Y Y C A T D D P P S
G T G S Y H V N W G Q G T L V T V S S G G G G S G G G G S G G G G S Q
S A L T Q P A S V S G S P G Q S I T I S C T G T S S D V G S Y N L V S
W Y Q Q H P G K A P K L M I Y E V S K R P S G V P D R F S G S K S G N
T A S L T V S G L Q A E D E A D Y Y C S S Y T S I T A A A V F G T G T
K V T V L G A A A H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.2 IYF5c_pMOD1

>IYF5c_pMOD1

M A Q V Q L V Q S G A E V K K P G A S V K V S C K A S G Y T F T S Y G
I S W V R Q A P G Q G L E W M G W I S A Y N G N T N Y A Q K L Q G R V
T M T T D T S T S T A Y M E L R S L R S D D T A V Y Y C A D G G N F D
Y W G Q G T L V T V S S G G G G S G G G G S G G G G S Q A V L T Q P S
S A S G T P G Q R V T I S C S G S S S N I G S N Y V Y W Y Q Q L P G T
A P K L L I Y R N N Q R P S G V P D R F S G S K S G T S A S L A I S G
L R S E D E A D Y Y C A A W D D S L S G P V F G G G T K L T V L G A A
A H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.3 IIIC2c_pMOD1

>IIIRC2c

M A Q V Q L Q E S G P G L V K P F G D P G P H C T V S G G S L S S V N
S Y W D F I R Q P P G K G L E W I G S I Y Y R G T T Y Y N P S L K S R
V T L S V D T S Q N Q I S L K L T S L T A A D T A V Y Y C A R E S V T
R G T F D M W G Q G T M V T V S S G G G G S G G G G S G G G G S Q S A
L T Q P A S V S G S P G Q S I T I S C T G T S S D V G G Y N Y V S W Y
Q Q H P G K A P N L M I Y D V S N R P S G V S N R F S G S K S G N T A
S L T I S R L Q A E D E G I Y F C S A Y T S S S S L G V F G T G T K L
T V L G A A A H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.4 IYC11c_pMOD1

>IYC11c

M Q L V E S G A E V K K P G A S V K V S C K A S G Y S F T A Y Y I H W
V R Q A P G Q G L E W M G W I N P N S G T T T Y A Q R F Q G R V T M T
R D T S I S T A Y M E L S R L R S D D T A V Y Y C A R D F G G Y W R W
G A F D I W G Q G T T V T V S S G G G G S G G G G S G G G S G S S Y V L
T Q P P S V S V A P G K T A R I T C G G N N I G S K S V H W Y Q Q K P
G Q A P V L V V Y D D S D R P S G I P E R F S G S N S G N T A T L T I

S R V E A G D E A D Y Y C Q V W D S S S D H Y V F G T G T Q L T V L R

A A A H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.5 IYC12c_pMOD1

>IYC12c

M A Q V N L R E S G G G L V Q P G G S L R L S C A A S G F T F S S Y A

M S W V R Q A P G K G L E W V S S I T Y S G T A T S Y A D S V K G R F

T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K G Y S T F

D Y W G Q G T L V T V S S G G G G S G G G G S G G S G S Q A V L T Q P

S S T S G T P G Q R V T I S C S G G S S N I G S N T V N W Y R H L P G

T A P K L L I Y I D D R R P S D I P D R F S G S R S G T S A S L A I S

G L Q S E D E A D Y Y C A A W D D S L N G L V F G G G T Q L T V L G A

A A H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.6 IYC3c_pMOD1

>IRC3c

M A Q V N L R E S G T T V T V S S G G G G S G G G G S G G G G S Q S A

L T Q P A S V S G S P G Q S I T I S C T G T S S D V G G Y N Y V A W Y

Q H H P G K A P K L M I Y D V S N R P S G V S N R F S G S K S G N T A

S L T I S G L Q A E D E A D Y Y C S S Y T S S S T L V V F G G G T K V

T V L G A A A H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.7 IYD1c_pMOD1

>IYD1c

M A Q V N L R E S G G G L V Q P G G S L R L S C A A S G F T F S S Y A

M S W V R Q A P G K G L E W V S A I T Y N G A S T A Y A D S V K G R F

T I S R D N S Q N T L Y L Q M N S L R A E D T A V Y Y C A K G Y S T F

D Y W G Q G T L V T V S S G G G G S G G G G S G G G G S S S K L T Q D

P A V S V A L G Q T V R I T C Q G D S L R S Y Y A S W Y Q Q K P G Q A

P V L V I Y G K N N R P S G I P D R F S G S K S G T S A S L D I S G L

Q S E D E A D Y Y C A A W D D S L N G V L F G G G T K V T A L R A A A

H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.8 IIRD5v_pMOD1

>IIRD5v

M A E V Q L V E S G T E V R K P G D S V K V S C K A S G Y T F T D Y Y

L H W V R Q A P G Q G L E W V G W I Y P K R G G T H S A Q K F Q G R V

T M T R D T S I N T A Y M E L T R L R S D D T A V Y F C A R D R D I E

D A F D I W G Q G T M V T V S S G G G G S G G G G S G G G G S Q S A L

T Q P A S V S G S P G Q S I T I S C T G T S S D V G G Y N Y V S W Y Q

Q H P A K A P N L L I Y D V S N R P S G V S N R F S G S K S G N T A S

L T I S G L Q A E D E A D Y F C S S Y T T S S T L V F G G G T K V T V

L G A A A H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.9 IIYB5v_pMOD1

>IIYB5v

M A Q V Q L V Q S G A E V K K P G S S V K V S C K A S G G T F S S Y A

I S W V R Q A P G Q G L E W M G G I I P I F G T A N Y A Q K F Q G R V

T I T A D E S T S T A Y M E L S S L R S E D T A V Y Y C A R D R E L P

G F D Y W G Q G T L V T V S S G G G G S G G G G S G G D G S S Y V L T

Q P P S A S G T P G Q R V T I S C S G S S S N I G S N T V N W Y Q Q L

P G T A P K L L I Y S N N Q R P S G V P D R F S G S K S G T S A S L A

I S G L Q S E D E A D Y Y C A T W D N R L N A E W V F G G G T K L T V

L R A A A H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.10 IIYD4v_pMOD1

>IIYD4v

M A Q V Q L V Q S G G G V V Q P G R S L R L S C A A S G F T F S S Y A

M H W V R Q A P G K G L E W V A V I S Y D G S N K Y Y A D S V K G R F

T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A R L D I T M

I P E G P D A F D I W G Q G T M V T V S S G G G G S G G G G S G G G G

S S N F M L T Q P H S V S E S P G K T V T I S C T R S S G S I A S N Y
V Q W Y Q Q R P G S A P T T V I Y E D N Q R P S G V P D R F S G S I D
S S S N S A S L T I S G L K T E D E A D Y Y C Q S Y D S S N V V F G G
G T K V T V L G A A A H H H H H H G A A G P E Q K L I S E E D L N G T
A *

C.2.11IIYG4v_pMOD1

>IIYG4v_pMOD1

M A Q V Q L Q Q S G P G L V K P S Q T L S L T C A I S G D S V S S N S
A A W N W I R Q S P S R G L E W L G R T Y Y R S K W Y N D Y A V S V K
S R I T I N P D T S K N Q F S L Q L N S V T P E D T A V Y Y C A R E R
M G G F D P W G Q G T L V T V S S G G G G S G G G G S S G G G S N F M
L T Q P H S V S E S P G K T V T I S C T R T S G S I A S N Y V Q W Y Q
Q R P G S S P T T V I F E D T A R P S G V P A R F S G S I D P F S N S
A S L T I S G L K A E D E A D Y Y C H S Y D V H N Q V F G G G T K L T
V L G A A A H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.12 IIYE5v_pMOD1

>IIYE5v_pMOD1

M A Q V Q L Q Q S G P G L V K P P Q T L S L T C A I S G D S V S S N T
A A W N W I R Q S P S R G L E W L G R T Y Y R S K W H N D Y A V S V N
S R I S I N P D T S K N Q F S L Q L D S V T P E D T A V Y Y C A R D R
Y Y G S G S Y Y R G F D Y W G Q G T M V T V S S G G G G S G G G G S G
G G G S N F M L T Q P H S V S G S P G K T V T L S C T R N S G S I A S
A Y V Q W F R Q R P G S A P T T V I Y E D N Q R P S G V P A R F S G S
I D S S S N S A S L T I S G L Q T E D E A D Y Y C Q S Y D F T N Y V F
G T G T Q L T V L G A A A H H H H H H G A A G P E Q K L I S E E D L N
G T A *

C.2.13 IIYG8v_pMOD1

>IIYG8v

M A E V Q L V E S G G G L V Q P G R S L R L S C A A S G F T F D D Y A

M H W V R Q A P G K G L E W V S G I S W N S G S I G Y A D S V K G R F

T I S R D N A K S S L Y L Q M N S L R A E D T A L Y Y C A K G G H R G

A F D I W G Q G T M V T V S S G G G G S G G G G S G G G G S S Y V L T

Q P P S A S G T P G Q R V T I S C S G G S S N I G G N T V N W Y Q V P

P R T A P K L L I Y N N N Q R P S G V P D R F S G S K S G T S A S L A

I S G L Q S E D E A D Y Y C A T W D D S L H G V V F G G G T K V T V L

R A A A H H H H H H G A A G P E Q K L I S E E D L N G T A *

C.2.14 R1_pMOD1 (R2_pMOD1)

> R1_pMOD1 (R2_pMOD1)

M A Q V N L R E S G T T V T V S S G G G G S G G G G S G G G G S D I V

M T Q S P S G P R L E I K R A A A H H H H H H G A A G P E Q K L I S E

E D L N G T A *

C.2.15 R4_pMOD1

> R4_pMOD1

Met A Q V Q L Q E S G T Met V T V S S G G G G S G G G G S G G G G S D

I V Met T Q S P G T K L E I K R A A A H H H H H H G A A G P E Q K L I

S E E D L N G T A *

C.2.16 R6_pMOD1 (R8_pMOD1)

> Y6_pMOD1 (Y8_pMOD1)

M A Q V N L R E S G N P G H R L L R W W W Q R G G G S G G G G G S D I Q

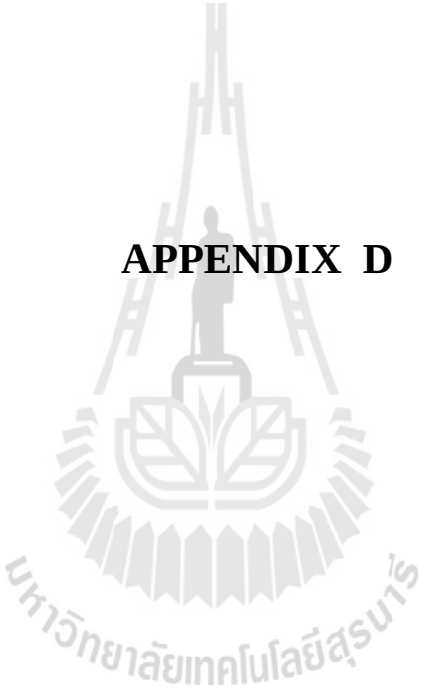
M T Q S P S S L S A S V G D R V T V T C R A S Q S I S S Y L N W Y Q Q

K P G K A P K L L I Y A A S S L Q S G V P S R F S G S G S G T D F T L

T I S S L Q P E D F A T Y Y C Q Q S Y S T P T F G Q G T R L E I K R A

A A H H H H H H G A A G P E Q K L I S E E D L N G T A *

APPENDIX D



APPENDIX D

LIST OF PATENTS APPLICATION

D.1 Thai Patent Application no. 1201005939

Yamabhai M and Pruksamethanan N. Twelve human scFv antibody specific to rabies virus. Thai Patent Application no. 1201005939

D.2 Thai Patent Application no. 1201005940

Yamabhai M and Pruksamethanan N. Light chain human antibody specific to rabies virus. Thai Patent Application no. 1201005940

D.3 Thai Patent Application no. 1201005941

Yamabhai M and Pruksamethanan N. Human scFv antibody specific to rabies virus strain FLURY_LEP 25. Thai Patent Application no. 1201005941

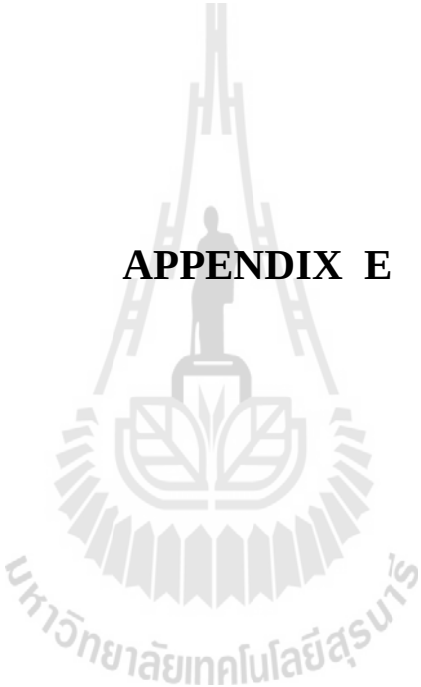
D.4 Thai Patent Application no. 1201005938

Yamabhai M Pruksamethanan N. Production of human scFv antibody fused with His-tag and Myc-tag for diagnostic of Rhabdoviridae Lyssavirus (rabies virus) by fluorescence in situ hubridization. Thai Patent Application no. 1201005938

D.5 Thai Patent Application no. 1301000990

Yamabhai M Pruksamethanan N and Yoohat K. Production of human scFv antibody conjugated with FITC for diagnostic of Rhabdoviridae lyssavirus (rabies virus) by fluorescence in situ hubridization. Thai Patent Application no. 1301000990

APPENDIX E



APPENDIX E

LIST OF AWARD

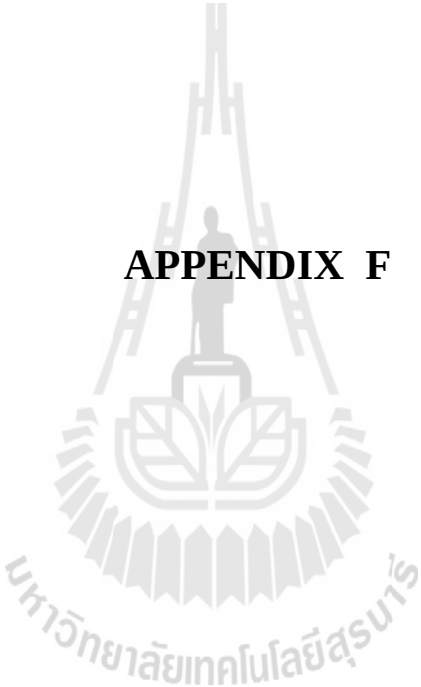
E.1 The recipient of Best Conference Paper Finalist Award

The 6th IEEE International Conference on Nano/Molecular Medicine and engineering, November 4-7, 2012, Imperial Queen's Park Hotel, Bangkok, Thailand (Oral presentation; in "Selection of Single Chain Human Monoclonal Antibody (scFv) Against Rabies Virus by Phage Display Technology"; recipient of Best Conference Paper Finalist Award).

E.2 The winner of the most popular vote for the oral presentation

She has been awarded as the winner of the most popular vote for the oral presentation at the SUT International Agricultural Colloquium in June 5-6, 2013 Suranaree University of Technology, Nakhorn Ratchasima, Thailand (Oral presentation; in "Production of Single Chain Human Monoclonal Antibody (scFv) Against Rabies Virus by Phage Display Technology").

APPENDIX F



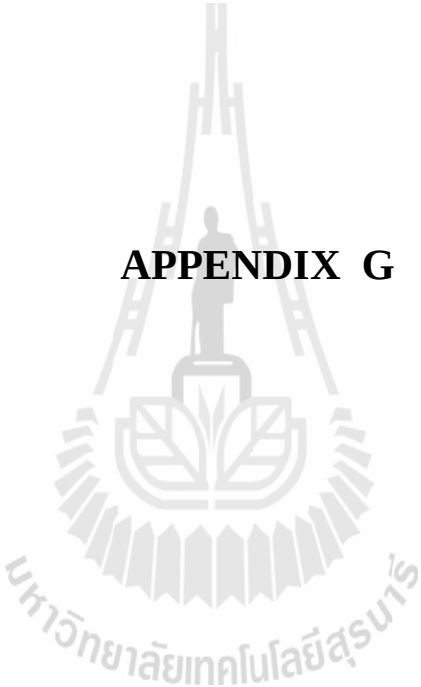
APPENDIX F

LIST OF PUBLICATION

F.1 Selection of Single Chain Human Monoclonal Antibody (scFv) Against Rabies virus by Phage Display Technology

Pruksametanan, N., Khawplod, P., Yamabhai, M., 2012. Selection of Single Chain Human Monoclonal Antibody (scFv) Against Rabies virus by Phage Display Technology. 6th IEEE Int. Conf. on Nano/Molecular Medicine and Engineering (IEEE-NANOMED 2012), Imperial Queen's Park Hotel, Bangkok, Thailand, November 4-7 Proceeding. (Recipient of Best Conference Paper Finalist Award).

APPENDIX G



APPENDIX G

RFFIT RAW DATA

G.1 *In vivo* neutralizing potency determined by RFFIT using phage scFv antibody (crude supernatant)

Sample	Dilution			TCID50	IU/ml
RAI	1/32	1/64	1/128	1/58.6882	1.0
Dilution;	8	3	0		
undilute	8	3	0		
1 IU	1/32	1/64	1/128	1/56.20005	0.96
Dilution;	8	3	0		
undilute	7	3	0		
10 IU	1/200	1/400	1/800	1/59.07305	10.065
Dilution;	8	6	2		
1:100	8	7	2		
IRA7c	1/4	1/8	1/16	1/7.025	0.119
Dilution;	8	3	1		(0.12)
undilute	7	2	0		

Sample	Dilution			TCID50	IU/ml
IYF5c	1/2	1/4	1/8		Negative
Dilution;	0	0			
undilute	0	0			
IIIRC2c	1/2	1/4	1/8	1/ 4.555	0.078
Dilution;	7	5	1		(0.08)
undilute	7	6	1		

G.2 *In vivo* neutralizing potency determined by RFFIT using phage scFv antibody (PEG precipitate)

Sample	Dilution			TCID50	IU/ml
RAI	1/32	1/64	1/128	1/76.10923	1.0
Dilution;	8	5	0		
undilute	8	6	1		
1 IU	1/32	1/64	1/128	1/79.4788	1.04
Dilution;	8	5	1		
undilute	8	6	1		
10 IU	1/400	1/800	1/1600	1/835.419	10.97
Dilution;	8	4	0		
1:100	8	5			

Sample	Dilution			TCID50	IU/ml
IRA7c	1/2	1/4	1/8	1/ 4.555	0.0598
Dilution;	8	4	1		(0.06)
undilute	8	4	2		
IYF5c	1/2	1/4	1/8		Negative
Dilution;	0	0			
undilute	0	0			
IIIRC2c	1/2	1/4	1/8	1/ 2.4837	0.0326
Dilution;	4	2	0		(0.03)
undilute	5	2	0		
IRC3c	1/2	1/4			Negative
Dilution;	0	0			
undilute	0	0			
IYC11c	1/2	1/4			Negative
Dilution;	0	0			
undilute	0	0			
IYC12c	1/2	1/4			Negative
Dilution;	0	0			
undilute	0	0			
IYD1c	1/2	1/4			Negative
Dilution;	0	0			
undilute	0	0			

Sample	Dilution			TCID50	IU/ml
IIRD5v	1/2	1/4			Negative
Dilution;	0	0			
undilute	0	0			
IIYB5v	1/2	1/4			Negative
Dilution;	0	0			
undilute	0	0			
IIYG4v	1/2	1/4			Negative
Dilution;	0	0			
undilute	0	0			
IIYE5v	1/2	1/4			Negative
Dilution;	0	0			
undilute	0	0			
IIYG8v	1/2	1/4			Negative
Dilution;	0	0			
undilute	0	0			
IIYD4v	1/2	1/4			Negative
Dilution;	0	0			
undilute	0	0			

G.3 *In vivo* neutralizing potency determined by RFFIT using soluble scFv

Sample	Dilution			TCID50	IU/ml
RAI	1/32	1/64	1/128	1/66.83352	1.0
Dilution;	8	3	1		
undilute	8	4	1		
1 IU	1/32	1/64	1/128	1/64	0.96
Dilution;	8	4	1		
undilute	7	4	1		
10 IU	1/400	1/800	1/1600	1/672.717	10.07
Dilution;	7	2	0		
undilute	7	3	0		
IRA7c	1/32	1/64	1/128	1/152.218	2.27
(500 ug/ml)	7	5	2		
	7	5	2		
IRA7c	1/400	1/800	1/1600	1/1345.43	20.13
(4900 ug/ml)	7	6	4		
	6	5	4		
IYF5c	1/2	1/4			Negative
(284 ug/ml)	0	0			
	0	0			

Sample	Dilution			TCID50	IU/ml
IIIC2c (500 ug/ml)	1/2	1/4	1/8	1/ 4.7568	0.07 (0.1)
	8	4	1		
	8	5	2		
IIYG4v (125.21 ug/ml)	1/2	1/4			Negative
	0	0			
	0	0			
3C1 (368 ug/ml)	1/2	1/4			Negative
	0	0			
	0	0			

BIOGRAPHY

Miss Natcha Pruksametanan was born on February 1st, 1985 in Khon kaen, Thailand. She graduated with the Bachelor degree in Biotechnology, Khon Kaen University, Thailand in 2007. During her Master degree enrollment in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology (2009-2013), she presented research the 6th IEEE International Conference on Nano/Molecular Medicine and engineering, November 4-7, 2012, Imperial Queen's Park Hotel, Bangkok, Thailand (Oral presentation; in "Selection of Single Chain Human Monoclonal Antibody (scFv) Against Rabies Virus by Phage Display Technology"; recipient of Best Conference Paper Finalist Award). The 2nd Thailand national Research University Summit, May 7-8, 2013 (Poster presentation; in "Human scFv Antibody Against Rabies Virus". She has been awarded as the winner of the most popular vote for the oral presentation at the SUT International Agricultural Colloquium in June 5-6, 2013 Suranaree University of Technology, Nakhorn Ratchasima, Thailand (Oral presentation; in "Production of Single Chain Human Monoclonal Antibody (scFv) Against Rabies Virus by Phage Display Technology"). While she was studies, she has an experience to improve her knowledge about Nuclear magnetic resonance (NMR) at University of Tsukuba, Tsukuba, Japan for 45 days. She conducted to research in the topic of Production of human monoclonal antibodies against Rabies virus using phage display technology.