



Institute of Health and Community Medicine

**Interaction of Phage Display Peptides to Chikungunya and Zika Virus
Recombinant Proteins**

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Interaction of Phage Display Peptides to Chikungunya and Zika Virus Recombinant Proteins

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulation of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any degree.



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ABSTRACT

Diagnosis by molecular methods may not be sensitive after viremic period while diagnosis by serological assay cross reactivity of antibodies might happen. Besides, serological assay usually requires specific monoclonal antibody which involves the use of animal thus raises animal welfare issues. Therefore, to overcome the traditional method in generating hybridoma for monoclonal antibody production, this study proposes to interrogate the phage display technology as an alternative to hybridoma screening technology for monoclonal antibody production. In this study, a Ph.D-7 Phage Display Peptide Library was used to screen high affinity ligands that could selectively bind to the purified recombinant E2 protein of CHIKV and the EDIII protein of the ZIKV. The viral antigens were immobilized to immunotubes and the phage-display library was added to the tubes to allow binding of peptide to the target molecules. The bound phage was amplified in a biopanning process. Following three rounds of biopanning, the amounts of bound phages were determined by titration. The phage clones that were positive against the viral protein in a plaque lift assay were selected and amplified. A total of 12 and 26 phage clones were amplified for potential binding with the recombinant CHIKV and ZIKV proteins respectively. The sequence analysis of the positive clones showed that, the selection with CHIKV recombinant protein generate a potential motif, NAALGT, in which 6 out of the 12 amplified clones contained the repetitive motif. However, the selection with ZIKV protein, did not show any repetitive motif sequence, the interacting peptide was further analyzed using CLUSTAL MSA software by MUSCLE suggests that these to be target-unrelated peptides.

Keywords: Chikungunya virus, Zika virus, phage display, biopanning, target unrelated peptides

Peptida yang Berinteraksi dengan Protein Rekombinan Virus Chikungunya dan Virus Zika

ABSTRAK

Diagnosis dengan kaedah molekular mungkin tidak sensitif setelah tempoh viremik sementara diagnosis dengan ujian serologi, kereaktifan silang antibodi boleh berlaku. Selain itu, ujian serologi biasanya memerlukan antibodi monoklonal tertentu yang melibatkan penggunaan haiwan yang boleh menimbulkan masalah kebajikan haiwan. Oleh itu, untuk mengatasi kaedah tradisional dalam menghasilkan hybridoma untuk penghasilan antibodi monoklonal, kajian ini mencadangkan untuk menggunakan teknologi paparan faj sebagai alternatif kepada teknologi saringan hybridoma untuk penghasilan antibodi monoklonal. Dalam kajian ini, Ph.D-7 Phage Display Peptide Library digunakan untuk menyaring ligan pertalian tinggi yang dapat mengikat protein E2 rekombinasi CHIKV dan protein EDIII ZIKV. Antigen virus telah dilancarkan kepada immunotub dan perpustakaan paparan faj telah ditambahkan ke tiub untuk membolehkan pengikat peptida ke molekul sasaran. Faj terikat diperkuatkan dalam proses biopanning. Setelah tiga pusingan biopanning, jumlah faj yang terikat ditentukan menggunakan titrasi. Klon faj yang positif terhadap protein virus dalam ujian peningkatan plak dipilih dan diperkuatkan. Sebanyak 12 dan 26 klon faj diperkuat untuk potensi pengikatan dengan protein CHIKV dan ZIKV. Analisis urutan klon positif menunjukkan bahawa, pemilihan dengan protein rekombinasi CHIKV menghasilkan motif yang berpotensi, AALGT di mana 8 daripada 12 klon yang diperkuat mengandungi motif berulang. Namun untuk pemilihan protein ZIKV tidak menunjukkan urutan motif berulang, peptida yang disintesis telah dikaji dengan menggunakan software CLUSTAL MSA oleh MUSCLE. Kesemua peptida yang dijumpai adalah merupakan peptida yang tidak berkaitan. Kajian lebih lanjut dapat dilakukan untuk

menganalisis lebih lanjut ciri peptide spesifik ini dalam menghambat penularan virus dan memunculkan perlindungan imun.

Kata kunci: *Virus Chikungunya, Virus Zika, paparan faj, biopanning, peptida tidak berkait*

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LIST OF ABBREVIATIONS

Ae.	Aedes
BSA	Bovine Serum Albumin
C	Celsius
CHIKV	Chikungunya virus
DENV	Dengue virus
DENV1	Dengue virus 1
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
E1	Envelope 1
E2	Envelope 2
ELISA	Enzyme linked immunosorbent assay
GBS	Guillain Barré syndrome
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	Isopropyl- β -D-thiogalactosidase
LB	Luria Bertani
LB+Tet	Luria Bertani + Tetracycline
μ l	Microliter
M	Molar
mAbs	Monoclonal antibodies

ml	Milliliter
mM	Mili molar
nm	Nanometer
PBS	Phosphate Buffered Saline
pfu	Plaque forming units
pfu/ml	Plaque forming units per milliliter
Ph.D.	Phage display
Ph.D.-7	Linear heptapeptide library
rAbs	Recombinant antibodies
rE2CHIKV	Recombinant E2 protein of CHIKV
rEDIII ZIKV	Recombinant E domain 3 of ZIKV
rpm	Revolution per time
RT-PCR	Reverse transcription polymerase chain reaction
SOC	Super Optimal broth with Catabolite repression
TBST	Tris-buffered saline with Tween-20
Tris-HCl	Trisaminomethane with hydrochloride
UHQ	Ultra High Quality
YFV	Yellow Fever virus
ZIKV	Zika virus

CHAPTER 1

INTRODUCTION

1.1 Study Background

Arthropod-borne virus or arbovirus infections are becoming an emerging medical issue mainly affecting endemic areas such as developing countries with upcoming economy such as China and India (Sautto et al., 2013). Also, CHIKV and ZIKV have attracted more attention due to the dramatic increase of incidence around 400% for CHIKV and 11-fold for ZIKV-associated microcephaly in northeast Brazil from 2014 to 2015 (Boletim Epidemiologico, 2015).

Chikungunya virus is transmitted by *Aedes (Ae.)* mosquitoes and is conserved in the human population by human-mosquito-human transmission (Cleton et al., 2012). Although CHIKV infection is rarely fatal and usually does not require close clinical supervision, some reported cases were found to be associated with meningoencephalitis and myelopathy (Kashyap et al., 2010; Chatterjee et al., 1965).

In 2016, ZIKV has become a main international health concern because the virus has been attributed as a cause of microcephaly, Guillain- Barré syndrome (GBS) and encephalitis (WHO, 2016). Aside from mosquito-dependent transmission, this virus can also be transmitted by the transplacental route, through blood transfusion and sexual intercourse (Lazear & Diamond, 2016), making the infection more alarming.

1.2 Problem Statement

At present, there are no specific treatment and commercial vaccine for CHIKV and ZIKV that are available for human use. Limited diagnostic capabilities in many regions where outbreaks occur is one of the major challenges in studying both viruses and to control outbreaks.

Additionally, the signs and symptoms of arboviruses such as CHIKV, ZIKV and DENV during the early stage of infection are similar. These include fever, headache, nausea, fatigue and joint pain. It is therefore quite challenging to clinically distinguish these infections without laboratory diagnostics, especially in regions where the viruses are co-circulating. Diagnosis by molecular methods may not be as sensitive after the viremic period. On the other hand, diagnosis by serological assays usually requires specific monoclonal antibodies, thus involves the use of animal and raises issues regarding ethics and animal welfare (Festing & Wilkinson, 2007). This study proposes the approach of phage display peptide technology for screening and isolating target peptides interacting to CHIKV and ZIKV recombinant proteins for potential detection and therapy of CHIKV and ZIKV.

1.3 Objectives

In this study, we are interested in investigating and identifying specific peptides that bind to the E2 protein of CHIKV and EDIII protein of ZIKV. We employed antibody phage-display technology to screen for the peptide-ligands that bind to the target antigen.

As CHIKV and ZIKV infections could lead to many complications, a rapid and reliable method in detecting and identifying both infections from other arboviruses is indeed crucial. Currently, the information about the exact peptides involved in the infection

mechanism of CHIKV and ZIKV are limited. The identification of these peptide-ligands has potential value for developing a more efficient method not only for the detection of CHIKV and ZIKV infections, but also in drug discovery and vaccine development.

Therefore, the objectives of this study are:

- i. To identify interacting peptides to CHIKV and ZIKV recombinant proteins
- ii. To analyze the interacting peptides of CHIKV and ZIKV

1.4 Summary

In this study, the focus is to identify the peptide ligands for potential detection and therapy of CHIKV and ZIKV. The viral antigens were coated to immunotubes and panned with phage-display library to allow binding of peptides to the targets. The bound peptides after 3 rounds of biopanning were automated-sequenced for identification and these interacting peptides can be further synthesized to be used in diagnosis of CHIKV and ZIKV infections. The identified bound peptides generated in this study will be useful in identifying bind target molecules that can be used in application such as B-cell and T-cell epitope mapping, selection of bioactives peptide bound to the disease specific antigens and development of peptide-mediated drug delivery system. Targeting peptides identified using phage display technology maybe also be useful for basic research and translational medicine.

CHAPTER 2

LITERATURE REVIEW

2.1 Arboviruses

2.1.1 Introduction to Arboviruses

Currently, more than 500 arboviruses or arthropod-borne viruses have been identified and out of that number, nearly 150 are associated with human infections (Lanciotti, 2016). In the past few decades, the total number of arboviral epidemics has significantly increased (Gubler & Vasilakis, 2016). In most cases, the emerging arboviral diseases were caused by viruses formerly judged to be under control or of little public health significance (Gubler & Vasilakis, 2016). The occurrence of some major breakouts is also caused by the introduction of viruses into new geographic areas such as West Nile Virus into the Americas, where native vertebrate and arthropod hosts were susceptible and able to sustain infections.

Arbovirus infections have gained much interest given the expanding globalization and tourism movement all over the world that could act as the starting point of epidemic events. The diagnosis and treatment of these infections are sometimes complicated when the same vectors transmit several different arboviruses simultaneously. Confusing combined epidemics have been reported, for example Yellow Fever Virus (YFV) with CHIKV, DENV with CHIKV, *Plasmodium falciparum* malaria with DENV1 and CHIKV on Madagascar's east coast (Cleton et al., 2012).

Infections that have recently risen in a population and are rapidly increasing in frequency or geographic range are described as emerging infectious diseases (Morse, 1995). Several factors are needed for such diseases, including the introduction of a pathogen and

its spread into the human population, along with its ability to be maintained in nature (Blitvich, 2016). Adaptation to emerge into a new environment is required by many pathogens. Two other factors that play important parts in the emergence of diseases are human behavior and ecology (May et al., 2001; Hahn et al., 2000; Schrag & Wiener, 1995). The geographical expansion of human populations, the growth of agriculture and the destruction of wild life habitats due to climate change or deforestation have facilitated the rise of several emerging viruses (Jones et al., 2008; Taylor et al., 2001).

The current global re-emergence of arboviruses such as CHIKV and ZIKV has been reported by the media as though it is a new phenomenon. This is not the case because Arboviruses have been re-emerging for centuries alongside other human microbial pathogens. The greatest difference today is that arbovirus emergence and dispersion are more rapid and geographically extensive. This is due to the rapid growth of global transportation systems, arthropod adaptation to increasing urbanization, failure to contain mosquito populations and land destruction (Saker et al., 2004).

The exposure frequency of humans to mosquitoes and the global mobility of humans have significantly increased as a consequence of the increases in population density through urbanization and the development of global transportation systems. Therefore, the patterns of virus-vectors-host interactions have changed during recent centuries. In parallel, agricultural capacity, animal husbandry and widespread deforestation have shot up in response to the demands of industrial development, urbanization and rising human population densities (Liang et al., 2015; Kilpatrick & Randolph, 2012; Gould & Higgs, 2009; Gubler, 2002). Humans have been increasingly violating natural habitats that were formerly a sanctuary for arthropods to interact and evolve with wild animals and plants. As a result, contemporary arthropods are regularly exposed to the modern human

environment, domestic animals and livestock, to which they quickly adapt. This adaptive process is defined as domestication (Powell & Tabachnick, 2013).

Chikungunya virus is an arbovirus that has notably widened its geographic range and spread into new territories. Despite the fact that it is an alphavirus, CHIKV shares epidemiological, ecological and biogeographical characteristics with the *Ae. aegypti*-associated flaviviruses YFV, DENV and ZIKV. It also shares the dependence on sylvan *Aedes* in the forest cycle and domestic *Aedes* in the human epidemic transmission cycle (Chen et al., 2016; Gould et al., 2009).

Apart from CHIKV, ZIKV is another arbovirus that is rapidly expanding its geographic distribution and has recently been introduced into areas not being previously reported. Isolations of the virus have been made from *Ae. africanus*, *Ae. luteocephalus* and *Ae. furcifer* (all tree-holes breeding mosquitoes implicated in the sylvan cycle of YFV) in Uganda, Senegal, Nigeria, Burkina Faso, Ivory Coast and the Central African Republic (Haddow et al., 2012). These investigations were interpreted as proof that ZIKV is maintained in forested areas of tropical Africa in a cycle similar to that of sylvan yellow fever between arboreal mosquitoes and non-human primates.

Outbreaks of YFV, DENV, CHIKV and ZIKV have been reported in recent years mostly in tropical and subtropical regions of the planet. In 2017, the major ones include CHIKV in France (WHO, 2017a) and ZIKV in India (WHO, 2017b). A more recent CHIKV outbreak occurred in Hat Yai, Thailand in January 2019 (New Straits Times, 2019).

2.1.2 Transmission Cycle of Arboviruses

In 1903, despite the long battle that was started more than a century ago by Oswaldo Cruz, Brazil still struggles to fight the mosquito vectors of DENV, CHIKV and ZIKV. There are over 135 arboviruses that have been recognized to cause human diseases. Arboviral infections can range from asymptomatic to fulminant fatal disease. The clinical symptoms are normally categorized as systemic febrile illness, hemorrhagic fever and invasive neurological disease (Gubler & Vasilakis, 2016).

Arboviral diseases are triggered by viruses that are maintained in transmission cycle between vertebrate hosts and blood-sucking arthropods such as mosquitoes, sandflies, midges and ticks. The most common arthropods vectors are *Aedes* mosquitoes including *Ae. aegypti* and *Ae. albopictus*. The virus must produce an adequately high level of viremia in the vertebrate host for a susceptible arthropod to become infected while taking a blood meal in order to complete the transmission cycle (Karabatsos, 2001). Arthropod viruses are sustained by vertebrate animal reservoirs and humans are usually the dead-end hosts because they do not develop the high viremia required to infect arthropods (Cleton et al., 2012).

Aedes mosquitoes reproduce in tree holes or other forms of vegetation and in man-made environments including houses, flower pots, disposed car tires, bottles, cans and so forth. Depending on the local habitat, they feed primarily on available non-human mammals (Powell & Tabachnick, 2013; Mattingly, 1967; Mattingly, 1957). *Aedes aegypti* evolved from the sylvan African *Ae. formosus* into an anthropophilic species that breeds in domestic urban and peridomestic environments and feeds primarily on humans (Harrington et al., 2001). These urban and peridomestic mosquitoes are acknowledged as the domestic

version of *Ae. aegypti*. These are some of the epidemiological history of emergent arboviruses that have been structured during recent centuries.

Most arboviruses are zoonotic, infecting a vast variety of arthropods and other animals including birds in their sylvatic habitats, with humans as incidental hosts. Arboviruses have evolved a balanced relationship with these sylvatic hosts over a long period of time. Hence, morbidity and mortality is rarely observed in these sylvatic animals when they are infected by arboviruses. For example, YFV rarely kills non-human primates in African forests (Gould & Solomon, 2008; Strode, 1951). In contrast, these balanced relationships have not been established in humans and consequently they show significant morbidity and mortality following infection by sylvan arboviruses because human infections by sylvan arboviruses are uncommon (Jentes et al., 2011). However, with the exception of epidemic arboviruses such as DENV, ZIKV and CHIKV, human infections are generally not essential to maintain the arbovirus life cycle as they are usually dead-end hosts for these viruses (Kenney & Brault, 2014).

Although more than 150 arboviruses are known to infect humans, only a certain group is globally dispersed. Most of them are zoonotic and belong to the families of *Flaviviridae*, *Togaviridae*, *Reoviridae* and *Orthomyxoviridae* (Cleton et al., 2012).

Infections of arthropod-borne virus are gradually becoming an emerging medical problem, striking endemic areas that are developing nations or upcoming economies like China and India. The source of major arbovirus outbreaks in endemic areas is mostly related to the appearance of the viruses in an animal reservoir and a following development in humans. Besides the presence of the viruses, the outbreaks also occur during seasons with increased disease activity due to climate variations. Additionally, with the global

grow of travelers and movement of big populations, explorers and researchers travelling to exotic and medically high-risk sites has enlarged this problem (Cleton et al., 2012).

2.2 Chikungunya Virus

2.2.1 Introduction of CHIKV

The name Chikungunya comes from the Makonde people of Tanzania who describe the stooped posture of CHIKV-infected persons suffering from severe joint pain that characterizes infection and loosely translates to “that which bends up” (Kucharz & Cebula-Byrska, 2012). This virus belongs to the *Togaviridae* family and the genus *Alphavirus* (Yadav et al., 1963), which is composed of viruses with an icosahedral nucleocapsid surrounded by a lipid envelope and glycoprotein spikes (Solignat et al., 2009). The virus was first identified in 1952 and was first isolated from an individual that showed the symptoms of fever in Tanzania (Peter & Dalrymple, 1990; Strauss & Strauss, 1986).

This virus is transmitted to humans by *Ae. aegypti* and *Ae. albopictus* mosquitoes of the genus *Aedes* (Salvador et al., 2009). The infection is characterized by a sudden onset of high fever, myalgia, vomiting, headache and multiple joint pains or polyarthralgia (Pialoux et al., 2007; Bodenmann & Genton, 2006; Peters & Dalrymple, 1990). Polyarthralgia may last for months or even years (Schwartz & Albert, 2010). CHIKV-induced arthritis is most frequently symmetrical, co-occurring with swelling and involves multiple joints (Sissoko et al., 2009; Simon et al., 2007). Other than that, CHIKV infection is also related with the inflammation of and damage to musculoskeletal tissues in humans (Suhrbier & La Linn, 2004).

Despite the fact that it is generally not fatal, CHIKV has sickened 1.6 million individuals in the Indian Ocean region since 2005 (Charrel et al., 2007). During these

outbreaks, a single mutation of E1 in the virus has enhanced its ability to replicate in *Ae. albopictus* mosquitoes, which greatly facilitated the transmission of the disease (Tsetsarkin et al., 2007). CHIKV infections although rarely but have been associated with complications such as encephalopathy and hepatic failure (Robin et al., 2010). Periodical deaths have also been reported over the last decade (Sam et al., 2010). With the introduction of CHIKV into the Americas and the ongoing spread of the virus in Southeast Asia and the South Pacific, interest has been renewed in further understanding the virus and its pathogenesis.

Current laboratory diagnosis of CHIKV infection is based on the isolation of the virus, serological and molecular methods. Serological tests are more desirable due to time and cost effectiveness. Generally most serological methods detect anti-CHIKV IgM and IgG (Pialoux et al., 2007). The frequently used serological tests for CHIKV antibodies detections include Haemagglutination Inhibition (HI) assay, Enzyme Linked Immunosorbent Assay (ELISA) and indirect immunofluorescent assay (IIFA). As for molecular methods, reverse transcription polymerase chain reaction (RT-PCR) is used most widely (Naze et al., 2009; Edwards et al., 2007; Santhosh et al., 2007; Pastorino et al., 2005).

Although readily available, the implementation of these assays is currently limited especially in many endemic regions due the high cost of commercial test kits. In many endemic regions, the development of an in-house assay is not accessible due to the limitation in the production capability of antigens and specific antibodies.

2.2.2 Epidemiology

Chikungunya virus was historically restricted to the Old World (Jupp & McIntosh, 1988). It is believed that the virus was maintained in an enzootic transmission cycle between non-human primates (NHP) and arboreal *Aedes* mosquitoes. This has led to indications that the virus was begun in sub-Saharan Africa (Volk et al., 2010; Powers et al., 2000). A continuous transmission cycle between humans and anthropophilic mosquitoes such as *Ae. aegypti* is a result of the spillover transmission to nearby human populations that has probably occurred multiple times (Diallo et al., 2012; Volk et al., 2010; Diallo et al., 1999). Travelers who became infected in endemic or epidemic areas returned home and were contributing to the establishment of autochthonous transmission, which presumably is the main factor for the emergence of CHIKV (Lanciotti et al., 2007; Taubitz et al., 2007; Hochedez et al., 2006).

Chikungunya virus is an arthropod-borne virus with major outbreaks in Africa, India and South-East Asia (Power & Logue, 2007). The first outbreak in Asia was reported in Bangkok, Thailand in the mid-1970 (Burke et al., 1985). Historically, CHIKV only caused outbreaks in confined regions within Africa and Asia. However, from the end of 2004 onwards, Chikungunya has been identified not only in Asia and Africa but also across Europe and the Americas, causing huge outbreaks in naïve human populations (Halstead, 2015).

Four genotypes of CHIKV have been recognized since its discovery in 1952, namely East-Central-South African (ECSA), West African, Asian and the Indian Ocean Lineage (IOL) (Volk et al., 2010; Powers et al., 2000). The different CHIKV lineages show distinct patterns of infectivity and transmissibility in the mosquito vectors (Arias-Goeta et al., 2013; Vega-Rua et al., 2013). The acquisition of specific mutations in the E1

(Tsetsarkin et al., 2007; Vazeille et al., 2007) and E2 (Tsetsarkin et al., 2014; Tsetsarkin & Weaver, 2011) envelope glycoprotein of emerging IOL strains allowed viral adaptation and consequent increased its transmission in the peridomestic mosquito *Ae. albopictus*. This adaptation may have caused the spread and continual transmission of CHIKV in tropical urban areas where *Ae. aegypti* is abundant, and to peridomestic and temperate habitats where *Ae. albopictus* is more adapted (Leisnham et al., 2014).

Since 2004, millions of infections in coastal Africa, islands within the Indian Ocean, India and countries in Southeast Asia are caused by CHIKV re-emerging in Africa and spreading throughout the Indian Ocean region (Sergon et al., 2008; Schuffenecker et al., 2006; Simon et al., 2006; Laras et al., 2005; Sergon et al., 2005). On the other hand, infected tourists returning to northern Italy, New Caledonia, China and the French Riviera commenced autochthonous outbreaks resulting in infection of local mosquito populations (Angelini et al., 2008; Angelini et al., 2007; Beltrame et al., 2007; Pialoux et al., 2007; Rezza et al., 2007). This shows the important role played by infected travelers in introducing CHIKV into new areas, as further indicated by the introduction and subsequent epidemic of CHIKV into the Caribbean and the Americas in 2013 (Leparc-Goffart et al., 2014; Van Bortel et al., 2014; Cassadou et al., 2013).

About 300 000 cases of CHIKV infection were reported out of 785 000 inhabitants in La Reunion island in 2005-2006, with a fatal prognosis for 237 patients (Schwartz & Albert, 2010). Chikungunya is considered a re-emerging virus with the large outbreaks in the islands in the Indian Ocean from the end of 2004 to 2007, which also played a major role in the transmission of the virus to the Western Hemisphere (Weaver et al., 2015).

An outbreak was recorded in 2006 in different regions of Maharashtra, India. Between February and March 2006, over 2000 cases of CHIKV fever have been reported from Malegaon town in Nasik district, India (Kumar et al., 2008).

As of October 2014, approximately 760,000 suspected and 14,000 confirmed cases of CHIKV in 36 countries or territories in the Caribbean, Central America, South America and North America were recorded by the U.S. Center for Disease Control and Prevention or the CDC (Centers for Disease Control and Prevention, 2014). In the same period of time, numerous cases of CHIKV have been brought back to the U.S. from the Caribbean, resulting in 11 instances of localized viral transmission in Florida (Centers for Disease Control and Prevention, 2014).

2.2.3 Clinical Presentation

Chikungunya virus was first isolated in Tanzania (formerly Tanganyika) in 1952-1953 (Ross, 1956; Robinson, 1955). The virus was isolated from an individual that showed the symptom of fever (Peter & Dalrymple, 1990; Strauss & Strauss, 1986). Patients suffer from this disease shows similar symptoms to dengue fever such as high fever, headache, chills, and myalgia. The disease presents itself with an acute febrile phase that lasts for 2-5 days, followed by a prolonged arthralgic disease that affects the joints (Saxena, 2007). The pain in the joints related with CHIKV infection continues for weeks or months and in some cases even for years. This dengue-like symptom affects 40% of infected subjects. However, prognosis is seldom fatal (Solignat et al., 2009).

An analytical study of the epidemic from 2004 to 2007 suggests that the re-emergence of CHIKV is also a cause for concern due to increased morbidity and mortality

related with infection (Economopoulou et al., 2009; Josseran et al., 2006). The greater the numbers of CHIKV infected persons, the more critical the forms of the disease, including neurological complications and fulminant hepatitis, while maternal-fetal transmission associated with neonatal encephalopathy was also reported (Chua et al., 2010; Economopoulou et al., 2009; Rampal et al., 2007; Rampal et al., 2007; Wielanek et al., 2007).

However, some cases of CHIKV meningoencephalitis and myelopathy were reported recently from the Reunion Islands (Rajapakse et al., 2010; Zhisheng et al., 2009). The first report of neurological complication with CHIKV infection was recorded in the 1960s. There were some cases that indicate the infection was associated with meningoencephalitis, myelitis, and choroiditis (Kashyap et al., 2010; Chatterjee et al., 1965).

Currently, there is no commercial vaccine and anti-viral treatment for CHIKV. However, some candidate vaccines have been tested in humans. The US Army carried out a Phase II clinical trial with a live-attenuated CHIKV vaccine (TSI-GSD-218) derived from a 1962 strain (15561) in an outbreak in Thailand in 2000. Out of 58, all patients developed neutralizing antibodies (Edelman et al., 2000; McClain et al., 1998).

Despite numerous advanced diagnostic methods, early and cost-effective diagnosis of these patients remains unavailable. A reliable diagnostic test that could be carried out in any standard pathology laboratory is essential to obtain definitive early diagnosis for CHIKV patients (Kashyap et al., 2010).

2.3 Zika Virus (ZIKV)

2.3.1 Introduction of ZIKV

Zika virus (ZIKV) is an emerging arthropod-borne virus or arbovirus with a single stranded RNA from the *Flavivirus* genus (Musso & Gubler, 2016). It is named after the Zika Forest in Uganda, the location where it was isolated from a sentinel monkey in 1947 (Dick, 1952). In 1948, the virus was isolated from an *Ae. africanus* mosquito (Dick et al., 1952).

This disease is described by a broad range of clinical symptoms including fever, rash, headache, retro-orbital pain, myalgia, arthritis or arthralgia, conjunctivitis and vomiting, which are clinical signs identical to dengue disease and many other viral diseases such as Chikungunya and Mayaro fevers and parasitic aetiologies such as scrub typhus and leptospirosis (Kutsuna et al., 2014; Foy et al., 2011; Duffy et al., 2009; Olson et al., 1981; Macnamara, 1954).

Zika virus is transmitted between humans through the bite of infected mosquitoes and the virus has been isolated from various *Aedes* mosquito species, particularly *Ae. aegypti*, which is widespread in the tropics and subtropics, and *Ae. albopictus*, which is established in many areas of Europe especially the Mediterranean countries (Grard et al., 2014). *Ae. polynesiensis* is also suspected to play a part in ZIKV transmission in French Polynesia. However, *Ae. aegypti* is considered the primary vector of ZIKV and *Ae. albopictus* the secondary vector (WHO, 2016).

Besides mosquito-dependent transmission, ZIKV can also be transmitted by the transplacental route, blood transfusion and sexual intercourse (Lazear & Diamond, 2016). Following the explosive outbreaks in Brazil and Central America since early 2015,

potentially risky complications such as GBS and congenital microcephaly linked to ZIKV infection have been reported.

Due to the increase in the number of ZIKV outbreaks, cases of congenital ZIKV infection syndrome and the expanding list of species of *Culicidae* in which the virus has been isolated from or that have demonstrated vector competence (*Ae. hensilli* and *Ae. vexans*) (Benelli & Romano, 2017), ZIKV has recently appeared as one of the most important arboviruses for public health. The concomitant appearance of neonatal neurological malformations and cases of GBS has led to a declaration of ZIKV infection as the Public Health Emergency of International Concern by the WHO on 01 February 2016 (WHO, 2016).

The standard diagnosis of ZIKV infection can be determined by direct methods that involve the isolation and detection of viral genome by RT-PCR from blood, saliva, urine and other body fluids such as cerebrospinal fluid, amniotic fluid, semen, vaginal fluid, breast milk, pharyngeal secretion. It could also be done via indirect methods based on the identification of Zika antibodies in the blood (Barzon et al., 2016; Charrel et al., 2016; Waggoner & Pinsky, 2016). Although diagnosis confirmation is easily achieved using indirect methods, the cross-reactivity between viruses of the *Flaviviridae* family makes the serological results not easy to interpret. The routine diagnosis of acute ZIKV infection depends essentially on the detection of IgM antibodies by IgM antibody capture (MAC)-ELISA and the detection of viral nucleic acid by RT-PCR. The RT-PCR provides a definitive diagnosis and is often considered as the gold standard. However, this test remains unavailable in most clinical settings because of the associated costs and shortage of experienced laboratory personnel. There is a clear lack of a ZIKV laboratory diagnostic capacities and access to standardized reagents remains difficult in most countries (Charrel

et al., 2016; Zumla et al., 2016). Furthermore, the unavailability of ZIKV-specific antiviral agents, vaccine and a practical rapid test make the situation even more critical.

2.3.2 Epidemiology

Zika virus was first isolated from the blood of a sentinel rhesus monkey (*Macaca mulatta*) in the canopy of Zika Forest in Uganda in 1947 during epidemiological studies of yellow fever (Dick et al., 1952). The first human case was reported in a 10-year-old female from Nigeria in 1952 (Macnamara, 1954; Dick et al., 1952). The virus was first discovered outside Africa in *Ae. aegypti* mosquito from Bentong, Pahang in 1951 (Marchette et al., 1969). ZIKV was isolated for the first time outside the African continent from *Ae. aegypti* mosquitoes which collected in Malaya in 1969 (Marchette et al., 1969) and the first human case was reported in neighboring Indonesia in 1977 (Olson et al., 1981).

In 2007, ZIKV surprisingly caused an epidemic involving ~73% of the population on Yap Island, Federated States of Micronesia (Duffy et al., 2009). This was the first large ZIKV outbreak reported from Yap State. No further epidemics were described on the Pacific Ocean islands until October 2013 on French Polynesia following the 2007 epidemic in Micronesia which was caused by the introduction of ZIKV from Malaysia.

A major epidemic of ZIKV was reported in French Polynesia in 2013, where human patients were showing dengue-like symptoms and rash. Interestingly, a small number of the affected patients presented severe neurological complications. Non-vector borne, sexual transmission and transfusion-associated cases were also reported (Musso et al., 2015; Cao-Lormeau et al., 2011; Laigret et al., 1967). In 2014, subsequent outbreaks were reported in New Caledonia in the South Pacific. A majority of the cases were derived

from individuals who have been in French Polynesia (Dupont-Rouzeyrol et al., 2015; ProMEDmail, 2014a). Besides New Caledonia, outbreaks were also recorded in the Cook Islands and Easter Island (Institute de Veille Sanitaire, 2014).

In 2015, ZIKV has spread to Columbia and Brazil, emerging rapidly across the American continent and the Caribbean, causing an epidemic with remarkable numbers of associated clinical cases of microcephaly and GBS (Musso & Gubler, 2016). This has led to the speculation that ZIKV was presented in Brazil through people attending in the 2014 World Cup, although many countries with reported cases of ZIKV did not participate in the competition (Salvador & Fujita, 2015). On the other hand, ZIKV infection was described in a German tourist who traveled to the state of Sabah in Malaysian Borneo in 2014 (Tappe et al., 2015).

Zika virus then continued to spread across the Pacific Ocean dispersing itself throughout the tropical and sub-tropical regions of the Americas in 2015-2016 (Pettersson et al., 2016). In September 2016, an autochthonous Zika case was identified in Malaysia, followed by another case in December in a 67-year-old man from Petaling Jaya (Tam, 2016). In February 2017, 57 Zika cases were reported by the Philippine Department of Health, including 7 cases in pregnant women (ABS CBN News, 2017). In Taiwan, 14 imported Zika cases had been recorded by the Ministry of Health and Welfare by May 2017 (TNIDSS, 2017). As of 2021, there are no confirmed report of Zika virus cases from United States territories (CDC, 2021).

2.3.3 Clinical Presentation

New Caledonia Health Authority declared an outbreak situation in February 2014. A total of 1,385 laboratory-confirmed ZIKV cases have been documented, including 35 imported cases from French Polynesia, Vanuatu and the Cook Islands (Direction des Affaires Sanitaires et Sociales de Nouvelle-Calédonie, 2014).

In French Polynesia, the number of patient consultations presenting symptoms of Zika fever was approximated to be 28 000, although the total number of confirmed cases remains unknown. A retrospective serosurveillance estimated the overall infection rate at 50-66% of the total population during the major epidemic of ZIKV in 2013 (Aubry et al., 2015).

Most clinical cases during the French Polynesia outbreak presented with mild disease characterized by low-grade fever, maculopapular rash, arthralgia and conjunctivitis. A patient presented with GBS, an autoimmune disease causing acute or sub-acute flaccid paralysis a week after a confirmed acute ZIKV infection (Oehler et al., 2014). Subsequent GBS cases were recognized, correlating temporally with the ZIKV outbreak. The frequency of GBS cases during the ZIKV outbreak was approximately 20-fold greater than expected given the size of the French Polynesia population and the established incidence rates of 1–2/100 000 population per year (Sejvar et al., 2011). No critical disease from ZIKV infection had been presented prior to the French Polynesia outbreak, but previous clinical characterization was based on a limited number of confirmed cases.

As of April 2017, 209 628 laboratory-confirmed infectious and 3,122 confirmed congenital syndrome with a total of 559,721 Zika cases, have been reported in the Americas (ProMed-mail, 2017).

We believe that ongoing and undetected ZIKV transmission in other Pacific island countries, and potentially beyond, is highly probable due to the typically mild clinical symptoms, limited ZIKV diagnostic capacity, and overlapping clinical features of ZIKV, dengue, and Chikungunya, which are also circulating in the Pacific. The study that serious clinical complications may occur foregrounds the demand to strengthen surveillance for this emerging virus, and, in the event of a ZIKV outbreak, establish meticulous clinical monitoring to detect GBS or other unusual clinical manifestations (Musso et al., 2014).

2.4 Phage display (Ph.D) Technology

2.4.1 Introduction and History of Ph.D

The combination of genetic materials from 2 or more sources can be done due to the advance of recombinant DNA technology, leading to the foundation for recombinant antibody production (Cohen et al., 1973; Lobban & Kaiser, 1973; Jackson et al., 1972). This technology was originally demonstrated by George P. Smith in 1985 (Smith, 1985). Later, McCafferty and colleagues (1990) introduced antibody DNA into phages genomes via vectors and showed that antibody fragments could be displayed on bacteriophages or phages, which are viruses that infect bacteria. Subsequently, researchers designed libraries of antibody genes to display on phages and developed methods to successfully isolate individual antibodies from the large phage display libraries. Ever since, many research groups have contributed towards crucial enhancements and adjustments of the method for its potential applications in basic and applied biological sciences. This procedure has become known as antibody phage display (APD).

Phage display is based on genetic operation of the surface proteins filamentous on phages such as M13 and fd. Foreign DNA is inserted into the genome of filamentous phage

and the foreign peptide is encoded as a fusion protein with a surface coating protein (Pande et al., 2010; Barbas et al., 2004; Smith, 1985). The filamentous bacteriophages infect bacteria such as *E. coli* that commonly used in this application. Inside the bacterial cells, the double stranded replicative form that is transformed from the single stranded phage genome serves as a template for the construction of single stranded DNA progeny and viral coat proteins. (Newton & Deutscher, 2008; Paschke, 2006; Hoess, 2001; Sidhu, 2000; Smith & Petrenko, 1997). Throughout its extrusion from the host cell, the viral coat proteins encapsulate the single stranded DNA. The filamentous bacteriophages have various useful applications as cloning vectors and for the display of the encoded entities.

The most commonly used filamentous phage is M13. This bacteriophage is 6-7 nm wide, 900 nm long and is a member of F positive family (Carmen & Jermutus, 2002). M13 has a flexible rod shaped structure with a circular genome of 6000-8000 bases surrounded in a coat constituted of 5 different proteins (Kehoe & Kay, 2005; Smith & Petrenko, 1997). Different from Lambda, M13 is not a lytic phage. Plaques are produced by diminished cell growth instead of cell lysis, are turbid and not clear. This is the reason Xgal/IPTG media was used in the current study to facilitate visualization of plaques (New England Biolabs, Beverly, MA, USA). The most crucial feature of the M13 is that in contrast to other phages, it can be easily purified and used. M13 phages will infect bacteria that contain pili as binding of the phage to the F-pilus receptor on the cell will initiate the replication of the phage (Carmen & Jermutus, 2002).

There are 3 varieties of peptide phage libraries: linear heptapeptide library (Ph.D.-7), loop-constrained heptapeptide library (Ph.D.-C7C) and dodecapeptide library (Ph.D.-12). The many applications of phage-displayed peptide libraries include drug discovery, recognition of ligand-binding activity, identification of biomarkers and epitope mapping

(Pasqualini & Ruoslahti, 1996; Gallop et al., 1994; Luzzago et al., 1993; Lam & Salmon, 1991; Scott & Smith, 1990).

2.4.2 Application of Ph.D

In phage display, the exogenous polypeptides expressed and presented by phage particles bind to different target molecules. It has transformed as a powerful technology in drug discovery as well as in identifying and engineering polypeptides with novel functions (Newton & Deutscher, 2008; Paschke, 2006; Hoess, 2001; Sidhu, 2000; Smith & Petrenko, 1997). This technique has helped enormously in the study of molecular mechanisms involved in protein-protein (Sidhu et al., 2003) or protein–non protein interactions (Yu et al., 2009). Ph.D technology refers to the use of phages with fusion proteins on their surface to display random peptides, foreign proteins or protein domains (McCafferty et al., 1990). Over 80% of cellular proteins may work in interconnected system with other proteins and their protein-protein interactions are controlled by several mechanisms (Berggard et al., 2007). Phage display has been used in various studies of protein-protein interactions (Caberoy et al., 2010; Voss et al., 2010; Hertveldt et al., 2009; James et al., 2009; Fuh et al., 2000; Kiewitz & Wolfes, 1997). This include the mapping of intracellular interactions of distinct protein domains like SH3, where PDZ phage display has been used (Fuh et al., 2000; Kiewitz & Wolfes, 1997).

Through the clonal selection of antibody fragments in a prokaryotic system, the isolation of human antibodies against nearly any antigen is enabled through the use of phage display technology. This facilitates both immunotherapy and *in vivo* diagnosis. Phage display of antibody fragments has been used successfully in producing target-specific antibodies that have multiple applications such as proteomics, specific drug

delivery and analysis of intracellular processes. There are many examples of success reported in current reviews on this subject (Bratkoviv, 2010; Hoogenboom, 2005; Benhar, 2001; Smith and Petrenko, 1997). Speed and an absence of necessity for immunizing animals, especially humans, are the vital advantages in using phage display for obtaining antibodies. The target-selected phages can be kept in reserve and used on demand to produce antibody for therapeutic or diagnostic applications.

On the other hand, phage display has been used in enzymology to discover substrate specificity and to establish modulators of both the active and allosteric sites of an enzyme (Diamond, 2007; Kehoe & Kay, 2005; Benhar, 2001; Kay et al., 2001). The same approach can also be used to study the mechanisms of action of displayed enzyme mutants (Verhaert et al., 2002; Ponsard et al., 2001; Vanwetswinkel et al., 2000). Filamentous phage has been used in the identification of substrates of various proteases since it is resistant to a broad range of proteases (Diamond, 2007; Matthews & Wells, 1993).

In virology, several peptides have been discovered as potential antivirals against hepatitis B virus (Ho et al., 2003), newcastle disease virus (Chia et al., 2006), influenza virus (Rajik et al., 2009), human immunodeficiency virus (HIV) (Welch et al., 2010), transmissible gastroenteritis virus (Zou et al., 2013) and classical swine fever virus (Yin et al., 2014) by using phage display technology. However, there is no report on screening of polypeptides that specifically bind to CHIKV and ZIKV in phage display libraries.

Phage display is a low-priced and speedy approach to map epitopes of the antigen that participates in a specific interaction with an antibody. The recognition of epitopes is necessary in diagnostics, immunotherapy and vaccine development. Phage display peptide libraries can assist in recognizing critical residues within a continuous epitope that are

involved in antibody binding. The screening of libraries may affinity-select peptides that completely complement the primary structure of the epitope since linear continuous epitopes are usually 6 amino acids in length (Fack et al., 1997; Geysen et al., 1988).

Biopanning is a selection technique which selects for peptides that bind to a given target from phage display libraries. In this affinity-driven technique, the phage library will be introduced to the target protein that coated to the surface or bead to allow binding between specific binders against a target of protein. Followed by washing step, amplification and re-selection of bound phage. Multiple rounds of biopanning are required to enrich the specific binders over a general background of unspecific binders (Ganten, Ruckpaul, & Birchmeier, 2006).

A study by Guo and his colleague, 36 enriched peptides were discovered by biopanning with antibodies from two COVID-19 patients. Four motif peptides with consensus residues corresponding to two potential B-cell epitopes on SARS-CoV-2 viral proteins (Guo et al, 2021).

CHAPTER 3

MATERIALS AND METHODS

3.1 Propagation of Chikungunya Virus

3.1.1 Sources of Chikungunya Virus

Chikungunya outbreaks were reported in Sarawak in April 2009. Serum samples were sent to the Institute of Health and Community Medicine (IHCM), UNIMAS for investigation. The sera were inoculated to Vero cells. The Chikungunya virus (CHIKV), BS5 used in this study is isolated from a serum from this collection.

3.1.2 Maintenance of Vero Cells

Vero cell-line was maintained in 1X Dulbecco's Modified Eagle's Medium (DMEM) media (Gibco, USA) supplemented with 5% deactivated foetal bovine serum, FBS (Gibco, USA), 100 U/ml Penicillin G and 100 µg/ml of Streptomycin sulfate. A confluent Vero cells in a T25 cell culture flask (TPB, Switzerland) was sub-cultured by removing the spent media from the flask. The cell monolayer was washed with phosphate buffered saline (PBS) by adding 5 ml of sterile PBS to the flask. The PBS was completely removed before adding 2 ml of 0.1% Trypsin-EDTA for 2-3 minutes or until the cells were starting to dislodge. At this point, the flask was gently tapped to add in the cells detachment before adding in 5 ml of 1X DMEM media to fully resuspend the cells. The cell suspension is sub-culture into a new flask at a ratio of 1:4 to 1:6. The cells were incubated in a 37 °C incubator supplemented with 5% CO₂.

3.1.3 Inoculation of Chikungunya Virus in Vero Cells

Vero cells at 70-80% confluency was inoculated with CHIKV. Briefly, the cells were inoculated with 100 µl of the stock virus in infection medium (1X DMEM with 3% FBS). Initially, after the growth medium is removed, 1 ml of infection medium was added to the flask prior to the addition of the 100 µl inoculum. The flask was then put on a rocker for 15 minutes before adding another 4 ml of infection medium. The cells were incubated at 37 °C with 5% CO₂. The cells were observed daily under light microscope for cytopathic effect (CPE). The virus was harvested once 90% CPE was observed, for this purpose the supernatant were transferred into centrifuge tube and subjected to centrifugation at 1200 rpm for 10 minutes. The clarified supernatant was aliquoted into several screw-capped tubes and stored at -80 °C until used.

3.2 Propagation of Zika Virus

3.2.1 Sources of Zika Virus

Zika virus (ZIKV) was obtained from WRCEVA, Arbovirus Reference Collection as a gift from the University of Texas Medical Branch, Galveston as part of research collaboration. The prototype strain of ZIKV, MR766 (accession number: LC002520) was propagated and maintained in C6/36 cells.

3.2.2 Maintenance of C6/36 Cell

C6/36 cells were maintained Leibovitz-15 (L-15) medium (Gibco, USA) supplemented with 10% FBS, 10% Tryptose Phosphate broth, TPB (Sigma, USA), 100 U/ml Penicillin G and 100 µg/ml Streptomycin sulfate. Confluent cells in a T25 cell culture flask (TPB, Switzerland) were sub-culture at a ratio of 1:4 to 1:6 for every 4-5 days.

The spent media was carefully removed from the flask. Then, the flask was gently tapped to dislodge the cells from the bottom of the flask. Next, 5 ml of growth media was added to the flask to resuspend the cells. Cell suspension were transferred to new T25 flask at the designated ratio. The cells were incubated at 30°C incubator.

3.2.3 Inoculation of ZIKV in C6/36 Cells

The C6/36 cells at 80-90% confluency was inoculated with ZIKV. Briefly, the cells were inoculated with 100 µl of the stock virus in infection medium (1X L-15 with 1% FBS). Initially, after the growth medium is removed, 1 ml of infection medium was added to the flask prior to the addition of the 100 µl inoculum. The flask was then put on a rocker for 15 minutes before adding another 4 ml of infection medium. The cells were incubated at 30 °C. The cells were observed daily under light microscope for cytopathic effect (CPE). The virus was harvested once 90% CPE was observed, for this purpose the supernatant were transferred into centrifuge tube and subjected to centrifugation at 1200 rpm for 10 minutes. The clarified supernatant was aliquoted into several screw-capped tubes and stored at -80 °C until used. Figure 3.1 presented the overview of the study on peptide-ligand interaction

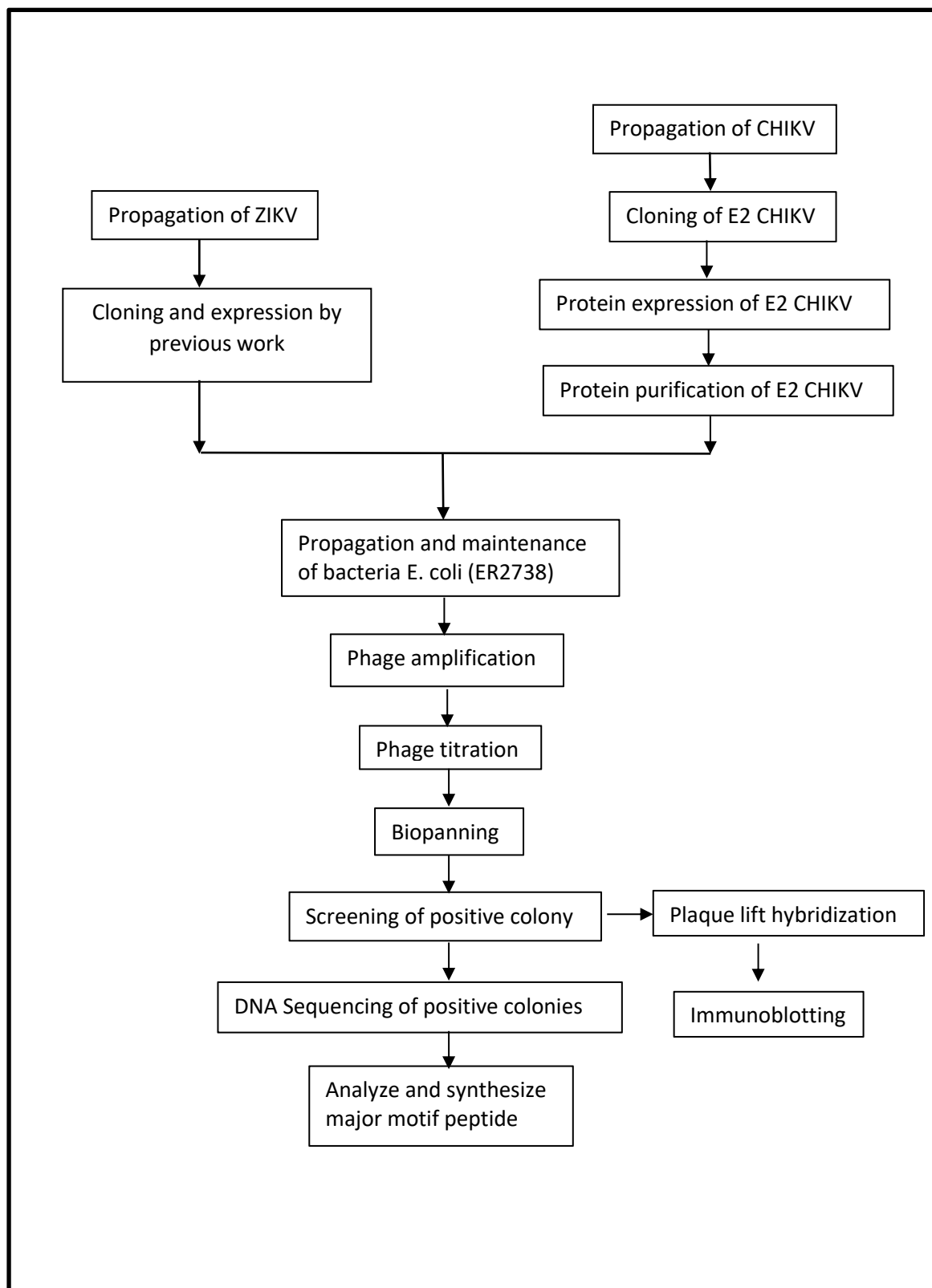


Figure 3.1: Overview of the Study on Peptide-Ligand Interaction

3.3 Cloning of CHIKV E2 and ZIKV EDIII Proteins

The cloning and expression work was done using the Champion pET SUMO Expression System (Invitrogen, Carlsbad, USA). The Champion™ pET SUMO Expression System is provided as a complete system that consists of linearized Champion™ pET SUMO vector, sterile water, dNTPs, 10X PCR Buffer, control template and primers, T4 DNA ligase, 10X ligation buffer, primers for sequencing or PCR screening, and an expression control that are stored at -20 °C. The systems also consist of the One Shot® TOP10 box that contains chemically competent E. coli cells stored at -80 °C (Mach1-T1 and BL21-DE3 competent cells), S.O.C. medium, and a control plasmid.

The cloning and expression of the CHIKV E2 recombinant protein was done as described in this study. On the other hand, the cloning and expression of the ZIKV DIII protein was done as a part of a colleague's study as mentioned in 3.3.1 below.

3.3.1 Cloning and Expression of ZIKV EDIII

The cloning and expression work was done in a previous study (Andrew, 2020). The cloning, expression, purification and characterization of the protein is described in that study. This work utilized the same ZIKV EDIII protein provided by my colleague.

3.3.2 Cloning and Expression of CHIKV E2 Protein

3.3.2.1 RNA Extraction

The extraction of the viral RNA was carried out using the High Pure Viral Nucleic Acid Kit (Roche Diagnostic, Germany) according to the manufacturer's procedures. Briefly, 200 µl of binding buffer, 20 µg/ul proteinase K and 4 µg/ul poly A were added into a nuclease-free 1.5 ml microfuge tube containing 200 µl of clarified virus culture

supernatant. The mixture was incubated in a water bath at 72 °C for 10 mins for lysis to occur. After the incubation, 100 µl isopropanol was added to the mixture and the content was transferred to a spin column and centrifuged at 13,400 rpm for 1 min (Minispin Eppendorf, Germany). The flowthrough was discarded and 500 µl of inhibitor removal buffer was added and spun again for 1 min. This is followed by 2 cycles of washing by adding 450 µl of wash buffer to the column followed by 1 min centrifugation to remove the wash buffer. After the washing step, the spin column is transferred to an oven at 80 °C for 5 mins. Finally, the nucleic acids was eluted with 55 µl of elution buffer. The eluate was either used immediately in RT-PCR or kept at -20 °C until used.

3.3.2.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed by incubating 6 µl of RNA template with 1 µl of 20 pmol downstream primer (CHIKE2/R 5' TTACGCTTTAGCTGTTCTTGAT 3') AT 70 °C for 10 mins in a thermocycler. The mixture was immediately placed on ice for 2 mins to prevent the reformation of secondary structure. A master mix containing 0.5 µl of 10 mM dNTPs, 2.0 µl of 5X RT buffer and 0.5 µl (100 U) M-MULV reverse transcriptase (MBI Fermentas, Amherst, USA) was prepared. The 3 µl mastermix was then added into the mixture and incubated for 37 °C for 1 hour followed by an enzyme inactivation step at 70 °C for 10 mins in the thermocycler (PTC100, MJResearch Inc, USA).

Two microliters of the cDNA generated in the reverse transcription step was used in a single 50 µl PCR reaction. The PCR master mix consisted of 34.5 µl sterile UHQ water, 5.0 µl of 10X Taq Buffer with KCl, 3 µl of 25 mM MgCl₂, 1.5 µl of 20 pmol/µl upstream primer (CHIKE2/F 5' AGCACCAAGGACAACTTC 3'), 1.5 UL OF 10 Mm

dNTPs and 1.0 μ l of *Taq* DNA polymerase (5 U/ μ l) were added into a PCR tube containing 2 μ l of cDNA. All reagents used in the PCR mixture were purchased from MBI, Fermentas (Amherst, USA). A water control (no cDNA) was included in each run and the PCR was carried out in cycling conditions 5 mins at 94 °C followed by 35 cycles of 20 sec at 94 °C, 30 sec at 55 °C, 2 mins at 72 °C and at the conclusion of the 35 cycles a final extension for 5 mins at 72 °C.

The PCR products were analysed on a 1% agarose gel electrophoresis with ethidium bromide. The electrophoresis was run in 1X Tris/Borate EDTA (TBE) buffer in an EasyCast Mini Electrophoresis System (Owl, NH, USA) and once completed the gel was viewed under UV transilluminator (UltraLum, Carlifornia) and the image was captured using Gel Doc XR (BioRad, CA, USA).

3.3.2.3 Gel Extraction and Purification

The band with the correct size was excised and purified using QIAquick gel extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, the gel was excised using a sterile blade and the gel slice was transferred into a 1.5 ml microfuge tube and 600 μ l of Buffer QG was added into the tube. The content of the tube was mixed gently and incubated in a water bath at 50 °C for 10 mins or until the gel slice has completely dissolved. Next, 100 μ l of isopropanol was added to the tube and whole mixture was transferred to the QIAquick spin column before subjected to centrifugation at 13, 400 rpm for 1 min (Minispin Eppendorf, Germany). The flowthrough was discarded and 500 μ l of Buffer QG was added to the column and spun again for 1 min. This is followed by a washing step by adding 700 μ l of Buffer PE to the column and spun as previous step. The QIAquick spin column was then kept at 80 °C oven for 5 mins to

completely remove all residue alcohol from the washing step. Finally, the bound DNA was eluted with 35 µl sterile UHQ water and stored at -20 °C until used. The concentration and the purity of the DNA was measured using Nanodrop 1000 spectrophotometer (Willmington, DE, USA).

3.3.2.4 Ligation of Insert (target DNA) into pET SUMO

The molar ratio of vector to insert used was 1:10. Briefly, a ligation reaction was set up by mixing 1 µl of pET SUMO vector (25 µg/µl), 1 µl of 10X ligation buffer, 1 µl T4 ligase (4 U/µl), purified DNA (to a ratio of 1:10 of the vector) and top up with sterile UHQ water to 10 µl. The ligation mixture was incubated overnight at 16 °C. The ligated product was then transformed into competent Mach1-T1 cells.

3.3.2.5 Transformation of Competent Mach1-T1 Cells with Recombinant Plasmid

The chemically competent Mach1-T1 *E.coli* cells supplied in the system functioned as a cloning host for a stable propagation and maintenance of the recombinant plasmid. For this purpose, 5 µl of the ligation mixture were added directly to a vial of 50 µl the competent cells kept on ice and mix gently with the pipette tip. The vial was kept on ice for 30 mins prior to a heat-shocked procedure (42 °C for 30 secs and immediately chilled on ice for 2 mins). Next, 250 µl of pre-warmed (at 37 °C) S.O.C medium was added to the vial and the culture was incubated with shaking in a rotary shaker (Innova 4000, New Brunswick, USA) at 225 rpm at 37 °C for 1 hour. Next, 100 µl of the cells were spread on LB plates (Appendix A7) with 50 µg/ml Kanamycin and incubated at 37 °C incubator. The plate was observed the next day for formation of colonies. The colonies were then screened for positive clones by colony polymerase chain reaction (PCR) as described below.

3.3.2.6 Colony Polymerase Chain Reaction (PCR)

Single colonies were picked using sterile toothpicks and each colony was mixed with 50 µl PCR reagent mixture containing 5 µl 10X Taq buffer, 3 µl 25 mM MgCl₂, 1.5 µl pE2_F1 primer (20 pmol/µl), 1.5 µl pET SUMO reverse primer (10pmol/µl), 1 µl 10 mM dNTP (Fermentas, NY, USA), 0.5 µl Taq polymerase (Fermentas, NY, USA) and 37.5 µl UHQ water. Colony PCR was performed to check the presence of the E2 protein in the colonies. The primers used are shown in Table 3.1. For the pET SUMO control, the colony picked was mixed with 50 µl PCR reagent mixture that contained 5 µl 10X Taq buffer, 3 µl 25 mM MgCl₂, 1.5 µl pET SUMO forward primer (10 pmol/µl), 1.5 µl pET SUMO reverse primer (10 pmol/µl), 1 µl 10 mM dNTP (Fermentas, NY, USA), 0.5 µl Taq polymerase (Fermentas, NY, USA) and 37.5 µl UHQ water. The PCR was performed in a thermocycler (PTC-200, MJ Research, NY, USA) with the conditions shown in Table 3.2. After that, samples were analyzed on 1.5% agarose gel that was electrophoresed at 120 V for 40 minutes (Thermo Scientific, NY, USA). The gel image was captured (Bio-Rad, CA, USA).

Table 3.1: Primers for Colony PCR

Primers	Primer sequences
pET SUMO Forward	5'- AGA TTC TTG TAC GAC GGT ATT AG – 3'
pET SUMO Reverse	5'- TAG TTA TTG CTC AGC GGT GG – 3'

Table 3.2: Condition for Colony PCR Reaction

Condition	Temperature (°C)	Duration	Cycles
Initial denaturation	94	8 minutes	1
Denaturation	94	20 seconds	35
Annealing	55	30 seconds	
Extension	72	2 minutes	
Final extension	72	4 minutes	1

3.3.2.7 Glycerol Stock Preparation for Positive Clones

Once the positive clones were selected, the culture was stored as glycerol stock for further work. For glycerol stock preparation, 1:10 dilutions of each culture was made by adding 200 µl of overnight culture into 1.8 ml of fresh LB medium containing 50 µg/ml Kanamycin. The fresh cultures were incubated in an incubator shaker at 225 rpm for 2 hours at 37 °C (Innova 4000, New Brunswick Scientific, NJ, USA). After that, 700 µl of each culture was mixed with 300 µl of 50% glycerol and stored at -80 °C.

3.3.2.8 Plasmid DNA Preparation

Plasmid DNA preparation was done by using PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA, USA). The positive clone was inoculated into 10 ml of Luria Bertani (LB) medium containing 50 ug/ml kanamycin. The culture was incubated overnight with shaking in an incubator shaker set at 225 rpm and 37°C. The next day, 6 ml of overnight culture was transferred into a microfuge tube and centrifuged (Minispin, Eppendorf, Germany) at 13 400 rpm for 5 minutes. After centrifugation, the supernatant was discarded. The pellet was resuspended completely in 250 ul of the lysis buffer (200

mM NaOH, 1% w/v SDS) and incubated for 5 mins at room temperature. Next, 350 μ l of the precipitation buffer was added to the tube and was centrifuged for 10 mins at 13 400 rpm. The supernatant was transferred to the spin column after the centrifugation and spun for 1 min. After the flow through was discarded, the column was subjected to 2 rounds of washing with the wash buffer provided. The column was then placed in a 80 °C oven for complete removal of excess ethanol. Finally, the DNA was eluted in 50 μ l sterile UHQ water. The plasmid DNA was stored at -20°C until used. The plasmid DNA was verified by sequencing (source out to Apical Scientific, Selangor, Malaysia) and the sequence was analysed using bioinformatics software (DNASTAR, LaserGene). The plasmid was then transformed into host for expression of recombinant plasmid.

3.3.2.9 Expression of CHIKV E2 Protein

3.3.2.9.1 Transformation into Host for Expression of Recombinant Plasmid

Escherichia coli strain BL21.DE3 expression host was used (Invitrogen, Carlsbad, CA). The plasmids containing the DNA of E2 protein (BS1/pE2/Mach1/COL3 and BS1/pE2/Mach1/COL5) and the vector plasmid (pET SUMO/Mach1/COL4) were transformed into *E. coli* BL21.DE3. For transformation of each sample, 2 μ l of the plasmid DNA was added into the tube containing *E. coli* BL21.DE3 competent cells. Transformation was done by heat shock where the tube was incubated on ice for 30 minutes and then transferred to a water bath at 42 °C for 30 seconds. The tube was immediately placed on ice for 2 minutes. Next, 250 μ l of prewarmed SOC medium was then added to the tube, followed by 1 hour of incubation in the incubator shaker at 225 rpm at 37 °C. After that, 50 μ l of the transformed cells was spread on LB agar containing 50 μ g/ml Kanamycin and the plates were incubated overnight at 37 °C.

3.3.2.9.2 Small Scale Protein Expression

For small scale protein expression, 1 ml of overnight culture was inoculated into 9 ml of LB broth containing 50 µg/ml Kanamycin. The culture was then incubated for 2 hours in an incubator shaker set at 225 rpm at 37 °C. Then, 3 ml of the culture was transferred into 2 different bijoux bottles labelled as “induced” and “uninduced” respectively. For the induced culture, 30 µl of 100 mM IPTG (5 Prime, MD, USA) was added. Both cultures were incubated at 37 °C with shaking for 3 hours (Innova 4000, New Brunswick Scientific, NJ, USA). This is followed by immediately placing the bottle on ice for 5 minutes to stop the reaction. Each of the 3 ml culture was divided into two 1.5 ml tubes labelled “reducing” and “non-reducing”, respectively. Both tubes were centrifuged at 5000 x g for 10 minutes at 4 °C (Minispin, Eppendorf, Germany). The supernatant was discarded and the pellet was resuspended in their respective reducing or non-reducing sample buffer and was stored at 4 °C.

3.3.2.9.3 SDS-PAGE and Western Blotting

Expression of the target protein was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). The samples were resolved on 12% resolving gel using the Mini Protean II electrophoresis cell (Bio-Rad, CA, USA). Samples were prepared by adding 30 µl of sample with 10 µl of 4X reducing or non-reducing sample buffer. This was followed by heating of the samples at 95 °C for 10 minutes (Benchmark, NJ, USA) and centrifugation at 13 400 rpm for 10 minutes (Minispin, Eppendorf, Germany). For each well of the gel, 10 µl of sample were loaded. Electrophoresis was performed vertically at a constant voltage of 120 V in 1X Tris-

glycine running buffer. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) R-250 (Bio-Rad, CA, USA).

For Western blotting, the proteins resolved on the gel were transferred onto nitrocellulose membrane (ADVANTEC, California, USA) according to Towbin *et al.* (1979). Using a Mini Trans-Blot module (Bio-Rad, CA, USA), gels from SDS-PAGE was placed onto a cassette containing a sandwich of filter papers and sponges. The gel was overlaid with nitrocellulose membrane and secured into place in the cassette. The transfer was performed at a constant current of 200 mA for 1 hour in cold Towbin buffer. After the run was completed, the membrane was transferred into a container containing the blocking buffer of 5% skimmed milk or 1% bovine serum albumin (BSA) in 1X phosphate-buffered saline (PBS). Blocking was done for 30 minutes at room temperature on a rocker (Edmund Buhler, WS-5). Next, the membranes were probed with either Nickel-HRP or specific antibodies against the target protein. Nickel-HRP (KPL, MD, USA) was used at 1:1000 dilution in 1% BSA-PBS and the membrane was incubated on the rocker in dark for 2 hours. A CHIKV-positive serum K336 (Sum, Andrew and Mailing, 2015) was diluted at 1:500 in PBS-skimmed milk and incubated with the membrane overnight on the rocker.

After incubation, the membrane was washed 3 times with 1X PBS at 10-minute intervals on the rocker. Then, protein bands on the blot that are reactive to Nickel-HRP were detected with the incubation of equal volume of chromogenic substrate 4-chloro-1-naphthol in methanol/H₂O₂ for 30 minutes on the rocker. Reactions were stopped by washing with RO water and the blot was air dried. As for the membrane that was probed with CHIKV serum, the blot was incubated with a polyclonal rabbit anti-human IgG/HRP (Dako, Denmark) at 1:2000 dilution in PBS-skimmed milk for 2 hours at room

temperature in dark. This was followed by another washing step and visualization of the reactive band as described with Ni-HRP.

3.3.2.10 Purification of CHIKV E2 Protein

3.3.2.10.1 Protein Purification

The glycerol stocks of BS1/Mach1/COL3/BL21/COL1 and pETSUMO/Mach1/COL4/BL21/COL1 were each inoculated into 20 ml of LB medium containing 50 µg/ml Kanamycin to prepare the cultures for cell lysate. The cultures were incubated overnight at 37 °C with shaking at 225 rpm. Next, 20 ml of the overnight culture was added into a 180 ml of LB medium containing 50 µg/ml Kanamycin. After 2 hours of incubation in the incubator shaker at 37 °C, 2 ml of 100 mM IPTG was added to each culture, followed by another 3 hours of incubation with shaking. Then, the cultures were placed on ice before being transferred into centrifuge tubes and centrifuged at 5000 rpm at 4 °C for 5 minutes (Avanti J-26S XPI, Beckman Coulter, CA, USA). The supernatant was discarded and the pellet of E2 culture was resuspended in 8 ml of guanidium lysis buffer and rocked at minimum speed (Biologix, KS, USA) for 5 to 10 minutes to lyse the cells. The cell lysate was sonicated (Cole Permer, IL, USA) on ice for 3 times with 5-second cycles. Meanwhile, the pellet of pET SUMO culture was resuspended in 8 ml of native binding buffer containing 0.016 g of lysozyme (Amresco, OH, USA). Both cell lysates were centrifuged at 3000 x g for 15 minutes at 4 °C (Avanti J-26S XPI, Beckman Coulter, CA, USA). The lysates, which were the supernatants, were transferred to fresh tubes. The cell lysate of E2 was stored at -20 °C and pET SUMO was stored at 4 °C.

ProBond Purification System (Novex, CA, USA) was used to purify the proteins of interest. ProBond resin was resuspended by inverting and gently tapping the

bottle, before 2 ml of the resin was transferred to the column and centrifuged at 800 x g (Avanti J-26S XPI, Beckman Coulter, CA, USA). The supernatant was discarded and the resin was washed with 6 ml of sterile UHQ water, followed by 6 ml of denaturing binding buffer for purification under denaturing condition or 6 ml of native binding buffer for purification under native condition. Washing steps were performed by centrifugation at 800 x g for 1 minute and discarding the supernatants.

For purification under denaturing condition, 8 ml of E2 lysate was added to the prepared purification column. Gentle agitation was applied by placing the column at a rotating wheel. Binding of proteins to the resin was allowed for 30 minutes at room temperature. The column was then washed with 4 ml of denaturing binding buffer (pH 7.8) and 4 ml of denaturing wash buffer (pH 6.0 and pH 5.3) with a cycle of gentle agitation and centrifugation for 1 minutes at 500 x g for each buffer (Avanti J-26S XPI, Beckman Coulter, CA, USA). The washing steps were done twice and the aspirated supernatants were collected in a fresh tube. Next, the column was fixed in a vertical position and 5 ml of denaturing elution buffer (pH 4.0) was added. The eluate was collected in 1-ml fractions. All the eluted proteins were stored at -20°C before analysis by SDS-PAGE as described in section 3.3.2.9.3.

For purification under native condition, 8 ml of pET SUMO lysate was added to the prepared purification column. Gentle agitation was applied by placing the column at a rotating wheel and binding of proteins to the resin was allowed for 30 minutes at room temperature. This was followed by 5 cycles of washing that include adding 8 ml of native wash buffer (pH 8.0) with gentle agitation and centrifugation for 1 minute at 500 x g (Avanti J-26S XPI, Beckman Coulter, CA, USA). The supernatants were gently aspirated and collected in a fresh tube. Next, the column was fixed in a vertical position and 8 ml of

native elution buffer (pH 8.0) was added. The eluate was collected in 1-ml fractions. All the eluted proteins were stored at -20°C and analyzed on SDS-PAGE as previously described.

3.3.2.10.2 Measurement of Protein Concentration

The concentration of the purified proteins was measured using Synergy H1 Hybrid Multi-Mode Reader (BioTek Instruments Inc., USA). For each measurement, 1.5 µl of the protein sample was used. The concentration was calculated as an average of two readings.

3.3.2.10.3 Gradient Dialysis

The dialysis tubing (Fisher Scientific, Pittsburg, PA, USA) with a pore size of 6 to 8 kDa was immersed in 1X PBS prior to use. Eluted fractions that were confirmed to contain the protein of interest from the SDS-PAGE analysis were pooled and 2 ml of the protein was pipetted into the dialysis tube. The protein was dialyzed in 6 M urea (Vivantis, CA, USA) for 2 hours at 4 °C. The concentration of urea in the buffer was gradually reduced to 4 M and 2 M in each round of buffer change. Finally, the protein was dialyzed in 1X PBS. The concentration of the dialyzed protein was measured as described in Section 3.3.2.10.2.

3.4 Propagation and Maintenance of *Escherichia coli* ER2738

3.4.1 Bacterial Stock and Maintenance

Escherichia coli ER2738 was purchased from New England Biolabs (Ipswich, MA, USA). The glycerol stock of ER2738 was streaked onto Luria Bertani agar plate containing 20 mg/ml Tetracycline (LB + 20 mg/ml Tet). The plate was inverted and incubated

overnight at 37 °C. The next day, the plate was sealed with parafilm and stored at 4 °C in the dark for a maximum period of 1 month. Fresh glycerol stocks of ER2738 were prepared from liquid cultures.

3.5 Initial Amplification of the Phage Library Ph.D.-7

3.5.1 Amplification of Phage Library

The Ph.D.-7 phage-displayed peptide library was purchased from New England Biolabs (Ipswich, MA, USA). The titer of this peptide library was up to 2×10^{11} plaque forming units (pfu/ml), containing about 2.5×10^9 independent random peptide sequences.

The glycerol stock of ER2738 was streaked onto a LB + 20 mg/ml Tet plate and incubated overnight at 37 °C. The next morning, a single colony was picked and inoculated into 5 ml of LB broth. The culture was grown in an incubator shaker (New Brunswick, Innova 4000, USA) for 4 to 5 hours at 37 °C. When the cells reached mid-log phase ($OD_{600} \sim 0.5$), 10 μ l of the phage display peptide library Ph.D.-7 (10^{11} phage/ml) was added to the culture. The culture was incubated further under similar conditions for 4-5 hours to allow the infection to occur.

After the incubation, the culture was chilled on ice for 10 minutes before being transferred to a centrifuge bottle (Beckman Coulter, IN, USA). The tube was centrifuged at 5000 x g for 20 minutes at 4 °C (Hermle, Z233MK, GA, USA). The supernatant was transferred to a fresh centrifuge bottle and 833 μ l ($1/6^{\text{th}}$ of the original culture volume) of 30% PEG/2.5 M NaCl solution was added. The mixture was then centrifuged at 5000 x g for 45 minutes at 4 °C. After that, the supernatant was discarded and the pellet was resuspended in Tris-Buffered Saline (TBS), followed by another round of centrifugation at 5000 x g for 10 minutes at 4 °C. The supernatant was transferred to another fresh tube and

833 μ l of 30% PEG/2.5 M NaCl was added. The precipitation was allowed to happen at 4 °C for 1 hour with rotation. Next, the mixture was centrifuged at 5000 x g for 20 minutes at 4 °C. Finally, the supernatant was discarded and the pellet was resuspended in 1 ml of TBS and this amplified phage was stored at 4 °C until use.

3.5.2 Phage Titration

Ten millilitre of LB broth was inoculated with ER2738 and the culture was grown to mid-log phase ($OD_{600} \sim 0.5$) in an incubator shaker for 4 to 5 hours at 37°C. While cells were growing, Top agar was melted in a microwave and 3 ml was dispensed into bijou bottles, one per expected phage dilution. The bottles were maintained at 45 °C to avoid the agar from hardening.

One LB/IPTG/Xgal plate per expected dilution were pre-warmed at 37 °C for at least one hour. Then, a 10-fold serial dilution (10^{-1} to 10^{-10}) of the phage in LB broth was prepared. A fresh pipette tip was used for each transfer to prevent cross-contamination and to avoid carrying virus particles from the pipette tip to the next dilution.

When the ER2738 culture has reached mid-log phase, 200 μ l was dispensed into a microfuge tube, one for each dilution. Next, 10 μ l of each of the diluted phage suspension was added to the respective tubes for infection. Each tube was vortexed briefly and incubated for 5 minutes at room temperature. The infected cells were then transferred to the bijou bottles containing Top agar and the suspensions were swirled several times. Each culture was immediately poured onto respective pre-warmed LB/IPTG/Xgal plate. The plates were gently tilted and rotated to spread the top agar evenly. The plates were allowed

to cool for 5 minutes before being inverted and incubated overnight at 37 °C. Figure 3.2 below depicts the workflow of the phage titration.

The plaques were counted manually and the results were calculated as below (Equation 3.1):

$$\frac{pfu}{ml} = \frac{number\ of\ plaques}{(volume)(dilution)}$$

Equation 3.1

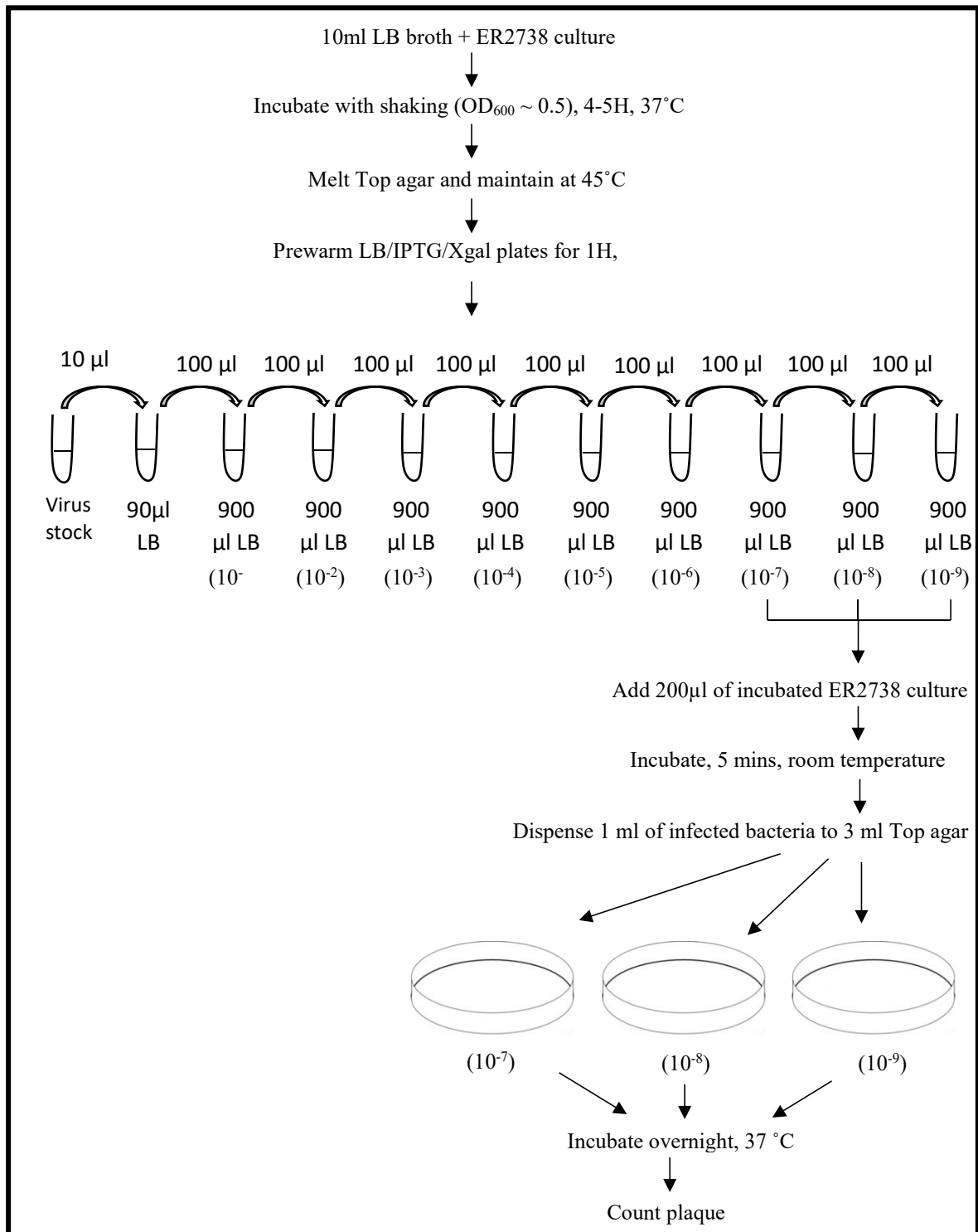


Figure 3.2: Workflow of Phage Titration

3.6 Biopanning and Enrichment Analysis of Phage Library against Target Antigen

3.6.1 Biopanning

In this study, Ph.D.-7 peptide library (Neb, New England Biolabs, USA, E8102A) was used according to the manufacturer's instructions with slight modifications to screen binding peptides of CHIKV E2 protein and ZIKV EDIII protein. The surface panning procedure was chosen as it is the most straightforward method of affinity partitioning or panning involving directly coating the tube surface with the protein of interest, washing away the excess and passing the pool of phages over the target-coated surface. Panning was carried out using individual sterile "Star Tubes" (Nunc, Denmark).

A subtraction step was carried out with the vector protein before biopanning was performed. Purified pET SUMO was diluted 1:10 in coating buffer (0.016M Na₂CO₃, 0.034M NaHCO₃; pH9.6) to a final volume of 1 ml coated into 2 different Star Tubes. The tubes were labeled as "pET SUMO A" and "pET SUMO B" respectively. Then, the target antigen diluted 1:10 in coating buffer to a final volume of 500 µl was coated into a new Star Tube and labeled as "Protein".

All three Star Tubes were incubated overnight at 4°C. On the next day, the solution in star-tube pET SUMO A was removed and the tube was washed 1 time with Tween 20-TBST (TBS + 0.1% [v/v] Tween-20). After washing, the tube was blocked with 2 ml Casein-PBS for 2 hours at 4°C. Next, the tube was washed 6 times with TBST and a 1:50 dilution of phage [input phage (10¹¹ phage/ml)] in TBST to a final volume of 500 µl was added and the tube was incubated for 5-6 hours at 4°C. Two hours before the incubation ended, the solution in the pET SUMO B tube was removed, followed by the same washing

and blocking steps as pET SUMO A. Then, the incubated phages in the pET SUMO A tube was transferred into pET SUMO B tube and the tube was incubated overnight at 4 °C.

On the next day, the solution in the Protein tube was removed, washed and blocked in the same way as pET SUMO A and B. Then, the incubated phages in the pET SUMO B tube was transferred into Protein tube and was incubated overnight at 4 °C to allow the binding between the phages and the target antigen. On the following day, 3 ml of LB broth was inoculated with ER2738 and cultured to $OD_{600} \sim 0.05$ in an incubator shaker for 30 minutes to 1 hour at 37 °C. While waiting for the cells to grow, the Protein tube was washed 10 times with TBST. Once ready, 1 ml of the ER2738 culture was added into the Protein tube and incubation with shaking was performed for 2 hours at 37 °C. Before the 2 hours was up, 20 ml of LB media was inoculated with ER2738 and grown to $OD_{600} \sim 0.05$ in an incubator shaker for 30 minutes to 1 hour at 37 °C. Then, the culture from the Protein tube was transferred into the ER2738 culture and was further cultured for 3 hours at 37 °C. After that, the culture was centrifuged at 12 000 x g for 10 minutes at 4°C. The supernatant was transferred to a fresh tube and 3.3 ml ($1/6^{\text{th}}$ of the culture volume) of 30% PEG/2.5 M NaCl was added and the mixture was incubated overnight at 4 °C.

The next day, centrifugation of the mixture was performed at 12 000 x g for 15 minutes at 4 °C. The supernatant was discarded and the pellet was resuspended in 1 ml TBS. The suspension was centrifuged at 14 000 rpm for 5 minutes at 4 °C and the supernatant was collected and 3.3 ml of 30% PEG/2.5 M NaCl was added. The mixture was chilled on ice for 20 minutes before being centrifuged at 14 000 rpm for 10 minutes at 4 °C. The final supernatant was discarded and the pellet was resuspended in 200 μ l TBS. This is the amplified eluate of the first panning.

The amplified eluate was tittered as described in Section 3.6.2. The second and third panning was being carried out under identical conditions except for the washing steps. Washing was done with higher concentrations of TBST (0.25% and 0.5% respectively). In between each round of panning, the titer of the eluate or output phages was calculated to assess the enrichment efficiency. Figure 3.3 summarizes the workflow of biopanning.

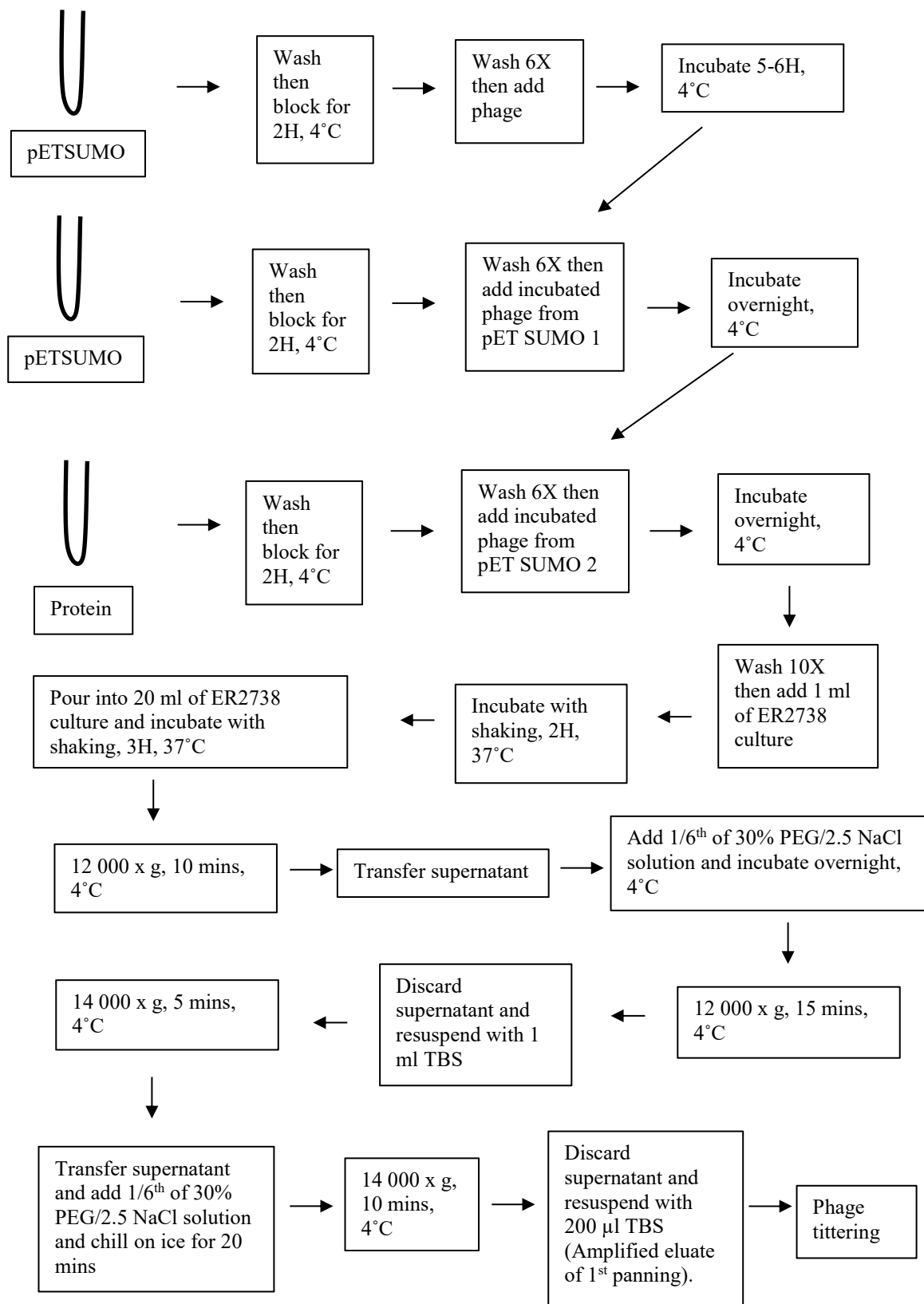


Figure 3.3: Workflow of Biopanning

3.7 Screening of Positive Colonies

3.7.1 Plaques Lift

The plaques that appeared on the surface of the LB/IPTG/Xgal plate were transferred onto nitrocellulose membrane (ADVANTEC, California, USA) for 30 minutes at room temperature to allow the DNA of the plaques to bind to the membrane.

3.7.2 Membrane Immunoassay

The nitrocellulose membrane was then blocked with 1X PBS containing 5% skimmed milk for 30 minutes on a rocker at room temperature. After blocking, the membrane was washed 3 times with 1X PBS at 10 minutes intervals. Next, the membrane was incubated with an anti-CHIKV serum diluted 1:200 in PBS-skimmed milk overnight on the rocker at room temperature. The next morning, the membrane was washed 3 times with 1X PBS at 10 minutes intervals. The membrane was then probed with a polyclonal rabbit anti-human IgG-HRP (Dako, Glostrup, Denmark) diluted 1:1000 in PBS-skimmed milk for 2 hours on the rocker at room temperature in the dark.

After incubation, the membrane was washed 3 times with 1X PBS at 10 minutes intervals. Positive clones were detected by incubation with the chromogenic substrate 4-chloro-1-naphtol in methanol/H₂O₂ on the rocker for 30 minutes at room temperature. The reactions were then stopped by washing the membrane with distilled water, followed by air-drying.

In the case of ZIKV, identical membranes were incubated overnight on the rocker at room temperature with two ZIKV positive sera (DKB/25/1 and DKB8/16/1) at 1:250 dilution and a DENV-positive serum pool (HPR) at 1:200. Another identical membrane was probed with a pan-flavi monoclonal antibody against the E protein (4G2) (Henchal et

al., 1982). For this membrane, incubation with polyclonal rabbit anti-mouse immunoglobulin-HRP (Dako, Glostrup, Denmark) was done instead of polyclonal rabbit anti-human IgG-HRP for the other membranes that were probed with human sera.

An identical membrane probed with M13-HRP (BioLabs[®] Inc., New England) at a 1:1000 dilution. The membrane was incubated in the dark for 2 hours on the rocker at room temperature. After that, the membrane was washed 3 times with 1X PBS at 10 minutes interval. Positive clones were detected by incubation with the chromogenic substrate 4-chloro-1-naphtol in methanol/H₂O₂ on the rocker for 30 minutes at room temperature. Finally, the reactions were then stopped by washing with distilled water and the membrane was air-dried.

After 3 rounds of panning, individual positive clones were picked based on the reactivity of the clones and were amplified in *E. coli* ER2738.

3.7.3 Plaque Amplification

Ten milliliter of LB media was inoculated with ER2738 and incubated overnight with shaking at 37 °C. On the next day, the culture was diluted 1:100 in LB broth and 1-ml aliquots were dispensed into culture tubes, one for each clone to be characterized. From the third round of panning, 10-20 positive clones were picked. A fresh sterile pipette tip was used to transfer each blue plaque from the titrating plate into the culture tubes containing the diluted culture. Well-separated plaques were selected to ensure each plaque contains a single DNA sequence.

The culture tubes were then incubated with shaking for 4-5 hours at 37 °C. After that, the cultures were transferred to microcentrifuge tubes and centrifuged at 5000 x g for

20 minutes at 4 °C (Hettich® MIKRO 200R, Germany). The supernatant was transferred to a fresh tube and 166 µl (1/6th of the volume of the culture) of 30% PEG/2.5 M NaCl solution was added to each tube. The content of the tubes were mixed and incubated overnight at 4 °C. The next day, the tubes were centrifuged at 5000 x g for 45 minutes at 4 °C. For each tube, the supernatant was discarded and the pellet resuspended in 100 µl TBS. Next, the tubes were centrifuged at 5000 x g for 10 minutes at 4 °C. The supernatant was transferred into fresh tube and 166 µl of 30% PEG/2.5 M NaCl solution was added. The tubes were incubated for 1 hour at 4 °C before being centrifuged again at 5000 x g for 20 minutes at 4 °C. Each of the final pellets was resuspended in 1 ml of TBS. The amplified plaques were stored at 4 °C up to several weeks with little loss of titer.

3.8 Extraction of DNA from Positive Colonies

3.8.1 Deoxyribonucleic Acid (DNA) Extraction

The extraction of the viral DNA was performed using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Germany). Briefly, 200 µl of binding buffer [6M guanidine-HCl, 10mM Tris-HCl, 20% Triton® X-100 (w/v), pH4.4], 20 µg/µl proteinase K and 4 µg/µl poly A were added into a nuclease-free 1.5ml microfuge tube containing 200 µl of clarified virus culture of CHIKV and ZIKV, respectively. The mixture was incubated in a water bath (Mettler, Germany) set at 72 °C for 10 minutes in which virus lysis occurred.

After the incubation, 100 µl of isopropanol (Merck, Germany) was added to the mixture and the content was transferred to a spin column and centrifuged at 13 400 rpm for 60 seconds (Minispin, Eppendorf, Germany). The flow-through was discarded and 500 µl

of inhibitor removal buffer (5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6) was added and the column was spun again for 60 seconds.

After discarding the flow-through, the spin column was washed twice with 450 μ l of wash buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5) followed by drying in an oven set at 80 °C for 5 minutes. Finally, the nucleic acids were eluted with 30 μ l of elution buffer (nuclease-free, sterile, double distilled water) provided. The eluate was either used immediately or stored at -20 °C until used.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Cloning of CHIKV E2 Protein

4.1.1 Growing of the Cultures

The positive clone was streaked on a LB-Kanamycin plate and a single colony from each culture plate was picked. Colony PCR was performed on these picked colonies to screen for plasmids that contain the E2 gene.

4.1.2 Confirmation of Clones by Colony Polymerase Chain Reaction (PCR)

A single colony was picked from each of the culture plates of E2 gene of CHIKV (BS1/pE2/Mach1/COL1, BS1/pE2/Mach1/COL3, BS1/pE2/Mach1/COL5a, BS1/pE2/Mach1/COL5b, BS1/pE2/Mach1/COL6, BS1/pE2/Mach1/COL7) and vector pET SUMO/Mach1/COL4. These were subject to colony PCR to screen for the presence of gene of interest as described in section 3.3.2.6. For the first round of the colony PCR, a band of 254 bp was detected in the vector pET SUMO/Mach1/COL4 as shown in lane 7 in Figure 4.1. In the second run, 5 colonies from 6 tested were positive with the expected size of CHIKV E2 protein of 850 bp as shown in lanes 1, 2, 3, 5 and 6 in Figure 4.2. Contamination was ruled out as no band was detected in the water control. Plates BS1/pE2/Mach1/COL3 and BS1/pE2/Mach1/COL5a were selected for protein expression.

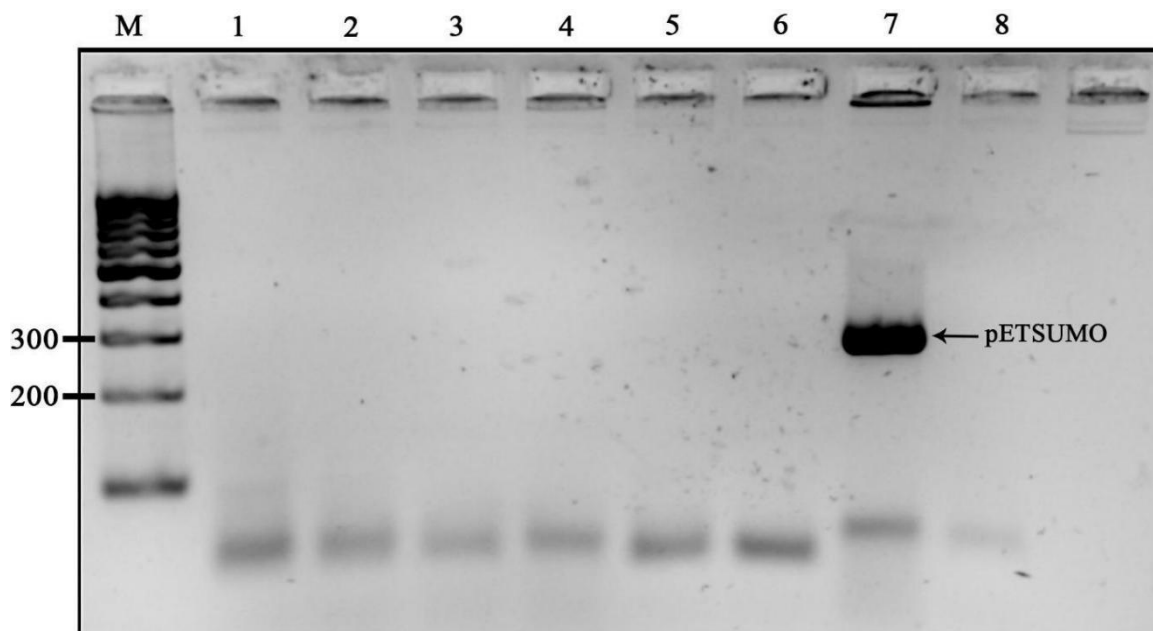


Figure 4.1: Colony PCR-1 (pETSUMO). M: 100 bp DNA marker, Lane 1: BS1/pE2/Mach1/COL1, Lane 2: BS1/pE2/Mach1/COL3, Lane 3: BS1/pE2/Mach1/COL5a, Lane 4: BS1/pE2/Mach1/COL5b, Lane 5: BS1/pE2/Mach1/COL6, Lane 6: BS1/pE2/Mach1/COL7, Lane 7: pETSUMO/Mach1/COL4, Lane 8: Water control (Negative control).

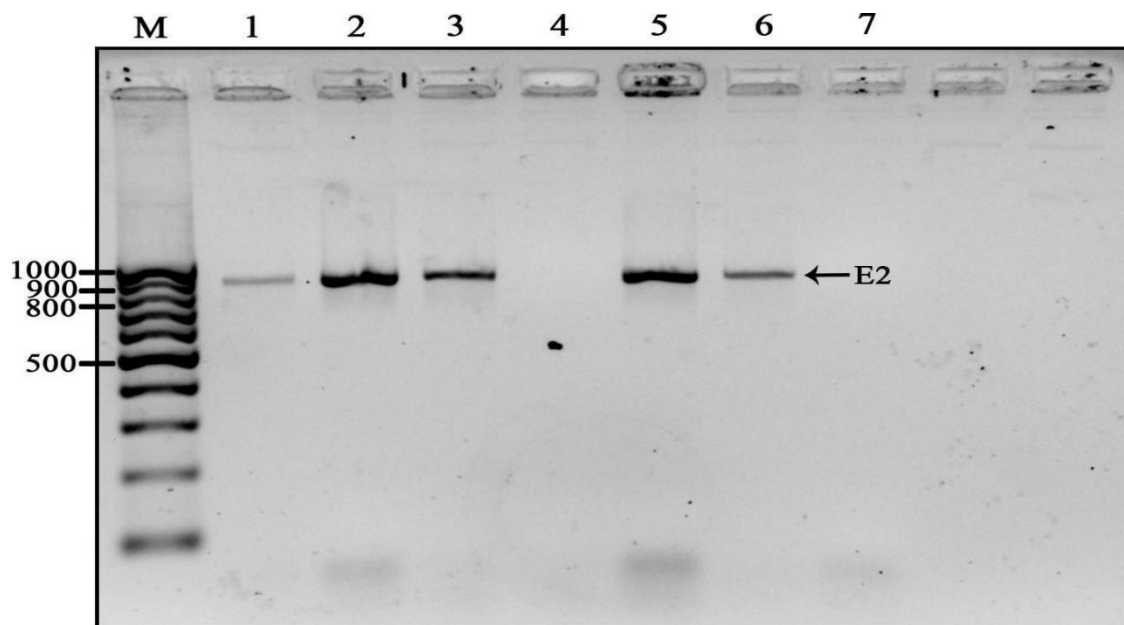


Figure 4.2: Colony PCR-2 (E2 protein). M: 100 bp DNA marker, Lane 1: BS1/pE2/Mach1/COL1, Lane 2: BS1/pE2/Mach1/COL3, Lane 3: BS1/pE2/Mach1/COL5a, Lane 4: BS1/pE2/Mach1/COL5b, Lane 5: BS1/pE2/Mach1/COL6, Lane 6: BS1/pE2/Mach1/COL7, Lane 7: Water control (Negative control).

4.2 Expression of CHIKV E2 Protein

4.2.1 Transformation of E2 Clone into BL21(DE3) One Shot Cells

The culture plates of E2 gene BS1/pE2/Mach1/COL3 and BS1/pE2/Mach1/COL5a and vector pETSUMO/Mach1/COL4 were selected for transformation into BL21(DE3) One Shot cells. Figure 4.3, Figure 4.4 and Figure 4.5 show the colonies for both E2 gene plates and the vector, respectively. All three plates contained more than 50 colonies.

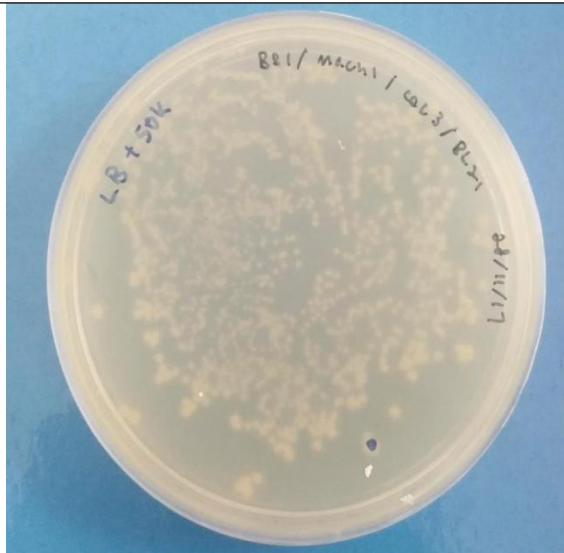


Figure 4.3: Culture plate of E2 BS1/pE2/Mach1/COL3/BL21

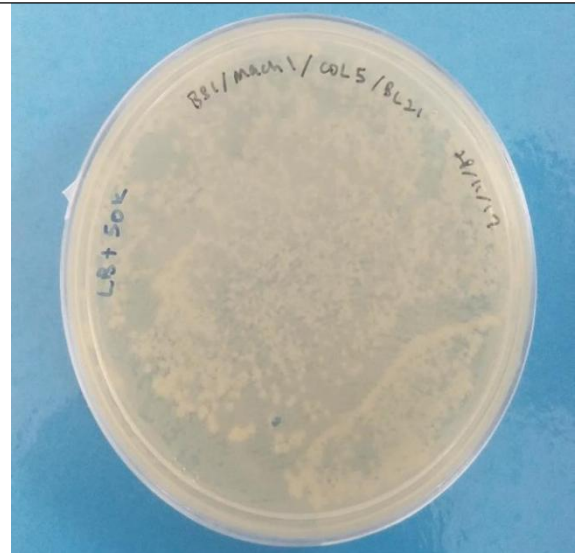


Figure 4.4: Culture plate of E2 BS1/pE2/Mach1/COL5/BL21



Figure 4.5: Culture plate of vector pETSUMO/Mach1/COL4/BL21

4.2.2 Small Scale Protein Expression

The expression of partial CHIKV E2 recombinant protein from COL3 and COL5, plus the pET SUMO vector was first analyzed on SDS-PAGE. On the CBB-stained gel, both recombinant proteins and the vector were expressed at the expected size of 35 kDa and 13 kDa respectively, as shown in Figure 4.6A and Figure 4.7A. Subsequently, Western Blots were prepared to verify the expression of the recombinant protein and vector. The blots identical to the CBB-stained gels were probed with Nickel-HRP for the detection of the recombinant protein by binding the N-terminal polyhistidine (6xHIS) tag. As shown in Figure 4.6B and Figure 4.7B, both partial CHIKV E2 protein and pET SUMO showed clear bands with their respective expected sizes. A duplicate blot for each of the proteins was probed with K336, a human serum that is CHIKV-positive, to check the reactivity of the recombinant proteins. Figure 4.6C and Figure 4.7C show the blots with a single faint band each in the induced culture that was treated with reducing sample buffer. This proves that COL3 and COL5 of partial CHIKV E2 recombinant protein and pET SUMO vector were successfully expressed.

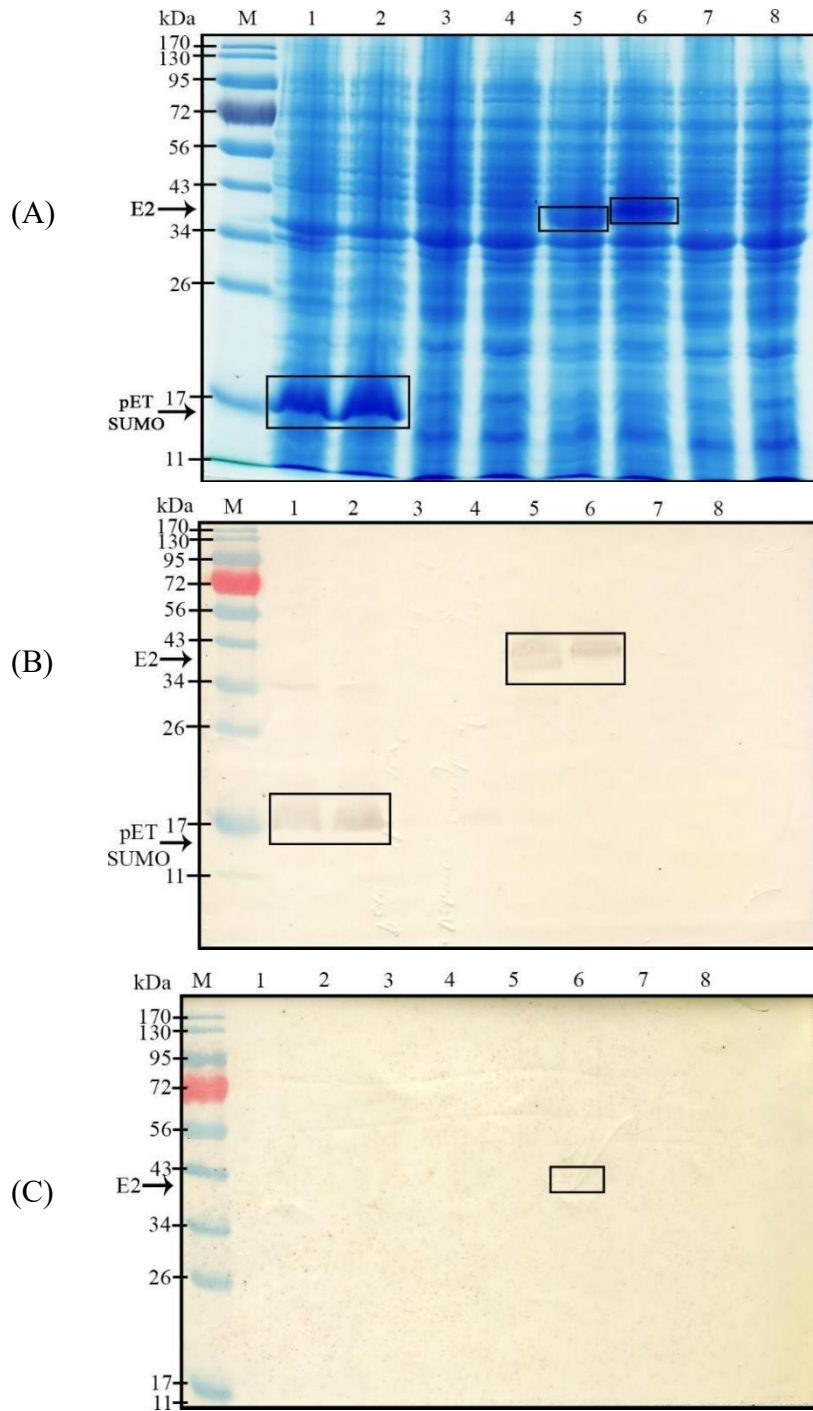


Figure 4.6: Expression of CHIKV E2 Protein under Different Conditions (COL3). Lane 1: pET SUMO, induced, non-reducing, Lane 2: pET SUMO, induced, reducing, Lane 3: pET SUMO, uninduced, non-reducing, Lane 4: pET SUMO, uninduced, reducing, Lane 5: CHIKV E2 (COL3), induced, non-reducing, Lane 6: CHIKV E2 (COL3), induced, reducing, Lane 7: CHIKV E2 (COL3), uninduced, non-reducing, Lane 8: CHIKV E2 (COL3), uninduced, reducing. (A) CBB-stained gel (B) Western blot probed with Nickel-HRP (C) Western blot probed with CHIKV positive serum (K336). M: molecular weight markers.

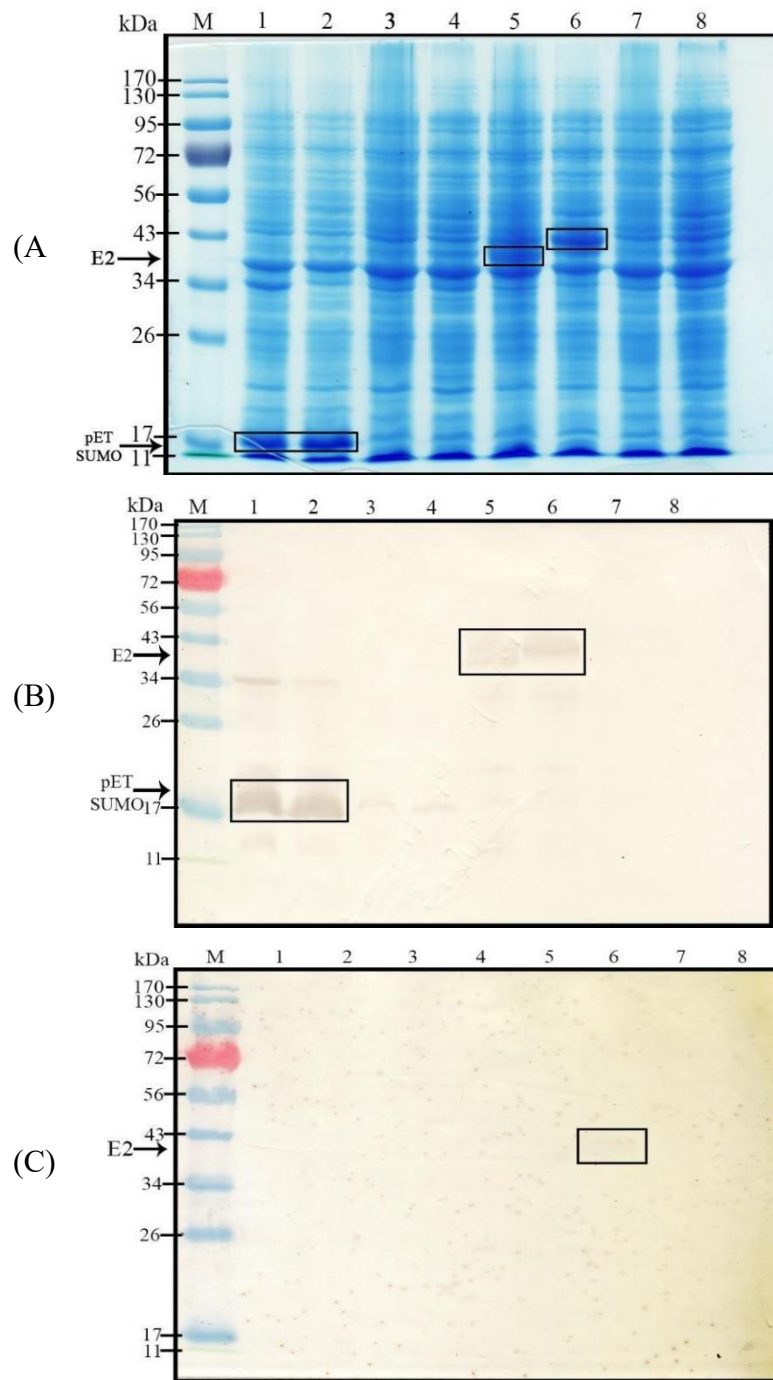


Figure 4.7: Expression of CHIKV E2 Protein under Different Conditions (COL5). Lane 1: pET SUMO, induced, non-reducing, Lane 2: pET SUMO, induced, reducing, Lane 3: pET SUMO, uninduced, non-reducing, Lane 4: pET SUMO, uninduced, reducing, Lane 5: CHIKV E2 (COL5), induced, non-reducing, Lane 6: CHIKV E2 (COL5), induced, reducing, Lane 7: CHIKV E2 (COL5), uninduced, non-reducing, Lane 8: CHIKV E2 (COL5), uninduced, reducing. (A) CBB-stained gel (B) Western blot probed with Nickel-HRP (C) Western blot probed with CHIKV positive serum (K336). M: molecular weight marker.

4.3 Purification of CHIKV E2 Protein

4.3.1 Protein Purification

The E2 protein selected as the target protein for ligand screening in this study is a structural protein involved in the mediation of virus-cell receptor attachment (Simizu et al., 1984; Strauss et al., 1991). The purification of the recombinant E2 protein and pET SUMO vector was performed using the Probond Purification System (Novex, CA, USA) as described in Section 3.3.2.10. The purification of rE2 protein was done twice, from 8 ml and 16 ml of cell lysate. The purified recombinant proteins were analyzed on SDS-PAGE. Figure 4.8A and Figure 4.8B show the purification profile of the 8 ml and 16 ml cultures respectively. Bands with the expected size of purified rE2 protein of 35 kDa were clearly observed on the CBB-stained gels. A band of 13 kDa for pET SUMO is also detected on the gel in Figure 4.9. However, the elution pET SUMO was contaminated with rE2 protein due to reusing of the purification column. This preparation of pET SUMO protein had to be discarded and the purified vector protein used for the subsequent studies was obtained from the IHCM lab. The concentration of the purified recombinant E2 proteins was quantified before further analysis.

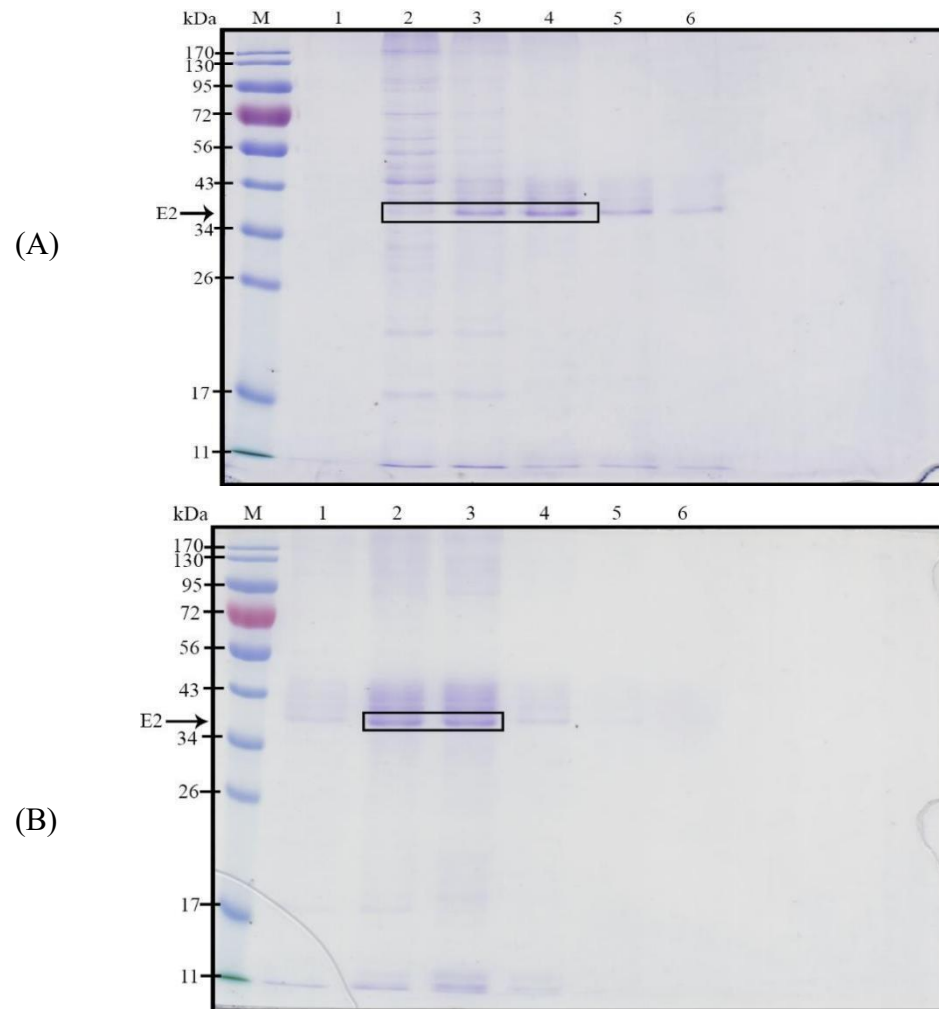


Figure 4.8: Purification of CHIKV E2 Protein. Lanes 1-6: Eluted fractions 1 to 6, M: molecular weight markers. (A) Purification of 8 ml lysate. (B) Purification of 16 ml lysate.

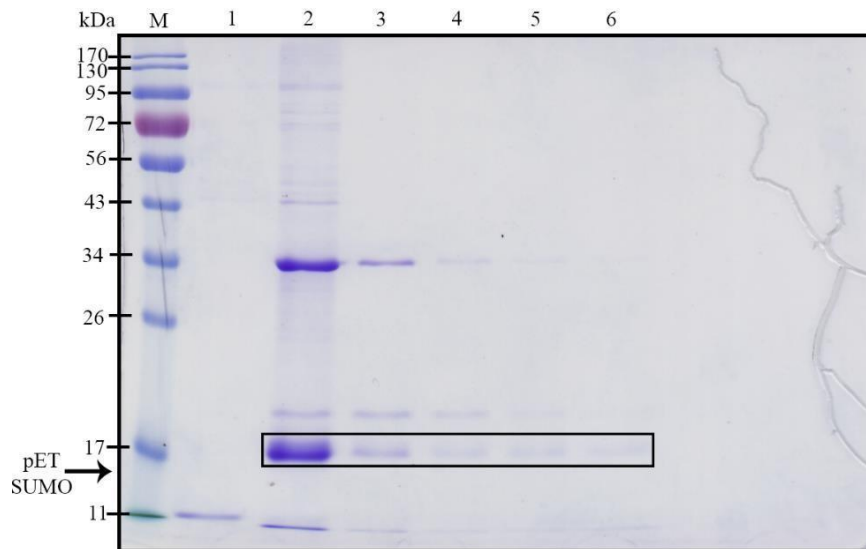


Figure 4.9: Purification of pET SUMO Protein. Lanes 1-6: Eluted fraction 1 to 6, M: molecular weight markers.

4.3.2 Protein Concentration

The concentration of the eluates of recombinant protein purification was measured as described in Section 3.3.2.10.2. The concentration of the purification of 8 ml and 16 ml cell lysate was shown in Table 4.1 and Table 4.2, respectively. Eluates 2, 3 and 4 of E2 protein purified from 8 ml of cell lysate are pooled as Sample 1. As for E2 protein purified from 16 ml of cell lysate, eluates 2 and 3 are pooled as Sample 2. The pools were dialyzed and the protein concentrations are shown in Table 4.3.

Table 4.1: Concentration of Purified CHIKV E2 Protein from 8 ml of Cell Lysate

Samples	Reading 1 (mg/ml)	Reading 2 (mg/ml)	Average reading (mg/ml)
Eluate 1	0.184	0.126	0.155
Eluate 2	0.211	0.081	0.146
Eluate 3	0.124	0.131	0.128
Eluate 4	0.052	0.011	0.032
Eluate 5	0.026	0.040	0.033
Eluate 6	0.024	0.021	0.023

Table 4.2: Concentration of Purified CHIKV E2 Protein from 16 ml of Cell Lysate

Samples	Reading 1 (mg/ml)	Reading 2 (mg/ml)	Average reading (mg/ml)
Eluate 1	0.192	0.119	0.156
Eluate 2	0.342	0.285	0.314
Eluate 3	0.223	0.225	0.224
Eluate 4	0.097	0.076	0.087
Eluate 5	0.04	0.059	0.050
Eluate 6	-0.034	0.074	0.020

Table 4.3: Concentration of Pooled Dialyzed E2 Recombinant Protein

Samples	Reading 1 (mg/ml)	Reading 2 (mg/ml)	Average reading (mg/ml)
1	0.082	0.067	0.075
2	0.194	0.170	0.182

4.4 Propagation and Maintenance of *Escherichia coli* ER2738

4.4.1 Strain Maintenance

The culture of ER2738, the host bacteria used in this study to amplify the phage provided in the library kit was streaked on LB + 20 mg/ml Tetracycline plate (Figure 4.10).

The propagation was successful and single colonies were picked and grown in LB broth to make glycerol stocks of the bacteria.



Figure 4.10: Culture Plate of ER2738

4.5 Amplification of the Phage Library (Ph.D.-7)

4.5.1 Initial Amplification and Titration of Amplified Phages

The phage library (Ph.D.-7) provided was amplified to increase the amount of the phage and to obtain the titer number. The phage was successfully amplified and was kept at 4 °C until use. The titer of the amplified phage was quantified to be 2.53×10^{11} pfu/ml, which fulfills the requirement of making sure the absence of bias in the pool. A study that utilized the same phage library used it at 2×10^{12} pfu/ml (Hou et al., 2018). The manufacturer's protocol suggests that a library with approximately 10^9 independent clones

is adequate to encode most if not all of the possible 7-mers peptide sequences (Neb, New England BioLabs). Figure 4.11 depicts the titration plate of the amplified phages.



Figure 4.11: Titration of Amplified Phages

4.6 Biopanning and Peptides Analysis

4.6.1 Biopanning against CHIKV E2 Protein

4.6.1.1 Identification of Peptides that Bind to CHIKV E2 Protein by Biopanning of a M13 Phage Display Peptide Library

In order to identify the binding peptides, a screening protocol using 7-amino acid peptide M13 peptide phage display library was utilized. The first cycle of screening of the phage peptide display library was performed by incubating the M13 phage peptide display library with E2 protein (0.2 mg/ml) immobilized on immunotubes, followed by amplifying the bound phages after washing off unbound ones. For each of the subsequent 2 cycles, the washing steps were performed under increasing concentrations of TBST (0.25% and 0.5% respectively).

The amount of input phages and output phages were determined by titration and the properties of phage recovery efficiency (output phages/input phages) in each cycle of biopanning were quantified and expressed as plaque forming unit per milliliter (pfu/ml). Following each cycle of screening, an increase in the ratio of output to input phages was observed, suggesting enrichment of phages targeting the E2 protein. Table 4.4 summarizes the enrichment of the phage peptides.

Table 4.4: Enrichment of Phage Peptides in each round of Biopanning against CHIKV E2 Protein

	1st round biopanning	2 nd round biopanning	3 rd round biopanning
E2 (μl)	100	100	100
Conc. of Tween20 (v/v)	0.10	0.25	0.51
Input (pfu/ml)	1×10^{11}	1×10^{11}	1×10^{11}
Output (pfu/ml)	1.7×10^{12}	1.7×10^{12}	3.8×10^{13}
Recovery (output/input)	1.7×10^1	1.7×10^1	3.8×10^2

As shown in Table 4.4, the recovery efficiency was 22-fold higher (from 1.7×10^1 to 3.8×10^2) after 3 rounds of panning compared to the first round, indicating that the phages specifically bound to E2 protein were successfully enriched.

4.6.1.2 Confirmation and Selection of Positive Clones

Confirmation of the plaques that successfully bound to the E2 protein was done by immunoassay. The membrane was tested against a panel of sera positive to CHIKV to confirm the expression of the plaques. The expressed plaques are clear indication that the phages had successfully bound to the E2 protein when the plaques are recognized by the positive sera. Figure 4.12 shows the expression of the plaques.

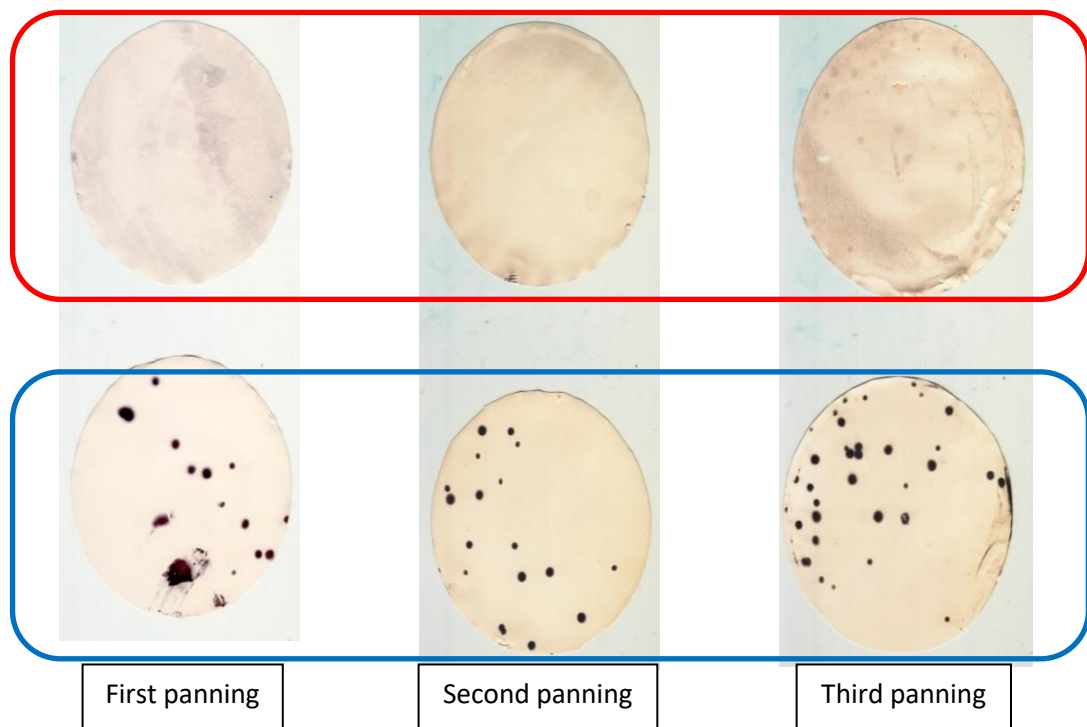


Figure 4.12: Membrane Immunoassay of CHIKV E2-Binding Plaques. The membranes in the top row were tested against a CHIKV-positive serum and the ones in the bottom row were tested against an anti-M13 antibody. The membranes on the left column are from the first panning, the middle column from second panning and the right column from the third panning.

Based on the results of the membrane immunoassay in Figure 4.12, the first and second rounds of biopanning showed negative reactivity when tested against CHIKV-positive serum because the target antigen (E2 protein) was not bound to the correct phage library. It could also be that the concentration of the bound peptides were too low to be detectable. However, the third round of biopanning showed positive reactivity against CHIKV-positive serum as the E2 protein had bound with the correct phage and the pool was enriched with the bound phage peptides. All 3 rounds of biopanning showed positive reactivity when tested with anti-M13 confirmed the presence of the phage vector. The colonies that showed positive reactivity when tested against positive serum of CHIKV were then picked and amplified.

4.6.1.3 Amino Acid Sequence of CHIKV E2-Interacting Peptides from Biopanning

The DNA of the selected positive phage colonies from Section 4.6.1.2 were extracted and sequenced. The colonies were amplified in *E. coli* ER2738 and extraction of DNA of these positive colonies was done as described in Section 3.8.1. A total of 12 individual plaques were selected and sequenced. The DNA sequencing reports showed the 7-mers peptides that were obtained from each of the positive phage clones. Table 4.5 displays the sequencing results of positive phage colonies that specifically bound to E2 protein of CHIKV.

Table 4.5: DNA Sequencing Results of the Positive Phage Colonies with Specific Binding to E2 Protein of CHIKV

Plaque names	Phage displayed peptide DNA sequence	Phage displayed peptide amino acid sequence
C1	TTTAATGCTGCGTTGGGGACTGGTGGAGGT	FNAALGT
C2	TTTAATGATGCGTTGGGGACTGGTGGAGGT	FNDALGT
C3	CCTTCGTATTAGAATAAGGCTGGTGGAGGT	PSYNKA
C4	TTTAAAGCTGCGTTGGGGAATGGTGGAGGG	FKAALGT
C5	TTTAATGCTGCGTTGGGGACTGGTGGAGGT	FNAALGT
C6	TTTAATGCTGCGTTGGGGACTGGTGGAGGT	FNAALGT
C7	TTTAATGCTGCGTTGGGGACTGGTGGAGGT	FNAALGT
C8	TTTAATGCTGCGTTGGGGACTGGTGGAGGT	FNAALGT
C9	CATTCGTATTAGAATAAGGCTGGTGGAGGT	HSYNKA
C10	TTTAATGCTGCGTTGGGGACTGGTGGAGGT	FNAALGT
C11	CATTCGTATTAGAATAAGGCTGGTGGAGGT	HSYNKA
C12	CATTCGTATTAGAATAAGGCTGGTGGAGGT	HSYNKA

Three of the 12 phages contained the identical peptide sequence HSYNKA, 6 phages contained identical peptide sequence FNAALGT, and the remaining 3 carried the peptide sequence PSYNKA, FNDALGT and FKAALGT respectively. The peptide amino acid sequence from the positive colonies was then analyzed using an alignment software to

```

Reference sequence (1): C9
Identities normalised by aligned length.
Colored by: identity

      cov      pid 1 [      ] 9
1 C9      100.0% 100.0% HSYNKA---
2 C11     100.0% 100.0% HSYNKA---
3 C12     100.0% 100.0% HSYNKA---
4 C3      100.0%  83.3% PSYNKA---
5 C4       66.7%  14.3% --FKAALGT
6 C1       66.7%  28.6% --FNAALGT
7 C5       66.7%  28.6% --FNAALGT
8 C6       66.7%  28.6% --FNAALGT
9 C7       66.7%  28.6% --FNAALGT
10 C8      66.7%  28.6% --FNAALGT
11 C10     66.7%  28.6% --FNAALGT
12 C2      66.7%  28.6% --FNDALGT
consensus/100% ..aPtA...
consensus/90%  ..aNTA...
consensus/80%  ..aNTA...
consensus/70%  ..aNTA...

```

[MView 1.63](#), Copyright © 1997-2018 [Nigel P. Brown](#)

Figure 4.13: Sequence Alignment of CHIKV E2-Binding Phage Colonies

identify the major motif. Figure 4.13 depicts the results of the alignment by using the CLUSTAL MSA software by MUSCLE. Three major motif were detected from the alignment of the 12 phages. A BLAST search in the NCBI database showed that these sequences shared no homology with known occurring peptides or proteins.

The phages were grouped based on their similarity in sequence. Phages C3, C9, C11, and C12 which are grouped as CHIKV 1 shared a motif of SYNKA. Meanwhile, phages C1, C2, C4, C5, C6, C7, C8, and C10 that had overlapping consensus sequence of AALGT are grouped under CHIKV 2. Phages C1, C2, C5, C6, C7, C8, and C10 are clustered under CHIKV 3 and bear NAALGT overlapping consensus sequence. From the results obtained, 3 major motifs were found to be potential peptide ligands of E2 protein of CHIKV. The motif of NAALGT was chosen to be synthesized as it contains a longer peptide sequence and 50% of the selected phages carried overlapping repetitive peptide sequence of NAALGT. Table 4.6 shows the grouping of the phages.

Table 4.6: Summary of Potential Major Motif Peptides

Groups	Plaques name	Potential major motif
CHIKV 1	C9, C11, C12, C3	SYNKA
CHIKV 2	C4, C1, C5, C6, C7, C8, C10, C2	AALGT
CHIKV 3	C1, C5, C6, C7, C8, C10	NAALGT

4.6.1.4 Summary of Biopanning against E2 CHIKV

The results obtained indicate that the synthetic peptide found in this study has led to the discovery of target unrelated peptides (TUPs). However, further studies are needed to confirm the peptides found in this study are target unrelated peptides.

Displayed peptides are divided into 3 main classifications: a substantial majority of peptides that do not bind to the target; a large number of peptides that bind non-specifically to the target; and small populations of peptides that bind specifically to the target (Lindner et al., 2011). Based on the DNA sequencing result, it is revealed that the displayed peptides

for E2 fall under the second category where a large number of peptides bind non-specifically to the target.

Target unrelated peptides may be enriched because of their binding to constant regions of the screening antibody or to common contaminants in the target antibody sample such as bovine immunoglobulins (Menendez & Scott, 2005). Selection-related TUPs is a category of TUPs that bind to contaminants in the target sample or components of the screening system instead of the target. Immobilization of the target structure is one of the crucial parameters that impacts TUP selection. The most frequently used approach for immobilization is direct adsorption of the target to the surface of microtiter plate wells or petri dishes (Adey et al., 1995). Blocking agents such as bovine serum albumin (BSA) and powdered skimmed milk are commonly used to coat the immobilized surface during biopanning. These blocking agents are utilized to hinder the interaction between the library phages and the surface of the solid phase, hence decreasing the possibility of selecting false-positive peptides. However, such blocking agents may be mistaken as bait and peptides binding to these blocking agents are favored during selection (Vodnik et al., 2011). This could explain the enrichment of the NAALGT peptide although there is no data available and further studies are needed to support this hypothesis.

4.6.2 Biopanning against ZIKV EDIII Protein

4.6.2.1 Identification of Peptides that Bind to ZIKV E2 Protein by Biopanning of a M13 Phage Display Peptide Library

Similar to CHIKV, a phage-displayed hepta-peptides library was employed to identify the ligands that bind to the EDIII protein of ZIKV. As described previously, EDIII protein (0.5 mg/ml) was immobilized on immunotubes and allowed to react with M13 phage display library. Unbound phages were removed by washing and the bound phages

were amplified. For each of the subsequent 2 rounds of biopanning, the washing steps were performed at increasing higher concentrations of TBST (0.25% and 0.5% respectively).

In each round of biopanning, a significant enrichment in the number of bound phages was determined by titration and the properties of phage recovery efficiency (output phages/input phages) was calculated and expressed as plaque forming unit per ml (pfu/ml). Following each round of panning, an increase in the ratio of output to input phages was observed, suggesting enrichment of phages targeting the EDIII protein. Table 4.7 shows the enrichment of the of the phage peptides.

Table 4.7: Enrichment of Phage Peptides from each round of Biopanning against of ZIKV EDIII Protein

	1st round biopanning	2 nd round biopanning	3 rd round biopanning
EDIII (μ l)	25	25	25
Conc. of Tween20 (v/v)	0.10	0.25	0.51
Input (pfu/ml)	1×10^{11}	1×10^{11}	1×10^{11}
Output (pfu/ml)	6.6×10^{12}	6.8×10^{13}	7.2×10^{13}
Recovery efficiency (output/input)	6.6×10^1	6.8×10^2	7.2×10^2

A significant enrichment in the number of bound phages and increased recovery efficiency was observed in each round of biopanning. Individual phage plaques from the final enriched population were selected and the sequence of the displayed peptides was determined. As shown in Table 4.7, the recovery efficiency was 11 folds higher (from 6.6×10^1 to 7.2×10^2) after 3 rounds of panning, suggesting that the phages specifically bound to EDIII protein were successfully enriched.

4.6.2.2 Confirmation and Selection of Positive Clones

Confirmation of the plaques that successfully binds to the EDIII protein was done by immunoassay. The membranes were tested against a panel of ZIKV serum to check the expression of the plaques. Due to limited volume of sera available in the laboratory, only plaques from the third round of panning were tested against selected ZIKV sera. The membranes were tested against HPR (dengue-positive sera pool), 4G2 (pan-flavivirus monoclonal antibody against E protein), DKB/25/1 (serum potentially positive for ZIKV), and DKB/8/16/1 (recently detected ZIKV-positive serum). Figure 4.14 depicts the results of the immunoassay.

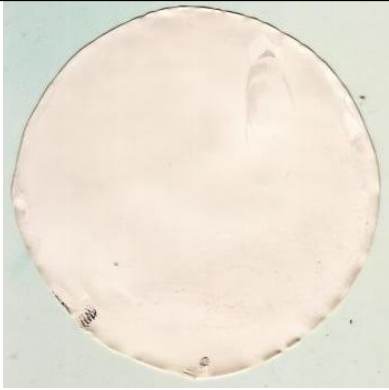
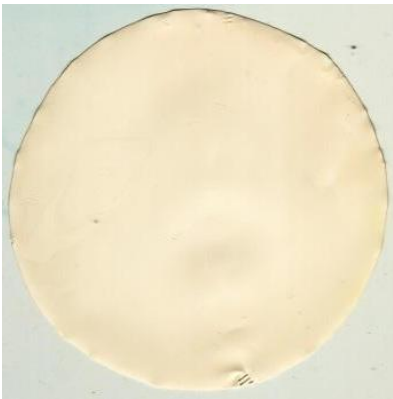
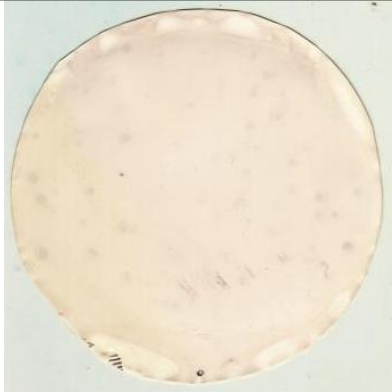
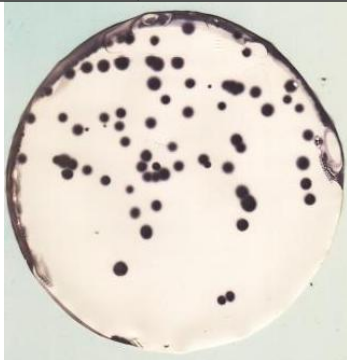
	
HPR	4G2
	
DKB/25/1	DKB/8/16/1
	
M13	

Figure 4.14: Membrane Immunoblot of ZIKV EDIII Protein-Binding Phages

Based on the results of the membrane immunoassay in Figure 4.14, only a single serum namely DKB/8/16/1 showed a weak positive reactivity while the other sera showed no activity. This proves that the plaques were successfully bound to the EDIII of ZIKV in the third round of panning. A positive reactivity when tested against anti-M13 was also obtained. The individual phage plaques that showed positive reactivity when tested against DKB/8/16/1 were selected and amplified.

4.6.2.3 Amino Acid Sequence of Displayed Peptides Isolated from Biopanning

The DNA of the selected positive phage colonies were extracted and sequenced. The colonies were amplified in the *E. coli* strain ER2738 and DNA extraction was performed as described in Section 3.8.1. The extracted DNA was sent for sequencing to determine the sequence of the displayed peptides. A total of 26 individual colonies were selected and sequenced. The DNA sequencing reports showed that 7-mers peptides were obtained from each of the positive phage colonies. Table 4.8 below shows the sequencing results of positive phage colonies that specifically bound to EDIII protein of ZIKV.

Table 4.8: DNA Sequencing Results of the Positive Phage Colonies that Specifically Bind to ZIKV EDIII Protein

Plaque names	Phage displayed peptide DNA sequence	Phage displayed peptide amino acid sequence
Z1	AATGATCTTCCGCGTAGGACTGGTGGAGGT	NDLPRRT
Z2	GGGTCGCCTCCGCGTACGCCTGGTGGAGGT	GSPPRTP
Z3	GTGAGGCCTCATACTTCTTCGGGTGGAGGT	VRPHTSS
Z4	TTGCCTTCTAATACTTCGAGTGGTGGAGGT	LPSNTSS
Z5	GCGAATACTCTGCGTTCTCCGGGTGGAGGT	ANTLRSP
Z6	TCTATTCTGCCGTATCCTTATGGTGGAGGT	SILPYPY
Z7	ACGCCTTGCCGCGTCTTAATGGTGGAGGT	TPWPRLN
Z8	AATCTTAATAATACTAGGCTGGGTGGAGGT	NLNNTRL
Z9	CATCTTACGTGGTTGCCGGATGGTGGAGGT	HLTWLPD
Z10	CTGCCGTTGACTCCGCTTCCGGGTGGAGGT	LPLTPLP
Z11	ACGATTACTAATCCGCGGCCTGGTGGAGGT	TITNPRP
Z12	TATCTTACGATGCCGACGCCTGGTGGAGGT	YLTMPPTP
Z13	ACGTTGACTACGCTGACGAATGGTGGAGGT	TLTTLTN
Z14	GCGGCGTGCCAGATTCATAGTGGTGGAGGG	AAWQIHS
Z15	GAGCCGCTGCAGCTGAAGATGGGTGGAGGT	EPLQLKM
Z16	ATTCAGTCTCCTCATTTTTTTTGGTGGAGGT	IQSPHFF
Z17	ATTCAGTCTCCTCATTTTTTTTGGTGGAGGT	IQSPHFF
Z18	AATCAGGATGTGCCGCTTTTTTGGTGGAGGT	NQDVPLF
Z19	AATCAGGATGTGCCGCTTTTTTGGTGGAGGT	NQDVPLF
Z20	TATGCTGGTCCTTATCAGCATGGTGGAGGT	YAGPYQH
Z21	ACTTTTGCTAAGTCTGCGTATGGTGGAGGT	TFAKSAY
Z22	GATAGGATGTCTGTTCGTA CTGGTGGAGGT	DRMSVRT
Z23	G TTCAGACTTATGCTCGTGT TGGTGGAGGT	VQTYARV
Z24	CATGCTATTTATCCGCGTCATGGTGGAGGT	HAIYPRH
Z25	CATGCTATTTATCCGCGTCATGGTGGAGGT	HAIYPRH
Z26	TCGACGGCGTCTTATACTCGTGGTGGAGGT	STASYTR

Based on the sequencing results, the phage colonies showed inconsistency in the peptide sequences, which is unlike the case of CHIKV. However, alignment of the peptide sequences reveals that 3 major motifs were found to bear overlapping consensus sequence of IQSPHFF, NQDVPLF and HAIYPRH. Figure 4.15 depicts the results of the alignment by using the alignment software CLUSTAL MSA by MUSCLE.

		cov	pid	1 [.] 16
1	Z1	100.0%	100.0%	---NDLERRT-----
2	Z27	85.7%	42.9%	---GSPERTP-----
3	Z12	71.4%	14.3%	---VRPHITSS-----
4	Z36	71.4%	42.9%	---LPSNTSS-----
5	Z4	85.7%	28.6%	--ANTLRSP-----
6	Z23	100.0%	28.6%	---SILPYPY-----
7	Z34	71.4%	14.3%	---TWPRLN-----
8	Z33	71.4%	14.3%	-NLNTRL-----
9	Z24	100.0%	0.0%	---HLTWLPD-----
10	Z25	85.7%	14.3%	--LPLTELP-----
11	Z39	85.7%	0.0%	---TITNPRP-----
12	Z26	85.7%	28.6%	---YITMPITP-----
13	Z20	71.4%	14.3%	-TLTTLTN-----
14	Z11	14.3%	0.0%	-----AAWQIHS
15	Z37	14.3%	0.0%	-----EPLQLKM
16	Z8	42.9%	0.0%	-----IQSPHFF--
17	Z22	42.9%	0.0%	-----IQSPHFF--
18	Z30	57.1%	0.0%	-----NQDVPLF--
19	Z35	57.1%	0.0%	-----NDQVPLF--
20	Z29	42.9%	0.0%	-----YAGPYQH--
21	Z28	57.1%	0.0%	-----TFAKSAY--
22	Z10	57.1%	0.0%	DRMSVRT-----
23	Z5	85.7%	0.0%	---VQTYARV-----
24	Z31	85.7%	0.0%	---HAIYPRH-----
25	Z38	85.7%	0.0%	---HAIYPRH-----
26	Z21	100.0%	0.0%	---STASYTR-----
	consensus/100%			
	consensus/90%			
	consensus/80%			
	consensus/70%			

Figure 4.15: Sequence Alignment of ZIKV EDIII Protein-Binding Peptides

The sequences of Phages Z16 and Z17 share a motif of IQSPHFF and were grouped as ZIKV1. Phage Z18 and Z19 were grouped under ZIKV2 with the overlapping consensus sequence of NQDVPLF. A third group of ZIKV3 with Phages Z24 and Z25 as members bears the repetitive peptide sequence HAIYPRH.

Table 4.9: Summary of Potential Major Motif Peptides

Groups	Plaques name	Potential major motif
ZIKV 1	Z16, Z17	IQSPHFF
ZIKV 2	Z18, Z19	NQDVPLF
ZIKV 3	Z24, Z25	HAIYPRH

4.6.2.4 Summary of Biopanning against EDIII

In summary, IQSPHFF, NQDVPLF and HAIYPRH were identified as possible peptides that interact with EDIII protein of ZIKV. Each of the groups carries 7% of the possible peptides. However, these peptides were not selected for further synthesis due to very low percentage of overlapping repetitive peptide sequence that would lead to a low percentage of successful findings.

Interestingly, several laboratories that conducted similar studies by using different target and different screening systems have reported the discovery of phage-displayed peptide HAIYPRH from the Ph.D.-7 library. This suggests that the phage displaying HAIYPRH is a target-unrelated peptide. A study by Brammer and colleague (2007) demonstrated that after serial amplification of the library, phage displaying HAIYPRH is enriched in the absence of introduction to the target. Furthermore, the same study found that the phage displaying HAIYPRH was discovered to be amplified dramatically faster

than the library itself. DNA sequencing revealed that there was a mutation in the Shine-Dalgarno (SD) sequence for gIIp (van Wezenbeek, Hulsebos & Schoemakers, 1980). gIIp is a protein that participates in phage replication (Webster, 2001; Horiuchi, 1997; Model & Rusel, 1988). As a consequence, the introduction of this mutation into a phage lacking a displayed peptide causes the propagation to accelerate. The mutation of SD sequence may change the expression of gIIp and as a result the propagation rate of phage is altered (Webster, 2001; Horiuchi, 1997; Model & Rusel, 1988). The study by Brammer and coworkers (2007) also offers proof that phage displaying the peptide HAIYPRH in the Ph.D.-7 library multiplies at a rapid rate compared to the most of phages in the library.

A type of the TUPs where fast-growing phage clones beat slow-growing ones is known as propagation-related TUPs. Interestingly, the high-throughput deep sequencing of clones from Ph.D.-7 uncovered that out of all the entire peptide sequences, more than one-fourth showed frequency higher than the theoretically estimated frequency for peptides of the library (Matochko et al., 2014). With an increase in growth rate, these oversupplied peptides are proposed to be presented on the surface of phage clones (Brammer et al., 2008; Nguyen et al., 2014). Aside from these overabundant peptides, there are clones that propagate fast in the library, known as parasitic clones (Matochko et al., 2014). Rivalry among phage clones that are present in a usual pool of bacterial cells are found to be triggered by different growth rates of different phage clones. A significant set of proof proposes that major contrasts in their growth advantage are led by the minor differences in the growth rate of phage clones of the library. Sequentially, huge differences in the concentration of phage clones are noticed after amplification (Derda et al., 2011). Hence, at the end of the phage display screening, fast-propagating phages are much more likely to come into view (Lindner et al., 2011).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Overall, the main objective of this study of identifying peptides interacting to CHIKV and ZIKV recombinant proteins was successfully achieved. The E2 protein of CHIKV and EDIII of ZIKV were successfully cloned, expressed and purified. In this study, a total of 12 and 26 phage clones were amplified for potential binding with the recombinant CHIKV and ZIKV proteins respectively. The sequence analysis of the positive clones showed that, the selection with CHIKV recombinant protein generate a potential motif, NAALGT, in which 6 out of the 12 amplified clones contained the repetitive motif. Further study may be carried out to further analyse the characteristic of these specific peptides in inhibiting viral infectivity and in eliciting immune-protection. However, the selection with ZIKV protein, did not show any repetitive motif sequence, the interacting peptide was further analyzed using CLUSTAL MSA software by MUSCLE suggests that these to be target-unrelated peptides (TUPs).

5.2 Future Recommendations

The potential binding peptides especially with the recombinant CHIKV protein can be further analysed for binding analysis in binding assay such as ELISA. The antiviral activity of the positive phage clones and the synthetic peptides can also be analysed by plaque reduction assay. The binding of the synthetic peptides to the antigen can be analysed in a pull-down assay.

The outcome of this study appears to be the discovery of TUPs. In the future, this study can be repeated with strategies to prevent TUP enrichment during biopanning. One

of the methodical tactics to reduce non-specific TUPs in phage display screening is by applying subtractive or negative biopanning. Through subtractive biopanning, a reduction in the phage number of the library can lower the background of unwanted TUPs. Other techniques could also be utilized to perform subtractive screening. For instance, if the target is an immobilized molecule, to immobilize the target the library is exposed to affinity chromatography.

Recognizing TUPs could be difficult and challenging. The binding assays that are used to confirm the affinity of peptides for the target usually employ the same components as the initial panning process. These include using the same molecule as the solid phase and capturing agent. There are cases where peptides are found to not actually have a strong affinity towards the target through control and binding competition (Menendez & Scott, 2005). Through this approach, TUPs are successfully recognized.

The reduction of noise and unwanted background is essential for a successful phage display selection. However, both positive and negative results make a remarkable contribution to our comprehension regarding the relationship between structure and function. The significance of negative result is that they help us learn about the range of conditions when specific structures in the ligand are malfunctioning (Matochko & Derda, 2013). Therefore, loss-of-function archetype may be understood in the framework of nonspecific binding peptides.

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APPENDICES

APPENDICES A: MEDIA, ANTIBIOTICS, REAGENTS AND SOLUTIONS

A.1 Small scale protein expression

100mM IPTG – 20 ml

Isopropylthio- β -D-galactosidase (Promega, USA) 0.476 g

UHQ water 20 ml

The solution was sterilized by positive pressure filtration through 0.22 μ l cellulose acetate filter (Milipore Corp, Bedford, MA, USA) and aliquoted into 1.5 ml tubes and stored at -20 °C until used.

A.2 Protein purification

Stock solution A: 0.1M Citric Acid

Citric acid 21.01 g

R.O water Top up to 1 L

Stock solution B: 0.2M disodium hydrogen phosphate, heptahydrate)

Na₂HPO₄·7H₂O 53.65 g

R.O water Top up to 1 L

Guanidium lysis buffer – 100 ml

UHQ water	60 ml
Stock solution A (10X)	0.58 ml
Stock solution B (10X)	9.42 ml
Guanidine hydrochloride	57.3 g

The solution was stirred until completely dissolved and the pH was adjusted to 7.8.

Native binding buffer – 100 ml

Sterile UHQ water	80 ml
5X native purification buffer	20 ml

The solution was mixed well and the pH was adjusted to 8.0.

Denaturing binding buffer – 100 ml

UHQ water	60 ml
Stock solution A (10X)	0.58 ml
Stock solution B (10X)	9.42 ml
Urea	48.1 g

The solutions were stirred with gentle heating (50 °C to 60 °C) until completely dissolved.

When cooled to room temperature, the pH was adjusted to 7.8.

A.3 Media and antibiotics used in strain maintenance of ER2738

Luria-Bertani (LB) medium – 1 Litre

Tryptone	10 g
Yeast extract	5 g
Sodium chloride (NaCl)	10 g

Dissolved all the powder above with UHQ water and the volume was adjusted to 1 L. The media was autoclaved and stored at 4°C until used. For the preparation of LB-antibiotic agar plate, 15g of bacto-agar were added and dissolved together with the powder above and was then autoclaved. The autoclaved LB agar was cooled to 50°C before adding antibiotic. The agar was immediately poured into sterile petri dishes.

Tetracycline (20mg/ml) – 20ml

Tetracycline	0.4 g
Ethanol	20 ml

The solution was sterilized by positive pressure filtration through 0.22 µm cellulose acetate filter (Milipore Corp, Bedford, MA, USA) and was kept in dark bottle at -20°C.

50% glycerol – 100 ml

Glycerol	50 ml
UHQ water	50ml

Mixed both of the solutions and autoclaved before used. Stored at room temperature.

A.4 Media and antibiotics used in initial amplification of the phage library

Luria-Bertani (LB) medium – 1 Litre

Tryptone	10 g
Yeast extract	5 g
Sodium chloride (NaCl)	10 g

Dissolved all the powder above with UHQ water and the volume was adjusted to 1 L. The media was autoclaved and stored at 4°C until used. For the preparation of LB-antibiotic agar plate, 15g of bacto-agar were added and dissolved together with the powder above and was then autoclaved. The autoclaved LB agar was cooled to 50°C before adding antibiotic. The agar was immediately poured into sterile petri dishes.

TBS (0.05M Tris, , 0.145M NaCl) pH7.6

Tris	6.055 g
NaCl	8.520 g

Tris and sodium chloride were first dissolve in 500 ml RO water. pH of the solution was adjusted to 7.6 with 1M HCl and finally the volume was top up to 1 Liter with RO water.

30% PEG/2.5 NaCl solution – 100 mL

polyethylene glycol-8000	20 g
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NaCl	14.6 g
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PEG-8000 and NaCl was first dissolve in 100 mL by stirring. Autoclaved and mix well to combine separated layers while still warm. Stored at room temperature.

A.5 Media and antibiotics used in titration

Luria-Bertani (LB) medium – 1 Litre

Tryptone	10 g
Yeast extract	5 g
Sodium chloride (NaCl)	10 g

Dissolved all the powder above with UHQ water and the volume was adjusted to 1 L. The media was autoclaved and stored at 4°C until used. For the preparation of LB-antibiotic agar plate, 15g of bacto-agar were added and dissolved together with the powder above and was then autoclaved. The autoclaved LB agar was cooled to 50°C before adding antibiotic. The agar was immediately poured into sterile petri dishes.

Top Agar – 1 Litre

Bacto-Tryptone	10 g
Yeast extract	5 g
Sodium chloride (NaCl)	5 g
Bacto-agar	7 g

Dissolved all the powder above with UHQ water and the volume was adjusted to 1 L. The media was autoclaved. The media was dispensed into 50 mL aliquots. Stored at room temperature and melt in microwave as needed.

IPTG (40 mg/ml) – 20ml

Isopropylthio- β -D-galactosidase (Promega, USA)	0.8 g
UHQ water	20 ml

The solution was sterilized by positive pressure filtration through 0.22 μ m cellulose acetate filter (Milipore Corp, Bedford, MA, USA) and was kept in dark bottle at -20°C.

Xgal (20 mg/ml) – 20ml

Xgal	0.4 g
N,Ndimethylformaldehyde	20 ml

The solution was sterilized by positive pressure filtration through 0.22 μ m cellulose acetate filter (Milipore Corp, Bedford, MA, USA) and was kept in dark bottle at -20°C.

LB/IPTG/Xgal agar plate

Tryptone	10 g
Yeast extract	5 g
Sodium chloride (NaCl)	10 g
IPTG/Xgal stock	1 ml

Dissolved all the powder above with UHQ water and the volume was adjusted to 1 L. The media was autoclaved and stored at 4°C until used. For the preparation of LB/IPTG/Xgal agar plate, 15g of bacto-agar were added and dissolved together with the powder above and was then autoclaved. The autoclaved LB agar was cooled to 50°C before adding 1ml of IPTG/Xgal stock. The agar was immediately poured into sterile petri dishes.

A.6 Media used in biopanning

Luria-Bertani (LB) medium – 1 Litre

Tryptone	10 g
Yeast extract	5 g
Sodium chloride (NaCl)	10 g

Dissolved all the powder above with UHQ water and the volume was adjusted to 1 L. The media was autoclaved and stored at 4°C until used.

TBS (0.05M Tris, , 0.145M NaCl) pH7.6

Tris	6.055 g
NaCl	8.520 g

Tris and sodium chloride were first dissolve in 500 ml RO water. pH of the solution was adjusted to 7.6 with 1M HCl and finally the volume was top up to 1 Liter with RO water.

30% PEG/2.5 NaCl solution – 100 mL

polyethylene glycol-8000	20 g
NaCl	14.6 g

PEG-8000 and NaCl was first dissolve in 100 mL by stirring. Autoclaved and mix well to combine separated layers while still warm. Stored at room temperature.

A.7 Media used in screening and amplification of positive colonies

Luria-Bertani (LB) medium – 1 Litre

Tryptone	10 g
Yeast extract	5 g
Sodium chloride (NaCl)	10 g

Dissolved all the powder above with UHQ water and the volume was adjusted to 1 L. The media was autoclaved and stored at 4°C until used.

TBS (0.05M Tris, , 0.145M NaCl) pH7.6

Tris	6.055 g
NaCl	8.520 g

Tris and sodium chloride were first dissolve in 500 ml RO water. pH of the solution was adjusted to 7.6 with 1M HCl and finally the volume was top up to 1 Liter with RO water.

Chromogenic substrate containing 4-chloro-1-naphtol in methanol/H₂O₂ substrate (600ml)

Substrate A

4-chloro-1-naphtol (SIGMA, St. Louis, USA)	0.75g
Methanol	200ml
*1X TBS (0.05 M Tris, 0.145M NaCl) pH7.6	400ml

Dissolve 4-chloro-1-naphtol with methanol and then add 1X TBS

Substrate B

1X TBS (0.05M Tris, , 0.145M NaCl) pH7.6	600ml
Hydrogen peroxide	1250µl

Mix the two solutions well in a bottle.

Equal volume of Subtrate A and Substrate B were used as a chromogenic substrate substrate in each reaction.

30% PEG/2.5 NaCl solution – 100 mL

polyethylene glycol-8000	20 g
NaCl	14.6 g

PEG-8000 and NaCl was first dissolve in 100 mL by stirring. Autoclaved and mix well to combine separated layers while still warm. Stored at room temperature.

A.8 Media used in SDS-PAGE

5X running buffer – 1 litre

Tris	15 g
Glycine	72 g
SDS	5 g

Dissolve the chemicals above with RO water and adjusted to 100 ml. The buffer was kept at room temperature and was dilute to 1X with RO water before used.

Acrylamide/bis solution – 500 ml

Acrylamide (Promega)	146 g
N’N’-bis-methylene-acrylamide (BIORAD)	4 g

Dissolved the powders with RO water and adjusted to a final volume of 500 ml. The solution was filtered through filter paper (whatman, Clifton, NJ, USA) and kept in a dark bottle at room temperature.

Resolving buffer (1.5 M Tris-HCl, pH 8.8) – 250 ml

Tris base	45.375 g
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Tris base was dissolved with 200 ml RO water and the pH was adjusted to pH 8.8 with 3M HCl, the final volume was adjusted to 250 ml. The buffer was kept at room temperature until used.

Stacking buffer (1.5 M Tris-HCl, pH 6.8)

Tris base	6 g
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Tris base was dissolved with 80 ml RO water and the pH was adjusted to pH 6.8 with 3M HCl, the final volume was adjusted to 100 ml. The buffer was kept at room temperature until used.

10% APS – 10 ml

Ammonium persulphate	1 g
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Was dissolved with 10 ml RO water and stored at 4°C until used.

10% APS – 100 ml

Sodium dodecyl sulphate	10 g
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Dissolved in RO water and adjusted the volume to 100 ml.

Coomassie brilliant blue staining – 1 litre

Coomassie brilliant blue (G250)	1 g
Methanol	400 ml
Glacial acetic acid	100 ml
RO water	500 ml

Dilute coomassie blue powder with RO water before adding methanol and glacial acetic acid. To prevent precipitation, the solution was filtered through filter paper (whatman, Clifton, NJ, USA) before used.

Destaining solution – 1 litre

Methanol	400 ml
Glacial acetic acid	100 ml
RO water	500 ml

Mixed well all the solution and stored at room temperature until used.

4X non-reducing sample buffer – 9.5 ml

RO water	3.55 ml
0.5 M Tris HCl, pH 6.8 (stacking gel buffer)	1.25 ml
Glycerol (amresco)	2.5 ml
10 % SDS	2.0 ml
0.5% bromophenol blue	0.2 ml

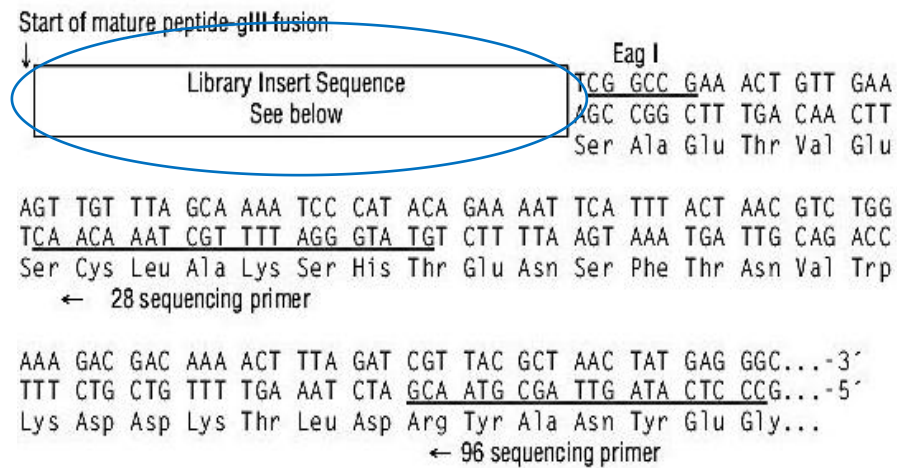
All solutions were mixed well and kept at 4 °C until used.

4X reducing sample buffer

50 µl of 2-mercaptoethanol (Merck) was added to 950 µl of 4X non-reducing sample buffer.

APPENDIX B: PHAGE DISPLAY LIBRARY INSERT

B.1 Library insert sequence



Library Insert Sequences

Ph.D.-7: (NNK)₇ GGT GGA GGT
(NNM)₇ CCA CCT CCA
Xxx₇ Gly Gly Gly

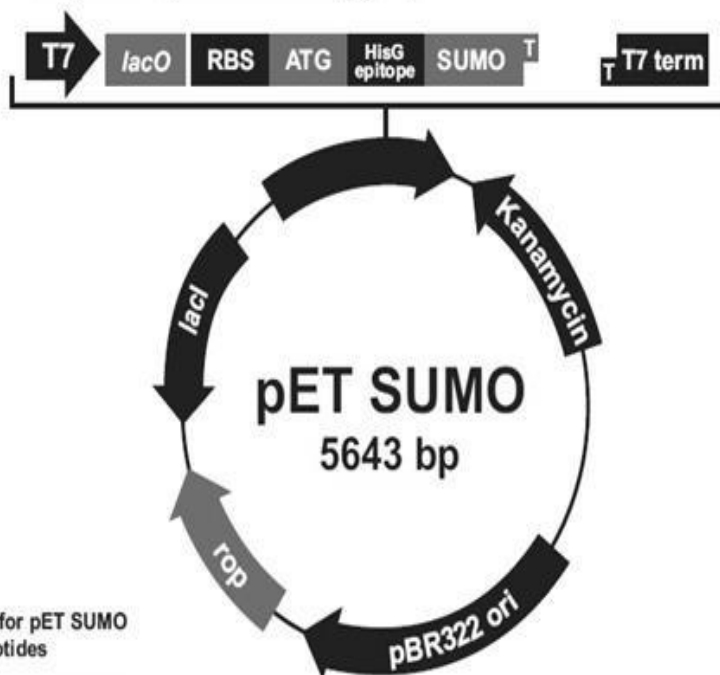
Sequence of random peptide library-gIII fusion. Adapted from Ph. D. Phage Display Manuals (BioLabs, New England)

APPENDIX C: VECTOR MAP AND FEATURES

C.1 Map and Features of pET SUMO

Map

The figure below shows the features of the pET SUMO vector. The vector is supplied linearized between nucleotides 653 and 654 with a one base pair 5' T-overhang on each strand as indicated. The complete sequence of pET SUMO is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 34).



Comments for pET SUMO 5643 nucleotides

T7 promoter: bases 209-225
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288
Initiation ATG: bases 297-299
HisG epitope: bases 309-329
SUMO ORF: bases 360-653
SUMO forward priming site: bases 549-571
TA Cloning site: bases 653-654
T7 reverse priming site: bases 783-802 (C)
T7 terminator: bases 744-872
Kanamycin resistance gene: bases 1431-2246 (C)
pBR322 origin: bases 2342-3015
ROP ORF: bases 3383-3574
lacI ORF: bases 4383-5474 (C)

(C) = complementary strand