Molecular Design and Functional Characterization Portfolio of Flavivirus Therapeutics

by

Jonathan Hurtado

A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved April 2014 by the
Graduate Supervisory Committee:

Qiang Chen, Chair
Holly Huffman
Kelly Steele

ARIZONA STATE UNIVERSITY

May 2014

ABSTRACT

Flavivirus infections are emerging as significant threats to human health around the globe. Among them West Nile(WNV) and Dengue Virus (DV) are the most prevalent in causing human disease with WNV outbreaks occurring in all areas around the world and DV epidemics in more than 100 countries. WNV is a neurotropic virus capable of causing meningitis and encephalitis in humans. Currently, there are no therapeutic treatments or vaccines available. The expanding epidemic of WNV demands studies that develop efficacious therapeutics and vaccines and produce them rapidly and inexpensively. In response, our lab developed a plant-derived monoclonal antibody (mAb) (pHu-E16) against DIII (WNV antigen) that is able to neutralize and prevent mice from lethal infection. However, this drug has a short window of efficacy due to pHu-E16's inability to cross the Blood Brain Barrier (BBB) and enter the brain. Here, we constructed a bifunctional diabody, which couples the neutralizing activity of E16 and BBB penetrating activity of 8D3 mAb. We also produced a plant-derived E16 scFv-CH_{1-3} variant with equivalent specific binding as the full pHu-E16 mAb, but only requiring one gene construct for production. Furthermore, a WNV vaccine based on plant-derived DIII was developed showing proper folding and potentially protective immune response in mice. DV causes severe hemorrhaging diseases especially in people exposed to secondary DV infection from a heterotypic strain. It is hypothesized that sub-neutralizing cross-reactive antibodies from the first exposure aid the second infection in a process called antibody-dependent enhancement (ADE). ADE depends on the ability of mAb to bind Fc receptors (FcγRs), and has become a major roadblock for developing mAb-based therapeutics against DV. We aim to produce an anti-Dengue mAb (E60) in different

glycoengineered plant lines that exhibit reduced/differential binding to FcγRs, therefore, reducing or eliminating ADE. We have successfully cloned the molecular constructs of E60, and expressed it in two plant lines with different glycosylation patterns. We demonstrated that both plant-derived E60 mAb glycoforms retained specific recognition and neutralization activity against DV. Overall, our study demonstrates great strives to develop efficacious therapeutics and potent vaccine candidates against Flaviviruses in plant expression systems.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my graduate committee, Dr. Qiang Chen, Dr. Holly Huffman, and Dr. Kelly Steele, for their valuable time, knowledge, and experience to help me provide the best version of my thesis possible. The thought provoking questions and suggestions during the defense have made me a better critical thinker, writer, and presenter which will allow me to continue my growth as a scientist.

I would like to highlight Dr. Qiang Chen involvement in my academic and professional career. If it was not for him I would not be in the path I am now. His support and encouragement throughout have not only made me a better scientist but also a better person. I would also like to thank him for the range information he has instilled in me varying from anecdotes to scientific lessons. His broad experience has prepared me both for career and life challenges.

Along with my graduate committee, I would like to thank my friends and lab members, Huafang Lai, Jake Stanhke, Kahlin Luezinger, and Matthew Dent for your patience in teaching me laboratory techniques and providing an excellent environment for me to work in. The long days in the lab always seem to get shorter when I was accompanied by them.

Finally, I would like to thank my family for their support and love. For my parents, your encouragement and efforts have allowed me to pursue my education to its fullest extent and accomplish my goals.

Some of the results reported in this thesis were obtained with the help of collaborators, including Huafang Lai for diabody binding analysis and yeast binding assay of pE60, Matthew Dent for purification and analysis of pE60 construct, and Dr. Michael Diamond's lab from Washington University School of Medicine for the neutralization assay of pE60.

TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER 1

OVERVIEW OF FLAVIVIRUSES

Flavivirus is one of the genera included in the *Flaviviridae* family that also includes the genera *Hepacivirus* and *Pestivirus*. Further subdivision of the *Flavivirus* genus is based on cross-neutralizing test results in eight antigenic complexes including Dengue, Japanese encephalitis, Tick-borne encephalitis, Rio Bravo, Tyuleniy, Ntaya, Uganda S, and Modoc antigenic groups (Calisher, 1989). Overall, within *Flavivirus* there are over 70 different viruses , and most noteworthy to human health include yellow fever virus (YFV), Dengue virus (DV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV) (Heinz, 2012). These viruses can cause a broad range of disease from mild discomfort to hemorrhagic fevers, flaccid paralysis and even encephalitis (Gould 2008). Other members of this genus are rarely encountered by humans and their reported case numbers are relatively small. Nevertheless, they have the potential to cause severe disease in humans (Heinz, 2012). Most of these viruses are arboviruses with YFV, DV, JEV, and WNV being transmitted by mosquitoes; and TBEV being transmitted by ticks. Humans are considered to be the "dead-end" or incidental host to these viruses, due to humans' inability to maintain high enough viremia titers to re-infect arthropod vectors. However, cases of human to human transmission, by means of blood transfusions and organ transplant have been reported (Nicole, 2012).

Flaviviruses are enveloped icosahedral viruses with a diameter of ≈500 Å and have a single positive strand RNA genome of 10.7 kb (Kuhn, 2002). The genome contains an open reading frame (ORF) that codes three structural proteins near the N-terminus along with seven non-structural proteins (NS) near the C-terminus. The three

major structural proteins encoded in the genome are the capsid protein (CP), pre-membrane/membrane protein (preM/M) and envelope protein (E). The CP (100 aa) is a dimer which surrounds positive strand RNA, housing the genome and creating the nucleocapsid core. Little is known about the preM/M protein (166/75 aa) but it is speculated to have a chaperone protein function for E protein, as well as serving as an indicator distinguishing the maturation transition from immature form to the infectious form of the viral particle (preM to M) (Zhang, 2004). The E protein (495 aa) is a dimer with each monomer made up of three domains (domain (D) I, II, and III). It is responsible for the binding of virus to cellular receptors, mediating the entry for virus, and cell membrane fusion (Chamber, 1990). DI carries out a structural rearrangement function in acidic conditions, DII is responsible for virus to cell membrane fusion due to its hydrophobic peptide region, and DIII serves as the binding site for cellular receptors with its C-terminal immunoglobulin (Ig)-like structure. The seven NS protein encoded in the genome are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. They function mainly on RNA genome replication and viral protein synthesis. Within the seven NS proteins, NS3 and NS5 are the most conserved proteins and they carry out the main functions while the rest of the NS proteins act as activators or support molecules. NS3 has bifunctional activity when coupled with NS2B, providing both protease and possibly helicase activity (Chamber, 1990; Pastorino, 2010). NS5 serves as a viral RNA polymerase with methyl-transferase activity (Chamber, 1990; Pastorino, 2010).

Flaviviruses follow a similar viral life cycle as other viruses. The cycle begins with attachment of the virion onto the surface of a host cell. This specific binding induces a receptor-mediated ($\alpha v\beta 3$ integrin and laminin-binding protein) endocytosis, granting

viral entry into the cell (Brinton, 2014). The low pH within the endosomal vesicle triggers conformational changes of the E protein, exposing Domain III. As a result, the viral and cell membranes fuse together (Thompson, 2009), nucleocapsid disassembles, and the RNA genome is released into the cytoplasm of the cell allowing both replication and translation of the genome to occur. Translation of the positive-sense RNA results in a single polyprotein with all 10 viral proteins being synthesized. Further processing incorporates host and viral proteases to cleave polyprotein into the ten mature proteins (Shi, 1996). The NS proteins (specifically NS5) construct a viral replication complex which uses the positive sense genome to generate complementary negative-sense intermediate strands (Chamber, 1990). The intermediate strands act as templates to synthesize the positive sense viral genome. Viral assembly of the viral RNA genome and structural proteins occurs on the surface of the endoplasmic reticulum (ER). The immature virions are transported through the trans-Golgi network (TGN) and pre-M is cleaved by the host protease furin, inducing rearrangement of E protein and activating the infectious form of the viral particle (Pastorino, 2010). After maturation, the virion along with subviral particles (that only contain M and E protein) are exocytosed and ready to infect other cells.

CHAPTER 2

WEST NILE VIRUS

1. Introduction

1.1 General Overview

West Nile virus (WNV) is an arbovirus that belongs to the *Flavivirus* genus of the *Flaviviridae* family. This neurotropic virus falls within the Japanese encephalitis virus antigenic complex; along with Murray Valley encephalitis virus, St Louis encephalitis virus, and Kunjin virus (Calisher, 1988). The virus is enveloped and icosahedral with a single positive stranded RNA genome (10.7 kb). Around the world, two lineages of WNV exist with the first lineage commonly responsible for avian, human, and equine disease.

1.2 Signs and Symptoms

In most cases, WNV infected patients are asymptomatic. Furthermore, 3-14 days after being bitten by an infected mosquito, only 20-30% of patients develop mild and acute symptoms (West Nile Fever) for which generally last 3-6 days (Goodman, 2012). Mild symptoms include a sudden appearance of flu-like symptoms, which may include rhinorrhea, cough, sore throat, malaise, fever, myalgia, nausea, vomiting, anorexia, eye pain, headache, and rash. In rare cases (less than 1%), severe neurological symptoms can arise with a 10% fatality rate (Peterson, 2003, CDC.gov). Severe neurological symptoms include encephalitis and meningitis with more than 50% presenting acute flaccid paralysis or other permanent neurological injury due to neurons inability to regenerate. Susceptibility of developing severe neurological symptoms increases with age (especially among those 60 to 89 years of age) with all ages and genders appearing to be equally susceptible to WNV infection (Hayes, CDC fig). Other than advanced age, people who

are immunosuppressed or suffer from other predispositions can develop severe neurological symptoms (Petersen, 2003). Predispositions include genetic mutations, such as C—C chemokine receptor type 5 (CCR5) and 2–5 oligoadenylate synthethase (OAS) mutations, or even hypertension, diabetes, and smoking (Sips, 2012; Diamond, 2009a).

1.3. Treatment and Prevention

Currently, there are no approved vaccines or specific antiviral treatments for WNV infections. People that develop symptoms rely on anodynes and supportive treatment until the infection is cleared. Treatment of WNV infection has been of growing importance with considerable research looking into immune γ-globulin, interferon α-2b, and antisense oligomers along with others as potential therapeutic treatments (Diamond, 2009b). Preliminary *in vitro* results of several candidates have shown prophylaxis, however *in vivo* and therapeutic studies have not had the same efficacy (Diamond, 2009b). Aside from the previous candidates, monoclonal antibodies (mAbs) therapies have also been explored as potential therapeutic treatments against viral infections with E16 showing both prophylaxis and therapeutic activity (Both, 2013; Kaufmann, 2006).

1.4. Ecology and Life Cycle

WNV is usually maintained and amplified in enzootic cycles between female mosquito vectors and bird hosts; predominantly, *Culex (Cx)* species of mosquitoes (e.g. *Cx pipiens*, *Cx tarsalis*, *Cx salinarius*, *Cx quinquefasciatus*) and passerine birds, although other species of mosquitoes and birds have been seen to carry the virus (Gea-Banacloche, 2004; Farajollahi, 2011). The female gender of *Culex* mosquito is the primary vector of many viruses, including WNV, due to the higher nutrient requirements needed for the energy demands of reproduction. While feeding on a blood meal they transfer saliva,

anticoagulants, and other pathogens into the host animal (Tolle, 2009). In most cases the host animals are birds, but in some instances other animals, including humans, can become an incidental/ "dead end" host. Birds are natural reservoirs for WNV with many avian species able to survive infection, become immune, and develop high-titer viremias to transmit to other feeding mosquitoes (Campbell, 2002). WNV infection cycle follows the mosquitoes' activity, which usually starts in the spring where mosquitoes are most active and abundant. The cycle ends early fall when conditions start to become unfavorable and mosquitoes go into diapause.

1.5. Epidemiology

Since the discovery of the virus in the West Nile district of Uganda (1937), major outbreaks have occurred all around the world, except for Antarctica (Kramer, 2008). From 1937 to 1990s, WNV infected people presented mild symptoms, e.g. fevers. Starting from 1990s, the frequency of outbreaks and number of cases presenting severe neurological symptoms, e.g. viral encephalitis, significantly increased. In Romania (1996–1997) an outbreak of WNV caused over 500 clinical cases with 10% of cases ending in fatalities (141). In Israel (2000), 417 cases were reported with 317 of the patients being hospitalized and 35 of them dying (case fatality rate 8.4%) (Weinberger, 2001). Along with these two cases, Algeria (1994), Morocco (1996), Tunisia (1997, 2003), Czech Republic (1997) reported WNV disease (Hayes, 2005).

The first documented occurrence of WNV in the Western hemisphere was in 1999 in New York City (United States (US)), speculated introduction from a single point, spreading throughout the eastern states along with westward migration, reaching as far as North Dakota in 2001 and California in 2002 (Petersen, 2003). Since the introduction of

WNV (1999-2012), over 37,000 cases of WNV infection have been reported (1,549 deaths) with 286 people dying in the US in 2012 [Centers for Disease Control and Prevention (CDC)]. Along with rapid expansion in the US, other western hemisphere countries and continents (e.g. Canada, Mexico, Central America, and South America) also reported cases of WNV (Campbell, 2002). In Canada (2001), there were reports of infected birds, horses, and mosquito pools with 462 human cases (10 deaths) (Dauphin, 2004). In Mexico (2002), WNV encephalitis-like symptoms in equine were reported in five different Mexican states from the border of Texas along the Gulf of Mexico to Southern Mexico (Tabasco) (Estrada, 2003).

As previously mentioned, female *Culex* mosquitoes transmit WNV to primarily passerine birds and cycle between each other, with vertebrates an incidental host. For that reason, high frequency of WNV transmission to humans directly correlates to the ecology and feeding behavior of the infected mosquitoes. People at risk of WNV infection include those living near flooded, high vegetation cover, low population density areas which are ideal mosquito habitats (Han, 1999). Rate of infection follows mosquitoes' activity with approximately 85% of human infections occurring in August and September when mosquitoes are most abundant (Peterson, 2004). Susceptibility increases with age, compromised immune systems, or predispositions.

1.6. Pathogenesis

After mosquito inoculation of the WNV through the skin cells, keratinocytes and most importantly Langerhan dendritic cells (LDC), get infected and replicate the virus. Initial recognition of nucleic acid intermediates by pathogen recognition receptors (PRR) (e.g. Toll-like receptors and RIG-like receptors) activates Interferon Regulation Factors

(IRF) 3 and 7 for production of type I interferons (IFN) (Diamond, 2003). IFNs work together to "interfere" with viral replication by increasing expression of Protein Kinase R (PKR) and 2'-5' oligoadenylate synthetase (OAS) and to warn nearby cells of viral infection (major function of INF-γ). Binding of PKR to viral dsRNA activates the complex resulting in phosphorylation and inactivation of Eukaryotic Initiation Factor 2α (eIF2α), inhibiting translation and leading to apoptosis (Lee, 1994; Szretter, 2011). OAS, which also binds to viral dsRNA for activation, converts ATP to 2'-5'oligoadenylate resulting in binding and activation of RNAse L. RNAse L cleaves both viral and host ssRNA, mRNA, and rRNA inhibiting virus replication and protein expression ending in apoptosis of infected cell (Silverman, 2007). Along with IFN innate response γδ T cells, NK cells, and macrophages are drawn toward site of infection for specific effector functions.

While INF response is occurring, infected LDC migrate to the nearest draining lymph node, whose primary function is to uptake and process pathogens (viruses) into linear antigens (epitopes) forming antigen presenting complex, better known as Major Histocompatibility Complex 1 (MHCI), and display assembled MHCI to B, T, and other immune cells for adaptive immune response. It is in the lymph node where initial viral amplification occurs, creating a high viremia and allowing easy access into the systemic circulation system, spreading to visceral organs where a second round of amplification occurs (Lim, 2011). During dispersion, naive B-cells are activated by MHCI containing WNV antigen for a primary antibody response (about 4 days into infection), which consists of naive B-cell production of low affinity but high avidity IgM functioning in enhancement of complement activation and immune complex formation (Samuel, 2006).

6-8 days into the infection (in mice), after CD4 T-cell activation and affinity maturation, higher affinity IgG antibodies are produced along with CD8+ cytolytic T-cell mediated response (Suthar, 2013). If spread of WNV is controlled, symptoms are generally mild and neurodisease does not present itself.

1.7. Neurodisease

At high viremia levels of WNV, production of tumor necrosis factor (TNF), metalloproteinases (Suthar, 2013) and interleukins (Cho, 2012; Welte, 2011) from immune response to periphery infection increases the permeability and chance of WNV crossing the Blood-Brain Barrier (BBB) into the brain. The BBB is made up of an extracellular matrix, endothelial cells, astroglia, neurons, and pericytes with tight junctions making it highly selective to small hydrophobic molecules; blocking access to large polar molecules and certain pathogens (Lawther, 2011). The exact mechanism via which WNV travels through the BBB is still unclear, but several theories have been presented: (1) a relay infection through choroid plexus epithelial cells, neurons, or olfactory bulb; (2) transport through infected immune cells in a "Trojan horse" mechanism; (3) or retrograde transport from infected peripheral neurons through their axons (Kramer, 2007; Welte, 2011; Lim 2011).

After breaching the BBB, infected neurons and other supporting cells contribute to the deterioration of CNS causing the inflammatory neurological disease by apoptosis, cell necrosis, and bystander cytotoxicity (Lim, 2011; Cho, 2012). Apoptosis is considered a normal defense mechanism against tumor cells and virally compromised cells by programmed cell death preventing them from spreading to uninfected cells. Apoptosis can be induced by CD8 T-cells and can take three mechanisms. The first is

perforin/granzyme cytolysis mechanism, which uses perforin to make pores in infected cells and transport granzyme B into the target cell. Granzyme B, in a series of signal cascades, activates caspase-3 (cysteine-dependent aspartate specific proteases) which cleaves the inhibitory site of caspase-activated DNAse (iCAD to CAD) leading to DNA fragmentation and apoptosis (Reed, 2000). Fas from infected cells and Fas ligand (fasL) from CD8 T-cells bind, activating death domains leading to the caspase-dependent apoptosis cascade (Wallach, 1999). The TRAIL dependent pathway has a similar ending with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) from CD8 T-cells binding to Death receptors (DR5) from infected cells and activating caspase-dependent apoptosis cascade. Other than CD8 T-cells initiating caspase-dependent apoptosis, WNV via NS2B-NS3 can also activate caspase-3 and other non-caspase proteases (Shrestha, 2012). The second mechanism by which WNV affects the CNS is by cell necrosis from extensive budding of WNV progeny losing and compromising membrane integrity. The last mechanism involves other cells (neural and non-neural) releasing cytotoxic factors, including inflammatory cytokines (T-cell chemoattractant Cxcl10, interleukins (ILs), and TNF), near a non-infected neuron resulting in degradation of healthy cells (Cho, 2012). The underlying conclusion is that regulation of inflammatory response needs to be controlled but can be difficult. For example, a minor inflammatory response results in uncontrollable infection, however, a major inflammatory response results in irreversible harm of both infected and uninfected neurons.

1.8. Antibody and Antibody-Based Therapeutics

A full IgG antibody is tetrameric protein with two light (25 kDa) and two heavy chains (LC and HC, respectively) (75 kDa) with each polypeptide held together by

disulfide bonds in the quaternary structure (Wang, 2007). The LC is made up of the light variable (VL) and the light constant (CL) domains. The HC is composed of the heavy variable (VH) and three separate heavy constant domains (CH1-3, respectively) (Harris, 1998). The IgG has two functional regions: the Fab and the Fc region (**Figure 1A**). The Fab region functions in specific antigen recognition and is composed of the LC coupled with VH and CH1 domains. The Fc region (CH2-3) is responsible for activation of many innate cells to perform specific functions and can also determine degree of antibody dependent enhancement (ADE), antibody dependent cell-mediated cytotoxicity (ADCC), and complement dependent cytotoxicity (CDC) involvement (Ying, 2012). Researchers have taken the variable domains and have created a variety of structural antibodies, such as Fab (**Figure 1E**), and single-chain variable fragments (scFv) (**Figure 1C**) (Eleniste, 2013; Re, 1988).

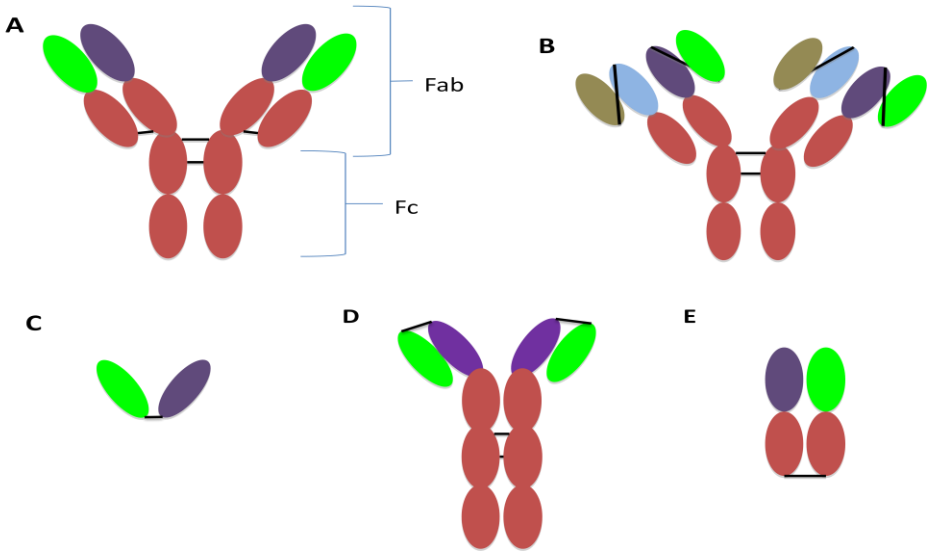


Figure 1: Different Antibody Structures. (A) Full IgG Antibody, (B) tetrameric bifunctional mAb (BsAb), (C) scFv, (D) E16 (scFv-CH1-3)$_2$, and (E) Fab

1.8.1 E16

Currently, the leading monoclonal antibody (mAb) candidate against WNV is a humanized murine mAb (Hu-E16), which demonstrates neutralizing activity of WNV infection in 90% of mice and hamsters when administered 5 days post inoculation (Morrey, 2006; Dowd, 2011). Hu-E16 recognizes and binds to Domain III (DIII) of the WNV Envelope (E) protein, specifically residues E302–E309 of the N-terminal region, residues E330–E333 (BC), E365–E368 (DE) and E389–E391 (FG) (Nybakken, 2005). As previously mentioned, the E protein is a dimer with each monomer made up of three domains; DI is responsible for conformational arrangement in low pH endosomes, DII provides the fusion between the virus and endosome membranes, and DIII acts as the binding receptor to trigger fusion (Oliphant, 2005). E16 functions in opsonization of WNV, preventing structural rearrangement of the E protein in the acidic environment (Kaufmann, 2006). Consequently, blocking DII fusion and denying the virus access to the cytoplasm of the cell where replication and translation occur. This inhibitory step contains the virus within the endosome ultimately for lysosomal degradation (Thompson, 2009).

1.8.2 8D3

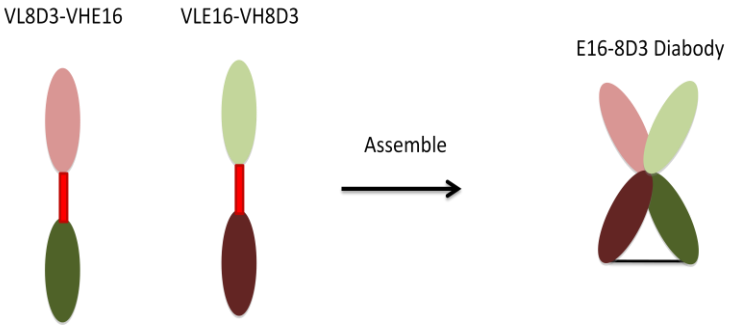


Figure 2: Assembly of two scFv (VL8D3-VHE16 and VLE16-VH8D3) into a diabody.

The Transferrin Receptor (TfR) is highly abundant in capillary endothelium of the BBB and functions in transcytosis of transferrin,

an iron carrying glycoprotein in blood plasma. 8D3 mAb, initially a rat IgG2a to the mouse TfR, mimics the specific binding of transferrin to TfR in mice (Kissel et al., 1998). Once 8D3-TfR binding occurs, receptor-mediated endocytosis engulfs the mAb into the cell. Acidic conditions within the endosome dissociate the mAb from TfR binding allowing release of the molecule and transportation across the BBB (Manich, 2013). Recent studies have shown 90% of the conjugated enzyme to 8D3 that entered the brain crossed the BBB into the brain parenchyma (Manich, 2013) with the rest targeting the liver and kidneys (Lee, 2000). Utilization of 8D3 mAb has the potential to carry other specific therapeutics across the BBB for treating neuro-pathogens and neurodisease.

The current challenge with WNV is that there is no known therapeutic that has the capability of crossing the BBB and still be able to retain its therapeutic activity to neutralize the virus. For example, E16 mAb has the neutralizing and therapeutic activity but does not cross the BBB. A recent study by our laboratory described the ability of plants to produce tetrameric bifunctional mAb (BsAb) that are able to recognize two different epitopes or binding sites within the Fab region (He, 2014) (**Figure 1B**). These BsAbs have the potential to cross the BBB and neutralize the virus. On the other hand, the large size of the molecule could decrease the transportation across the BBB membrane, thus decreasing the efficacy of the treatment. In a Dengue study, Brien et al utilized diabody structures to synergistically complement two mAb varying avidities to all four DV serotypes with result showing specific binding of individual mAb with no neutralizing enhancement. A diabody (DiAb) consists of two scFvs containing both VH and VL regions of two different mAbs with a short linker attaching each domain (**Figure 2**). To get around the BBB impediment, we constructed a bifunctional diabody

coupling the E16 mAb WNV neutralizing activity with 8D3 capability to cross the BBB. The molecule was characterized and its bi-specificity was tested for retention of specific binding.

2. Results and Discussion

2.1 Development of a Diabody-Based Therapeutic to WNV

2.1.1 Molecular design

The assembly of a diabody depends on two separately synthesized scFvs, which dimerize to form one molecule with antigen binding sites (Fv) facing away from each other. The first scFv contains the 8D3 variable light (VL) and E16 variable heavy (VH) domain linked together by a short Gly-Ser linker (GGGSGGGG) to connect and prevent intramolecular association. The size of the linker is critical for proper assembly of Fv regions. A Kozac sequence is added to enhance translation initiation at the 5' of the first scFv coding sequence and is followed by a leader sequence for protein secretion by mammalian cells. After the E16 VH coding region, sequence for a kappa LC derived C-terminus pairing domain (FNRGEC) followed by the stop codon is added. The construct is surrounded by a *Hind*III and a *Not*I restriction site (5' and 3', respectively) for ease of cloning (**Figure 3A**). The second scFv is composed of the complimentary domains, E16 VL and 8D3 VH domain also linked together by a Gly-Ser linker. The second scFv also has a similar molecular arrangement except for a substitution of the LC pairing sequence by an IgG1 hinge-derived pairing domain (VEPKSC) (**Figure 3B**). A Sac1 restriction site was integrated on the end of the VH sequences of both constructs for exchange of the pairing domains. Both constructs were cloned into both pcDNA3.1/Hygro (+) and pcDNA3.1-zeo (+) vectors for mammalian cell expression.

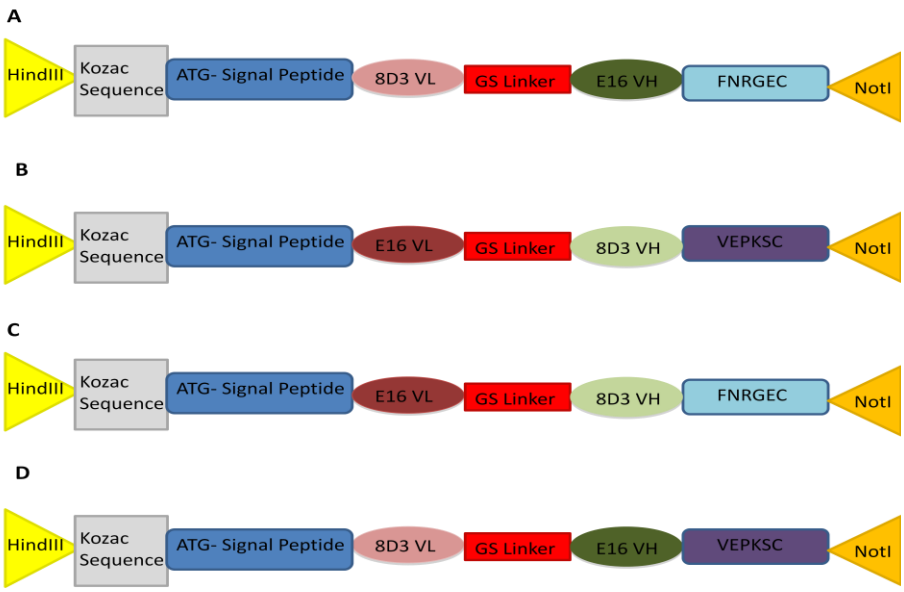


Figure 3: Schematic of E16-8D3 diabody gene constructs; (A) VL8D3-VHE16-FNRGEC, (B) VLE16-VH8D3-VEPKSC, (C) VLE16-VH8D3-FNRGEC, and (D) VL8D3-VHE16-VEPKSC

The design will seek to improve CNS penetration while maintaining WNV neutralizing activity by reducing the size of the molecule into a diabody format. Theoretically, the small size of the diabody allows for several advantages including ease of production and enhanced ability to pass the BBB. The transferrin mechanism allows for a one way passage through the BBB further supporting entrance into TfR saturated areas. On the other hand, lack of an Fc region makes purification difficult and glycosylation impossible. Also the small size of the molecule could result in rapid clearance from the blood through the kidneys meaning a higher concentration might be needed to have a comparable therapeutic effect as the full antibody.

2.1.2 Molecular Cloning

2.1.2.1 Liquid Culture

The E16-8D3 DiAb constructs genes (VL8D3-GS linker sequence-VHE16 - FNRGEC and VLE16- GS linker sequence-VH8D3-VEPKSC) were synthesized, cloned into pJ201 cloning vector, and transformed into DH10B *Escherichia coli* (*E. coli*) strain by DNA2.0. Both DH10B strains were grown in 3 mL of sterilized Luria- Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl), plus the addition of 100 µg/ml kanamycin at 37°C in a shaker (300 rpm) for 16 hours.

2.1.2.2 DNA Isolation

After 16 hours, the 3 mL of DH10B culture were centrifuged in a microfuge in 1.5 mL increments for 30 seconds at 12 kG. The supernatant was removed with the pellet resuspended in 50 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and vortexed until the pellet is resuspended. 300 µl of NS (0.1 N NaOH, 0.5% SDS, and sterile water) was added and gently inverted four times to lyse the cells releasing the gene of interest (GOI) containing plasmid. 200 µl 7.5 M ammonium acetate was immediately added (within a minute after adding NS), mixed and incubated for 5 minutes on ice to neutralize the reaction. After the time had elapsed, the sample was centrifuged (12 kG) at 4 °C for 5 minutes. The resulting supernatant was transferred to clean tube. 330 µl of isopropanol was added, incubated at room temperature (RT) for 3 minutes, and microfuged (RT) for 10 minutes at 12 kG to precipitate plasmid DNA. The supernatant was removed and the pellet was washed with 70% ethanol. After the wash, the DNA pellet was left to dry until the all of the ethanol evaporated. The DNA was then resuspended in 25 µl of sterilized

water. The same protocol was used to isolated pcDNA3.1/Hygro (+) and pcDNA3.1-zeo (+) vectors from DH5α (*E. coli* strain).

2.1.2.3 Restriction Endonuclease Digestion

The isolated plasmid DNA was digested with *Not*I and *Hind*III restriction enzymes (New England Biolab (NEB)). This was prepared by mixing of 2 µL of 10X buffer 2 (500mM NaCl, 100mM Tris-HCl, 100mM MgCl2, 10mM DTT, pH 7.9@25°C), 13 µL of the isolated DNA, 2 µL of Bovine Serum Albumin (10 mg/ml), 1 µL of RNAse A (10 mg/ml), and 1 µL of each restriction enzyme into a microfuge tube. The tube was picofuged for 3 seconds and placed in an incubator (37 °C) for 90 minutes.

2.1.2.4 Agarose Gel Electrophoresis of DNA

To separate the digested DNA fragments, they were subjected to agarose gel electrophoresis. 0.32 grams of agarose was melted in 40 mL of 1× TAE buffer (40 mM Tris-HCl, 40 mM acetic acid, 25 mM EDTA pH 8.0) by applying heat (1 minute in a microwave). The agarose was poured into a 7×5 cm agarose gel tray with an eight well comb and set to solidify. After the gel solidified, it was placed on electrophoresis tank and submerged in 1× TAE buffer. 3 µL of GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) and each of the 20 µL digest were pipetted into individual wells. The gel was ran at 80 Volt (constant voltage) for 90 minutes. After the time elapsed, the gel was stained with 0.5% ethidium bromide for 10 minutes and destained with DI water for 20 minutes. After 20 minutes of destaining, the gel was placed in a UV box for analysis and excision of appropriately sized DNA fragments. The DNA was then extracted with Qiagen QIAquick Extraction kit and resuspended in 20 µL of sterile water.

2.1.2.5 DNA Ligation

The next step requires the ligation of the DNA insert fragment to the vector. After extraction of DNA fragment, an analytical gel (using the same protocol but utilizing a subset of extracted DNA) was ran to estimate the concentration of insert and vector by comparing the intensity of the DNA bands with those of the molecular weight marker (**Figure 4A**). Depending on the amount of DNA recovered the quantities will vary, but an effective ligation recipe consist 1:3 molar ratio of vector to insert, T4 ligation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, pH 7.5 at 25 °C), and 1 unit of T4 DNA ligase. The ligation mixture was mixed and incubated (16°C) overnight.

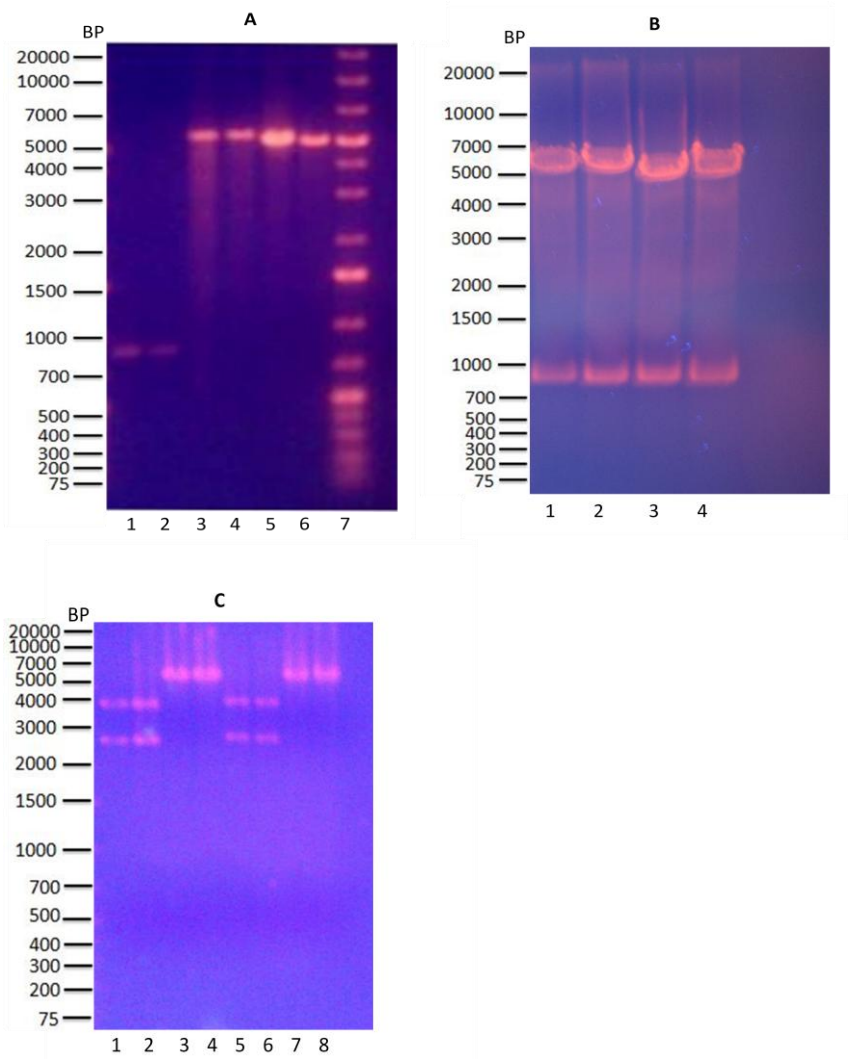


Figure 4: Analytical agarose gels (A) NotI and HindIII restriction enzyme digestion inserts and vectors. Lane 1: VLE16-VH8D3-FNRGEC; Lane 2: VL8D3-VHE16-VEPKSC insert; Lanes 3 and 4: 232 vector; Lane 5 and 6: 263 vector; Lane 7: Generuler 1kB Plus DNA Ladder (Thermoscientific). (B) NotI and HindIII restriction enzyme digestion of transformants. Lane 1: 458; Lane 2: 459; Lanes 3: 460; Lane 4: 463. (C) PvuI restriction enzyme digestion of transformants. Lane 1-4: transformants from figure 4B, respectively; Lane 5: 467; Lane 6: 468; Lane 7: 469; Lane 8: 470.

2.1.2.6 Electroporation Transformation

The newly ligated DNA was transformed into DH5α by electroporation. First, 2 μL of ligated DNA was mixed with electro-competent cells and incubated on ice for 1

minute. The mixture was transferred to an ice cold 0.2 cm electroporation cuvette, making sure the cells create a circuit between the plates. The cells were pulsed by MicroPulser Electroporator (BioRad) at approximately 2.49 kV with a time constant of 5.5 milliseconds. After the pulse, the cells were immediately transferred into 1 ml of SOC media (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) in a round bottom (RB) tube. The cells were incubated at 37°C with shaking at 250 rpm for 45 minutes, allowing the cells to recover from the electric shock. After the 45 minutes of incubating, the cells were concentrated by transferring cells to a microfuge tube, centrifuging them at 12 KG for 2.5 minutes and removing 800 µl of the supernatant. The cell pellet was then resuspended and spread on LB + ampicillin plates (pcDNA3.1/Hygro (+) and pcDNA3.1-zeo (+) vectors carry ampicillin resistance marker gene) and incubated for 24 hours at 37°C.

Colonies were screened for the expected insert and vector size following steps 2.1.2.1-2.1.2.4 (**Figure 4B**). Since the *Not*I-*Hind*III digest yielded similar size between pcDNA3.1/Hygro (+) and pcDNA3.1-zeo (+), a second restriction enzyme digestion (PvuI) was performed to distinguish the two vectors. pcDNA3.1/Hygro (+) contains two PvuI restriction sites, while pcDNA3.1-zeo (+) only contains one (**Figure 4C**). Analytical gel demonstrates the expected bands for all strains (**Table 1, in Appendix A**). After GOI containing plasmids were identified by gel analysis, the DNA insert in these plasmids was sequence to confirm the identity of the construct and that no mutations had occurred during the cloning process (**Figure 5, in Appendix B**). Our results clearly indicate that we have successfully constructed the intended diabody constructs.

2.1.3 Expression in Mammalian Cell Culture

After confirmation, the DNA was transformed into mammalian cells and screened for expression (performed by Huafang Lai a collaborator in the lab from Arizona State University (ASU)). Mammalian cell-produced E16-8D3 DiAb was examined for its ability to bind DIII antigen. This mammalian cell experiment was aimed to preliminarily test functionality of the diabody design before moving forward in plant expression. Since signal peptide should direct DiAb to be secreted out of mammalian cell, the cells were pelleted and conditional media which should contain DiAb was kept for assessment to DIII binding. We also examine the cell pellets for DiAb.

2.1.4 Diabody Binding Assay

As previously mentioned, the DiAb structure only contains two binding sites (E16 and 8D3) without an Fc region. This makes their characterization more difficult due to lack of secondary detection antibodies that usually bind to the Fc region. The obstacle was overcome by binding the DiAb directly to a high-binding ELISA plate. Thereafter, DIII was incubated in the wells to bind with exposed E16 sites. A rabbit anti-DIII antibody (He, 2014) was used to bind DIII. Subsequently, an HRP-conjugated goat anti-rabbit antibody was used for detection. Hu-E16 mAb was used as a positive control. While the positive control demonstrated specific DIII binding, no signal was observed from either DiAb contained in the conditional media or the cell pellet. To confirm these results, DIII was conjugated with a fluorochrome and used for detection of E16 binding. Again, no signal was observed.

2.1.5 Discussion of Binding Results

Here, we synthesized a gene construct for E16-8D3 DiAb for the goal of increasing BBB penetration for therapeutic development against WNV neurodisease. Constructs were first produced in mammalian cells to test the binding of the molecule with the future goal to be expressed in plants. Unfortunately, the E16 moiety failed to show any binding to the DIII antigen. We hypothesize the GS linker is not the optimal candidate in size which may lead to improper assembly of the E16 binding site unable to recognize the WNV DIII. Finding the correct linker size would take empirical experimentation, which demands time and resources that are not supported by our current funding. Therefore, we put the DiAb strategy on hold and are pursuing an alternative strategy in the form of Fabs.

2.2 Characterization of Other Anti-WNV Antibody Variants and Antigen

2.2.1 Plant-Produced mAb Therapeutics

There are several culture systems for the production of monoclonal antibodies, including mammalian, bacterial, and insect cell-based systems (Chen, 2011a). More advantageous is the utilization of plant-based expression systems, specifically agrobacterium-mediated vacuum infiltrated transient expression, which offers faster production times and greater scalability for commercial production. Aside from commercial benefits, plants offer greater safety with reduced risk of transmitting human pathogens, perform mammalian-like post-translational modifications (Chen, 2011a; Faye, 2010) and have capability of assembling multimeric proteins (Chen, 2009; Huang, 2010). Several plant produced multimeric proteins include mAbs, virus-like particles (VLPs), and subunit vaccines (Lai, 2012; Negrouk, 2005; Chen, 2009)

Historically, people have been afraid that genetically modified organisms (GMOs) would contaminate and decrease biodiversity of naturally occurring species. However, plant transient expression has minimized public concerns over GMOs due to the utilization of non-transgenic plants for production of biologics with transiently transgenic plants being stored within a confined area, eliminating exposure to the environment (Lai, 2012). In the laboratory setting, *Nicotiana benthamiana* (*N. benthamiana*) has been the main species of plants utilized for transient expression due to flexibility for both small scale analytical studies and commercial use, the broad range of expression vectors accessible, high biomass/seed bank yield, and extensive use within the scientific community (He, 2012). Although, tobacco is frequently used, other plants (such as lettuce, potatoes, tomato, etc.) have been used for transient expression (Wrobleski, 2005; Negrouk, 2005; Sohi; 2005).

Plant transient expression involves delivery of a GOI into the host plant's genome for short-term production of the target protein. Delivery of the transgene into plant cells is carried out by *Agrobacterium tumefaciens* (*A. tumefaciens*) in a process called agroinfiltration. The bacterium fills the interstitial space between the mesophyll, delivering GOI containing deconstructed viral vectors into the plant cell (Chen, 2011b; Leuzinger, 2013). Deconstructed viral vectors, such as MagnICON and geminiviral, utilize virus replicative genome without the structural genes necessary for the construction or infectivity of a full live virus, allowing larger transgene inserts to be expressed (Gleba, 2004). MagnICON expression system is based on the tobacco mosaic virus (TMV) and the non-competing potato virus X (PVX) genomes, each consisting of three separately housed components: 5' module (pICH15879 (TMV) and pICH21380

(PVX)) containing the promoter and other genetic elements, 3' module (pICH11599 (TMV) and pICH21595 (PVX)) containing the GOI, and integrase module (pICH14011) for integration of the 5' and 3' modules (Chen, 2013). Utilization of both TMV and PVX vectors depends if target protein contains multimeric subunits.

With all the benefits MagnICON expression offers, the production of mAb requires 5 *A. tumefacien* strains to be co-infiltrating at once. Entailing higher control parameters for proper expression of both HC and LC, thus increasing the cost to produce these molecules. To overcome this hurdle pE16 scFv-CH_{1-3} was produced in GnGn transgenic *N. Benthamiana* (**Figure 1D**) with the intention of lowering the amount and number of strains of *A. tumefaciens* being infiltrated into the host plant for better control of anti-WNV therapeutic production. pE16 scFv-CH_{1-3} was characterized and tested for proper folding and retention of binding site with SDS PAGE/Western Blot and ELISA analysis, respectively. Along with the pE16 scFv-CH_{1-3}, a DIII vaccine was developed in hopes to evoke a specific and sufficiently strong immunological response to provide long term immunity against WNV disease. DIII was also produced in *N. benthamiana* and characterized utilizing E16 antibody, known to bind to conformational epitopes (Nybakken, 2005), as indication of proper folding. Anti-DIII mouse serum was tested to see if DIII produced E16 like antibodies. Finding a prophylactic and/or therapeutic vaccine candidate for treatment of WNV requires variable attack strategies with a production method that is economically viable for meet the demand. Production of antibody variants as well as WNV DIII proteins in plant expression systems offers a cost effective and scalable option.

2.2.2 Methods, Results, and Discussion

2.2.2.1 Structural Characterization of Plant-Produced E16 scFv-CH_{1-3}

pE16 scFv-CH_{1-3} was expressed in *N. benthamiana* plants, and purified by protein A chromatography. pE16 scFv-CH_{1-3} and pE16 mAb (positive control) were ran on a 4-10% SDS PAGE under reducing (5% v/v β-mercaptoethanol) conditions and stained with Coomassie Blue (**Figure 6A**). For Western Blot, pE16 scFv-CH_{1-3}, pE16 mAb, and uninfiltrated GnGn leaf extracts (negative control) were ran on a 4-10% SDS PAGE under reducing (**Figure 6B**) and non-reducing (**Figure 6C**) conditions for 2 hours at 100V. Gels were transferred to PVDF membrane at 80 mA overnight in 4 °C. After transfer, the membrane was blocked with 5% milk in PBST for 2 hours and washed after with PBST. Membrane was probed with 1% milk diluted HRP-conjugated goat anti-human gamma for an hour intended to detect the light and HC, respectively. ECL Western blotting detection reagent (ECL) was added to the membrane and developed on x-ray film. These results demonstrate that pE16 scFv-CH_{1-3} was expressed in plants with the expected LC and HC components and efficiently assembled into its tetrameric form.

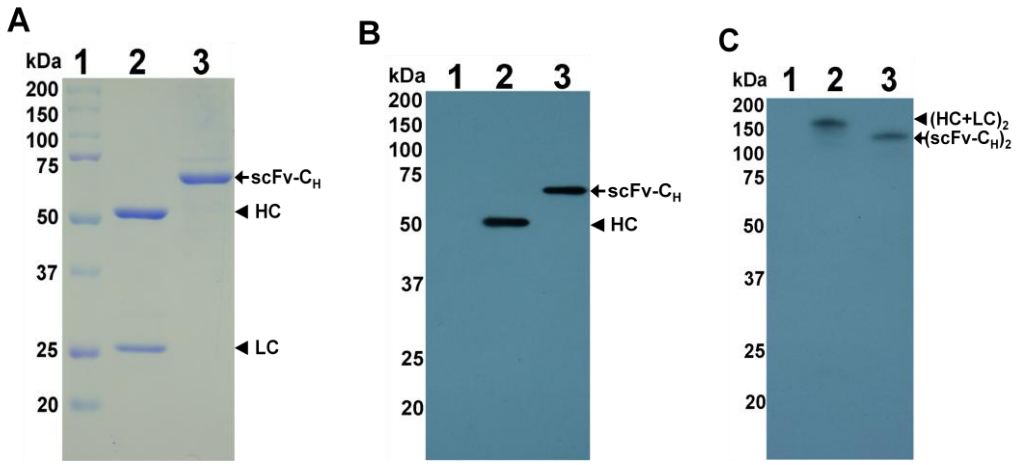


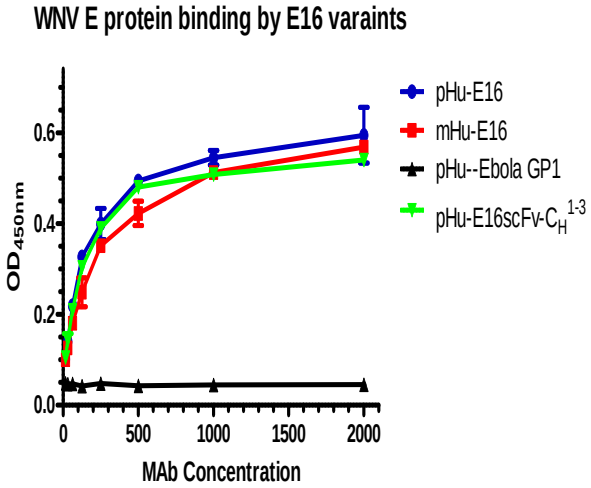
Figure 6: SDS PAGE and Western blot analysis of the pE16 scFv-CH1. (A) Molecular weight marker (lane 1), pE16 mAb (lane 2) and pE16 scFv-CH1 (lane 3) were separated on SDS-PAGE gels under reducing conditions (lanes 1 and 2) and stained with Coomassie Blue. (B and C) uninfiltrated GnGn leaf extracts (lane 1, negative control), pE16 mAb (lane 2), and pE16 scFv-CH1 (lane 3) were separated on SDS-PAGE gels under reducing (B) or non-reducing (C) conditions and transferred onto PVDF membranes. The membranes were incubated with a goat anti-human gamma chain antibody for detection.

2.2.2.2 pE16 scFv-$CH_{1\text{-}3}$ E Protein Binding by ELISA

96-well high-binding ELISA plates were coated with 1 µg/mL of WNV E protein in 0.1 M Na carbonate buffer and incubated for 4 hours at 37 °C. After the time had elapsed, the plates were washed three times with PBST (PBS with 0.1% Tween-20) and blocked with 5% milk in PBST for an hour at 37°C to reduce non-specific binding. All samples (pE16 scFv-$CH_{1\text{-}3}$ GnGn, pHu-E16 (plant-produced, positive control), mHu-E16 (mammalian cell-produced, positive control), and Hu-IgG Anti-Ebola (negative control)) were diluted in the following concentrations (ng/ml): 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625 in 1% milk in PBST. Samples were added to appropriate wells and incubated for 2 hours at 37 °C. After a wash step, 50 µL of HRP-conjugated anti-human-kappa antibody was added as the detection antibody for 1 hour at 37 °C. The ELISA plates were then thoroughly washed four times with PBST to remove unbound detection antibody

and developed with tetramethylbenzidine (TMB) substrate (KPL Inc). To stop the reaction, 50 µL of 1 M phosphoric acid was added after 2 minutes and read at 450 nm. ELISA analysis revealed similar binding of pE16 scFv-CH_{1-3} to WNV E protein as those of pHu-E16 and mHu-E16 (**Figure 7**). This result demonstrates that pE16 scFv-CH_{1-3} retains the antigen binding specificity of the full pHu-E16 antibody, indicating it is a promising therapeutic candidate against WNV infection.

Figure 7: Specific binding ELISA of pHu-E16 scFv-CH1-3 to WNV E protein was incubated in the sample wells coated with WNV E protein and detected with an HRP-conjugated anti-human kappa antibody. pHu-E16 and mHu-E16 were used as positive control. pHu-Ebola Gp1 was used as a negative control. Mean± SD of samples of samples from triplicate values.



2.2.2.3 Characterizing Plant-Produced DIII Antigen's Binding to E16 mAb

High-binding ELISA plates were coated with of *E. coli* or plant produced (extracts 1 and 2) DIII (1 µg/mL) in 0.1 M Na carbonate buffer and incubated for 4 hours at 37 °C, separately. E16 mAb was diluted (1% milk) in following concentrations (ng/ml): 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625 and added in duplicates into wells, followed by incubation for 2 hours at 37 °C. Detection was carried out by HRP-conjugated anti-human-kappa antibody for 1 hour in 37 °C. The plates were developed with TMB substrate and 1M phosphoric acid. The plates were read at 450 nm. Results show plant-produced DIII specifically recognizes E16 mAb. Since E16 has been shown

to only recognize properly assembled DIII antigen (Lai 2010), this result indicates that plant-produced DIII was properly folded in the tertiary structure (**Figure 8**).

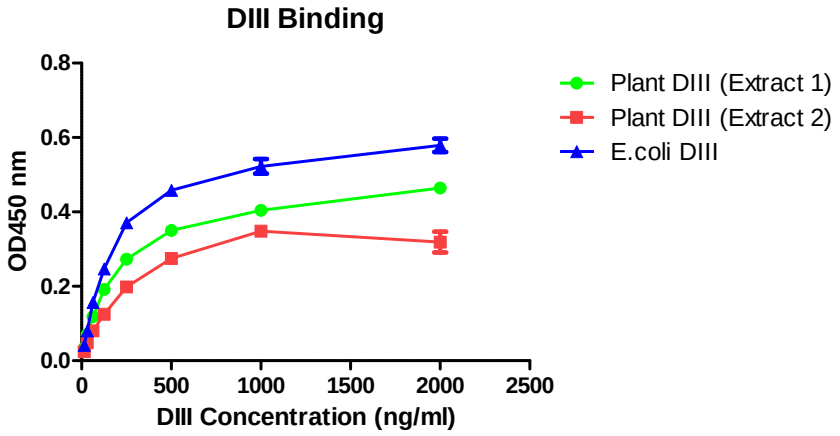


Figure 8: Specific binding ELISA of pHu-E16 to plant and E. coli produced DIII. Serial dilutions of pHu-E16 were incubated in sample wells coated with plant and E. coli produced DIII and detected with an HRP-conjugated anti-human kappa antibody. Mean ± SD of samples from duplicate values.

2.2.2.4 Characterize the Antigenicity of Plant-Produced DIII Antigen by Competitive ELISA

Plant-produced DIII antigen was examined for its immunogenicity in inducing the production of WNV DIII specific antibodies in mice. 25ug of plant-DIII was injected into groups of mice with PBS as a negative control. Serum samples were collected 12 weeks after the immunization. Two batches of plant-produced DIII were used in the animal study. High-binding ELISA plates were separately coated with *E. coli* or plant produced DIII (batch 1 and 2) (at 1 μg/mL) and incubated for 4 hours at 37 °C. After blocking and three washes, serum samples from DIII or PBS immunized mice were diluted 1:100 and incubated in the wells for 2 hours at 37 °C. Plates were washed and then incubated with E16 (32.25 ng/mL) for plant-DIII batch 1 and *E. coli* DIII-coated wells, and 62.5 ng/mL for plant DIII-batch 1-coated wells. Detection and analysis were carried out similarly as

ELISAs described above. Inhibition was calculated by the percent difference in OD450 absorption (formula between anti-DIII or preimmune serum samples and anti-PBS serum. Results indicate that antibodies in DIII-immunized serum efficiently compete with pHu-E16 in binding coated DIII antigen, and in turn, inhibit its binding to DIII by ~50% (**Figure 9**). This indicates that plant-produced DIII antigen elicited E16 like antibodies in mice, suggesting this antigen can be used as a potential vaccine candidate.

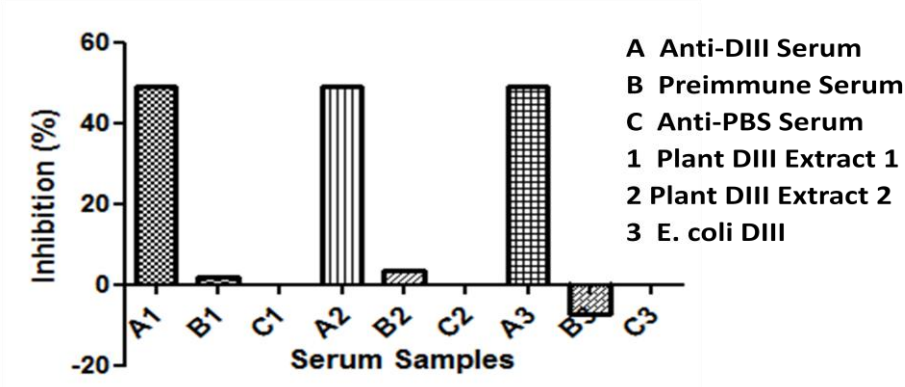


Figure 9: Competitive ELISA of plant and E. coli produced DIII binding by pHu-E16 and antibodies in anti-DIII serum. Plates coated with respective DIII were pre-incubated with 1:100 dilution of sera and subsequently with pHu-E16. Detection was carried out by anti-human kappa antibody. Mean ± SD represent triplicate samples.

Overall, structural and binding characteristics of pE16 scFv-CH_{1-3} as well as plant produced DIII were tested with western blot and specific binding ELISAs. Proper assembly into its tertiary structure was confirmed along with specific binding for both pE16 scFv-CH_{1-3} and DIII, demonstrating plants capacity to produce a great structural array of functional proteins. Furthermore, anti-DIII serums from mice presented competition (over 40% inhibition) against E16 in binding DIII antigen, indicating plant-DIII vaccine was able to generate a specific and potentially protective immune response in mice.

3. Conclusions

WNV is a growing epidemic all around the world with no approved prophylactic or therapeutic vaccines on the market. Great strides have been made to treat and prevent WNV disease especially those that develop into neurological symptoms. E16 mAb is the leading candidate able to prevent death up to 5 days after WNV infection (Morrey, 2006; Dowd, 2011), however lacks the ability to cross the BBB to neutralize CNS bound WNV. The diabody configuration that consists of both E16 and 8D3 scFv moieties was explored to enhance BBB penetration of E16. We successfully developed the molecular constructs of the diabody. However, further work need to be done to optimize the functionality of such design. Our results also demonstrate that the plant-produced E16 scFv-CH_{1-3} has the specific functionality in recognizing and binding DIII antigen with comparable affinities as pHu-E16 and mHu-E16. Also, the DIII-vaccine candidate was demonstrated to elicit production of E16 like antibodies that has a high likelihood to prevent severe WNV disease. Although, further research is still needed, vast progress has been made for the development of a prophylactic vaccine and an efficacious therapeutic against WNV with innovative strategies and promising results.

CHAPTER 3

DENGUE VIRUS

1. General overview

Dengue virus, also called break-bone fever and the 5-day or 7-day fever, is known as the most encountered *Flavivirus* in the world. Affecting close to a third of the world's population, all four or possibly five (Normile, 2013) distinct enough serotypes (DV 1-4) are capable of causing life-threatening disease.

1.1 Signs and Symptoms

Dengue infection can be asymptomatic or manifest itself in Dengue fever (DF) or Dengue Hemorrhagic Fever (DHF), which involves a rapid onset of capillary leakage leading to Dengue Shock Syndrome (DSS). Severity of DF symptoms usually depends on age of the patient and recurrence of inoculation with a different DV serotype. Younger children and people who have not had a DV infection often encounter the milder symptoms but the disease could still be fatal. Symptoms include high fevers (40 °C), headaches, myalgia, arthralgia, nausea, lymphadenopathy, and rash (Whitehorn, 2011). High fevers result in dehydration and could cause convulsions or other neurological trouble. A severe case of DF presents thrombocytopenia (low platelet counts) and leucopenia (low white blood cell count), which can be observed with hemorrhage complications usually present in the gums, epistaxis (nose), and gastrointestinal tract. Symptoms usually emerge 4-10 days after DV inoculation and last 2-7 days.

DHF typically presents itself in people that are immunocompromised and are exposed to a second infection with a different DV serotype (Guzman, 2003; Effler, 2005). Patients suffering from DHF often present high fevers, severe abdominal pain with

persistent/bloody vomit, plasma leakage resulting in high hematocrit concentration (increase of 20%), and hepatomegaly. Although, both DHF and DF share certain symptoms, severity among them differs. Symptoms usually progress together leading to dehydration, fluid accumulation, and eventually circulatory system failure reaching the critical stage (DSS). At that time, temperatures usually decrease (37.5-38 °C) and depending on degree of capillary permeability patient might recover or go into shock. After DSS episode, the patient usually dies within 12-24 hours or recovers with appropriate intravenous fluid replacement therapy. Patients experiencing DSS have a 20% mortality rate in places with insufficient resources or expertise but can be as low as 1% when treatment is applied early (Anne, 2013).

1.2 Treatment and Prevention

Currently, there are no approved vaccines or therapeutic treatments for DV infections; although, there are some promising vaccine candidates in phase 2 of clinical studies. These vaccines aspire to produce immunogenicity against all four DV serotypes (tetravalent), while decreasing DHF/DSS susceptibility to second DV different serotype infection (del Angel, 2013) and being relatively inexpensive to produce due to socioeconomic status of endemic areas. The leading candidate (phase 3 clinical trials) is a Chimeric vaccine (known as ChimeriVax or CYD TDV), which swapped the preM and E genes of yellow fever 17D virus for those of DV 1-4 (Guy, 2011). Other candidates include live attenuated, inactivated, and subunit (protein/DNA/viral-vector) vaccines with decreasing immunogenicity response, respectively (Webster, 2009; Lee 2012). Until approval of a specific and effective vaccine or therapeutic treatment, the recommended treatment regime consists of rest, plenty of water and paracetamol for fever and pain

alleviation. Preventative measures involve mosquito control strategies by environmental control (minimizing mosquito breeding areas), biological control (predation), and if necessary chemical control (insecticides) (WHO, 2014).

1.3 Life cycle

Dengue virus is transmitted primarily by *Aedes* genus mosquitoes with *Aedes aegypti* (*A. aegypti*) being the most prominent and other mosquitoes (e.g. *Aedes albopictus*) less frequent vectors. *Aedes aegypti* is highly successful in urbanized areas utilizing both man-made and natural sites as breeding areas (Rott, 2010). These mosquitoes are highly resourceful vectors with immature development occurring in man-made containers (e.g. jars, tires, flowerpots, cans) and adult females residing inside houses where they feed exclusively on human blood. For one gonotrophic cycle female mosquitoes require multiple blood meals. Due to this necessity and behavior, *A. aegypti* is able to transmit DV to multiple human hosts (Mosquitoes, 2009). DV cycles between the vector to humans and other non-human primates with vertical transmission to mosquito progeny. An uninfected *A. aegypti* can become infected by feeding on a blood meal with high titers of DV; in humans high titers develop 4-7 days post inoculation. This results in replication of the virus in the epithelial cells of the midgut and eventual spread to the salivary glands where it can infect the next blood meal after an extrinsic incubation time of 10-14 days, due to inefficient replication in *A. aegypti* (Dengue, 2009).

1.4 Epidemiology

The first documented cases of Dengue goes back to the Chin Dynasty (265-420 A.D.) believed to be attributed to flying insects from "poisoned water" (Gubler, 1998). Since then, the virus has been spread to more than 100 countries in the tropical and

subtropical areas of the world (e.g. Southeast Asia, Latin America, the Caribbean, and Mediterranean). Annually, there are ~50 to 100 million reported cases of infection per year with 500,000 of them developing DHF symptoms (Guzman, 2010). The geographical range of the virus is attributed to the spread of the vector, *Aedes* genus mosquitoes, which dispersed from Africa to Asia and later to the rest of the world through increased globalization and global travel (Simmons, 2012). It is thought that rapid development and weak infrastructure in healthcare increases the fitness of *A. aegypti* mosquitoes by providing a breeding sites and dense population of humans to feed on. Although, these mosquitoes are adapted for highly dense urban settings, there has been a movement toward rural areas due to improper vector control.

In Southeast Asia and the Pacific, more than half of the world's population is at risk of DV infections (leading cause of death in children) with several epidemics already occurring in tropical areas (e.g. Indonesia, Thailand, Cambodia) and spreading to deciduous areas (e.g. Bangladesh, India, etc). It is important to mention that all four serotypes are native in this region (DV-1 (1977), DV-2 (1981), DV-4 (1981), and DV-3 (1994)) (Gubler, 1998). In Indonesia (2007) and western pacific countries (2001-2008) 150,000 and approximately 1 million cases were reported, respectively. In both examples, fatality rates were no greater than 1% (WHO, 2009). Outbreaks of Dengue have been documented in African countries but surveillance data is incomplete.

Early eradication campaign of *A. aegypti* (1960-1970s) slowed the dispersion of DV in the Americas. Currently, outbreaks are prominently seen in the tropical regions where mosquito control is not maintained (Guzman, 2002; Kay, 2005). Throughout 2001 to 2007, 30 countries in the Americas reported about 4.3 million cases of dengue

infection with 106,037 developing into DHF (fatality rate 1.2%). Brazil, a highly urbanized country surrounded by rainforest, reported about 64% of the total dengue infections and had the highest fatality rate (WHO, 2009) with 700,000 cases reported per year (Figueiredo, 2012). The Andean countries (19%) reported the highest cases of DHF (61,341) with Central American (13%), Caribbean (3.9%), and North American (<0.1%) reporting lower case numbers (WHO, 2009). Aside from small epidemics occurring in Hawaii, most cases of DV infections in North America are imported from travelers.

The epidemiology studies (Guzman, 2003; Effler, 2005; Halstead, 2007) in Hawaii, Singapore, and Cuba show outbreaks of Dengue in island systems. Within these virgin systems, first exposure (DV-1 in Cuba) generally resulted in the mild disease of DF. After 4 years, a different serotype (DV-2) was introduced and resulted with higher incidences of DHF/DSS, especially previously exposed to DV-1, with long-interval secondary exposures resulting in more severe symptoms. Also, there were higher incidences of DHF/DSS in infants that were never exposed to DV but where the mother had been infected and produced antibodies against the specific DV serotype (Whitehorn, 2011; Kliks, 1988). Conclusions from these results support the theory of ADE and progressive loss of cross neutralizing antibodies with different serotypes of dengue.

1.5 Pathogenesis

Mosquito inoculation of DV eventually infects and replicates in LDC, which transports the DV antigens to the nearest lymph node, presenting to and activating passing innate cells. Innate cells (specifically monocytes and macrophages) travel to the infection site in attempts of killing the virus and any infected cells, but instead get infected by the virus (Ubol, 2010; Murphy, 2011). Throughout the innate immune

response infected cells produce interferons and other cytokines to prepare defenses of other cells causing initial DV symptoms. Infected innate cells disseminate the virus throughout the body, while infecting other cells and increasing DV viremia. After activation of adaptive immune system, CD8+ cytolytic T-cells along with B-cell production of IgM and IgG antibodies help kill and neutralize specific DV serotype. Both memory B and CD4+ T-cells recognize and can neutralize to homotypic (same serotype) DV infections for a lifetime and heterotypic (different serotype) DV infections for 3-4 months (Beltramello, 2010; Anne, 2013).

After the short period of protection, secondary heterotypic DV infections have been seen to produce more cases of the severe symptoms (DFH/DSS) in a phenomenon coined ADE (Halstead, 1988). In secondary heterotypic DV infections, antibodies from the first infection are able to recognize and bind to DV but fail to destroy the virus. These sub-neutralizing antibodies facilitate binding to monocytes through the Fcγ regions of the antibodies and Fcγ receptor (FcγR) on immune cells, increasing virus uptake and replication, consequently increasing severity of disease (Wan, 2013). Similarly, activation of memory T-cells generates a non-specific inflation of cytotoxic T-cell response incapable of clearing current DV serotype infection. In addition, heighten T-cell activation results in an exaggerated cytokine production, such as IFNγ, TNFα, and IL 2, 6, 8, and 10 (Guzman, 2002), which is suspected of increasing vascular permeability and tissue damage leading to plasma leakage.

1.6 Antibody-Based Therapeutics Against DV: E60

Initially raised against WNV, E60 mAb demonstrated cross reactivity and neutralizing activities against DII fusion loop on the E protein of DV (Oliphant, 2006; Williams, 2013; Brien, 2013). The fusion loop is highly conserved among all DV serotypes (Gromowski, 2008; VanBlargan, 2013). Early work modified E60 from a mouse IgG2a mAb into an E60 chimeric human IgG1 mAb with a mutation in position 297 (asparagine to glutamine) in Fcγ region (Balsitis, 2010). Administration of E60-chimeric Hu-IgG1 mAb (50 μg) in addition to anti-DV1 serum (to simulate secondary heterotypic DV exposure) 48 hours after initial infection resulted in 80% survival in mice demonstrating therapeutic efficacy (Balsitis, 2010). Similar to E16 mAb, E60 inhibits fusion of the virus to the endosomal membranes after initial binding to receptors for endocytosis (Costin, 2013; Lok 2008). This inhibitory step prevents the virus from gaining access to the translational and replicative machinery of the cell by holding it within the endosome (Thompson, 2009).

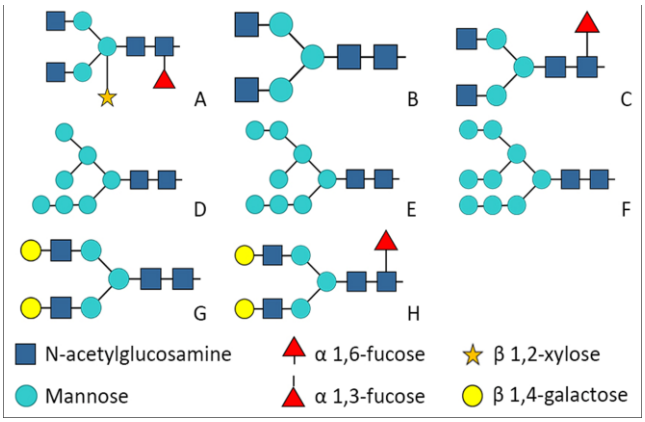


Figure 10: Plant and mammalian glycosylation forms for pE60 in transgenic plants. (A) wild type GnGnXF6, (B) GnGn, (C) GnGnF6, (D-F) high mannose forms Man7-9, (G) AA, (H) AAF6.

The glycosylation patterns and structure of an immunoglobulin molecule have a great impact on its effector function (Arnold, 2007). Specifically, different N-glycosylation patterns to the Fcγ region of CH2 of IgG1 can determine its CDC and ADCC, and ADE activity (Zheng, 2011). Aglycosylated mAb can prevent the binding of

IgG1 to the FcγR, and in turn, eliminating ADE. However, aglycosylated mAb also lost all its CDC activity that may be necessary for its full therapeutic activity. Furthermore, aglycosylation can also cripple the stability of the mAb, thus decreasing the half-life of the molecule (Zheng, 2011; Kayser, 2010). Wild type (WT) and glycoengineered *N. benthamiana* plant lines were used to test the hypotheses that specific glycosylation patterns can reduce or eliminate ADE response; while maintaining the necessary CDC activity for mAb efficacy against DV. Analyzing effects of glycosylation of IgG on ADCC/CDC activity will also provide answers for the basic question of how carbohydrate moieties affect antibody effector functions. Thus, different glycoforms of E60 including WT GnGnXF3, GnGn, GnGnF6, high mannose, AA, AAF6 are being produced in *N. benthamiana* plants (**Figure 10**). Plant-produced E60 was characterized as well as investigated for specific binding (*in vitro* and *in vivo*) to E protein and neutralization activity with the aim of developing an effective of anti-DV therapeutics with enhanced safety.

2. Results and Discussion

2.1 Molecular Design

The humanized E60 VH coding sequence was optimized with tobacco codons and fused with human IgG1 CH1-3 sequence and VL to human kappa CL. Both molecular constructs were preceded with a Kozac and leader sequence to enhance translation and for ER targeting, respectively. In both constructs *Eco*RI and *Bam*HI (5' and 3', respectively) restriction sites bordered the gene for ease of sub-cloning. The longer HC was cloned into 3' TMV vector while the shorter LC was cloned into 3' PVX vector for

MagnICON plant expression due to 3'TMVs has a stronger promoter compared to 3'PVX (**Figure 11**).

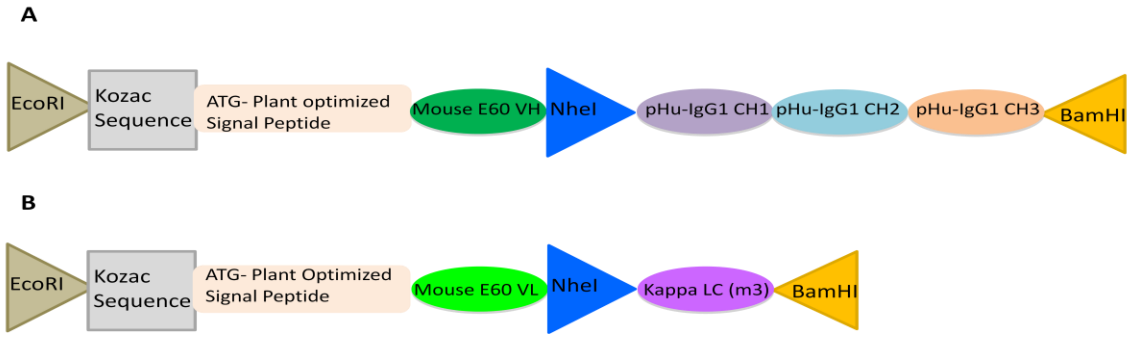


Figure 11: Schematic of pE60 gene constructs. (A) pE60 HC-IgG1, and (B) pE60 LC-IgG1.

2.2 Molecular Cloning

Overlap PCR was utilized to add in the *Eco*RI site, Kozac sequence, start codon, intronless signal peptide to the 5' end, and a *Nhe*I site to the 3' end of both E60 VH (pCI neo-chE60hG1) and E60 VL (pCI neo-chE60Lc). PCR products were cloned into Topo2.1 vector (antibiotic resistant against ampicillin) and transformed into DH5α (Topo VH and Topo VL).

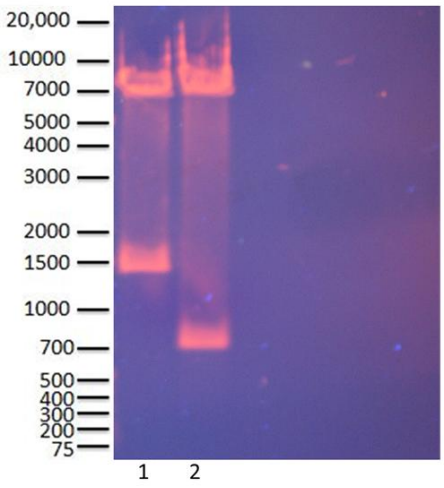


Figure 12: Analytical agarose gel of constructed E60 IgG1 cut with EcoRI and BamHI. Lane 1: E60 IgG1 HC in 3' TMV; Lane 2: E60 IgG1 LC in 3' PVX.

The construction of the LC involved a three way ligation between TOPO VL (*Eco*RI/ *Nhe*I), plant codon optimized human Kappa LC (*Nhe*I/*Bam*HI) and the 3' PVX vector (*Eco*RI/*Bam*HI). For the HC, the three

way ligation was between TOPO VH (*Eco*RI/ *Nhe*I); plant optimized human IgG1 CH1-3 region (*Nhe*I/*Bam*HI) and the 3' TMV vector (*Eco*RI/*Bam*HI). The only difference in the ligation method is the molar ratio (1:2:2, vector: insert 1: insert 2). Once the constructs were verified through gel analysis (**Figure 12**) and sequence confirmation (**Figure 13, in Appendix C**), the DNA was transformed into *A. tumefaciens* (electroporator settings: 2.20 kV and 5.80 milliseconds) (new strains 485 (E60 HC) and 486 (E60 LC)).

2.3 Plant expression

All MagnICON modules were cultured individually in YENB media (0.75% Bacto yeast extract, 0.8% Nutrient Broth, and pH 7.5) plus appropriate antibiotics in RB tubes at 30 °C in a shaker (300 rpm). Depending on the type of agroinfiltration (syringe or vacuum) and the amount of plants that are going to be infiltrated, volumes will vary with optimal concentrations determined by empirical experimentation (He, 2014). For the following pE60 analysis results, the *A. tumefaciens* concentration used was 0.12 for each construct, totaling a final concentration of 0.60 OD_{600}. Both WT and GnGn six week old plants were infiltrated for p E60 expression. After 4 days post infiltration (dpi), necrosis of the leaf became apparent so samples were gently cut from infiltrated leaf tissue. For small scale estimation of expression levels, samples were weighed to 0.3 gram leaf fresh weight (LFW), placed in microfuge tubes with a scoop of copper beads for plant leaf homogenization, and were immediately stored in -80 °C until usage. For larger scale extraction, leaves were either stored in -80 °C or processed with 30 min of harvest.

2.3.1 Extraction of pE60 from *N. benthamiana* Leaves

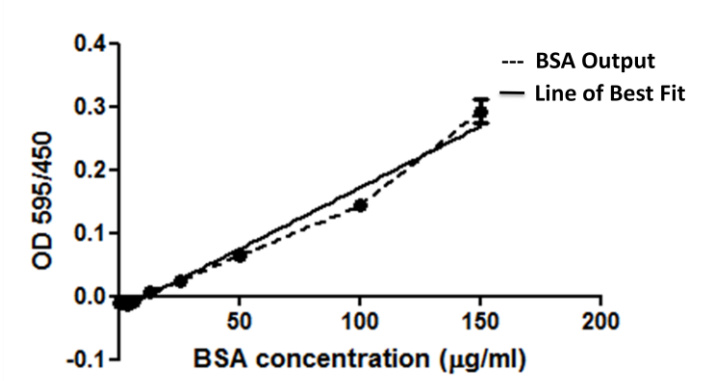
Leaf samples were homogenized at 4 °C (to reduce protein degradation) in extraction buffer (100mM Tris-HCl, pH 8.0, 150mM NaCL, 1mM PMSF, tablet protease

inhibitor cocktail) at a 1:1.5 ratio of LFW (g) to buffer (mL). Samples were repeatedly centrifuged at 10 kGs for 15 minutes with supernatant transferred to clean microfuge tubes until extract was clear. Clear extracts were used to initially estimate total protein with Bradford protein assay, characterize pE60 with Western Blot analysis, and binding specificity with E protein binding ELISA. pE60 was purified by ammonium sulfate precipitation and protein A chromatography (by Matthew Dent from ASU). Purified samples were used for yeast binding (performed by Huafang Lai) and neutralization assays (performed by Dr. Michael Diamond lab from Washington University School of Medicine).

2.3.2 Expression Level Quantitation by ELISA

To analyze the total soluble protein we utilized a Bradford protein assay. Regular (non-high binding) ELISA plates were loaded with diluted plant extracts (40x) with 5 μL going into each well. BSA (positive control) was diluted with PBS (137mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4,) at the following concentrations (μg/ml): 150, 100, 50, 25, 12.5, 6.25, 3.125, and 0. The Bradford reagent was also diluted (specified by the manufacturer) and 160 μL was added to each well. The samples were read at 450 and 595 nm with the ratio (595/450) plotted against the known concentrations of BSA (μg/mL). The crude pE60 ratio was 0.05725 which when calculated suggests the

total protein concentration of pE60 is about 4 mg/mL (**Figure 14**).



Line of Best Fit Formula	A	B	R^2
Y=A*X+B	0.001944	-0.02177	0.98

Figure 14: Bradford assay OD595/450 versus BSA concentration. Dilutions of BSA as well as crude pE60 extract (1:40 diluted) was pippetted into plate. Total protein was detected with Bradford's Reagent. Mean ± SD represents duplicate samples.

2.4 Structural and Functional Characterization of Plant-Produced E60.

2.4.1 Proper Assembly of pE60 Analyzed by SDS PAGE and Western Blot

The pE60 samples were ran on a 4-10% SDS PAGE under reducing and non-reducing conditions for 2 hours at 100V. Gels with purified pE60 were stained with Coomassie Blue (**Figure 15A)** and gels loaded with crude pE60 extracts were transferred to PVDF membrane at 80 mA overnight in 4 °C. After transfer, the membranes were blocked with 5% milk in PBST for 2 hours. After blocking, the membranes were washed with PBST and probed with 1% milk diluted HRP-conjugated goat anti-human kappa LC (Southern Biotech) for an hour intended to detect the light chain. ECL reagent was added and developed on x-ray film (**Figure 15B**). Our results indicate that E60 was produced in plants with the expected HC and LC, and they efficiently assemble into the tetrameric

structure. In addition, they can be purified to high homogeneity by a two-step processing scheme.

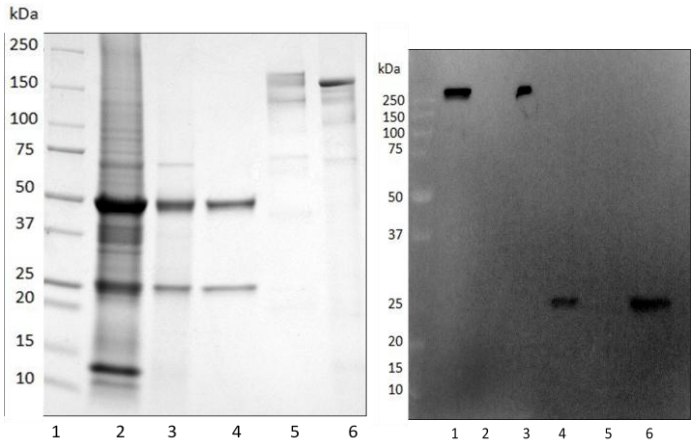


Figure 15: Characterization of pE60 with SDS PAGE (A) and Western blot (B) under reducing (R) and non-reducing (NR) conditions. (A) Molecular weight marker (lane 1), flow through (lane 2), pE60 (lane 3), mE16 (lane 4), mE16 (NR) (lane 5), and pE60 (NR) (lane) were ran on SDS-PAGE gel and stained with Coomassie Blue. (B) pE60 (NR) (lane 1), negative control (lane 2), mE16 (NR) (lane 3), pE60 (R) (lane 4), negative control (lane 5), and mE16 (R) (lane 7) ran on SDS PAGE and transfer to PVDF. The blot was incubated with HRP-conjugated goat anti-human kappa for detection.

2.4.2 Specific Binding to its Antigen Measured by ELISA

To test if pE60 specific binding to its antigen, high binding ELISA plates were coated with 1 μg/mL of purified WNV E protein in carbonate buffer (100 mM Na_2CO_3, pH 9.6) overnight at 37 °C for 4 hours. The plates were washed three times with PBST and blocked with 5% milk in PBST. Plant extracts were diluted 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 with 1% milk in PBST. Purified plant E16 ((ng/ml): 2000, 1000, 500, 250, 125, 62.5, and 31.25) was used as a positive control to generate the standard curve and a generic IgG was used as the negative control. All samples were incubated for 2 hours at

37 °C. After washing, the plates were incubated with HRP-conjugated anti-human-kappa LC antibody. The plates were then developed with TMB substrate, stopped with 1 M phosphoric acid, and read at 450 nm. The results show specific binding of pE60 mAb to WNV E protein. It appeared that the binding affinity of pE60 was lower than that of pHu-E16 mAb (**Figure 16**). However, it is most likely that this discrepancy is due to the different concentration of the two mAbs used in the assay.

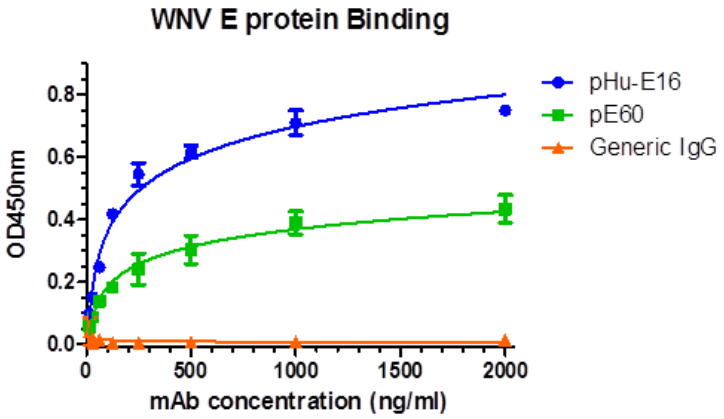


Figure 16: Specific binding ELISA of pE60 to WNV E protein. Serial dilutions of pE60 were incubated in sample wells coated with WNV E protein and detected with an HRP-conjugated anti-human kappa antibody.pHu-E16 was used as positive control and a generic human IgG was used as a negative control. Mean ± SD of samples from triplicates values.

2.4.3 Specific Binding to its Antigen Measured by Flow Cytometry

To confirm the ability of pE60 in recognize DV DII in its native conformation, as expressed in DV, recombinant yeast cells expressing DV2 DI-DII were taken from the log phase and incubated in tryptophan free media containing 2% galactose to induce expression of DI-DII. Positive yeast cells were incubated in mE60 (positive control), pE60 (WT and GnGn), stained with Alexa Fluor 467 (Invitrogen), and subject to analysis by BD FACS Calibur flow cytometer. The yeast binding assay demonstrated that pE60 (WT and GnGn) has the same specific binding as mE60 (**Figure 17**).

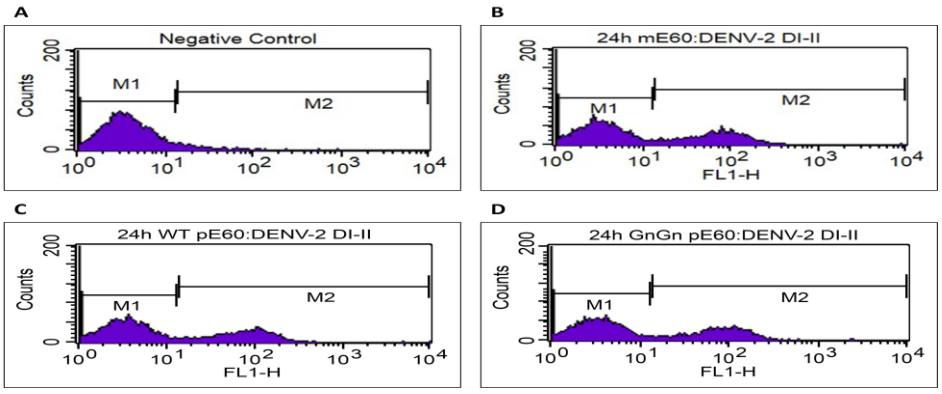


Figure 17: Binding of E60 mAb to DI-DII displayed on yeast cell surface. Recombinant yeast cells were incubated in (A) PBS, (B) mE60 (positive control), (c) WT pE60, and (D) GnGn pE60. After incubation, yeast cells were stained with an Alexa Fluor -467 and analyzed with FACS Calibur flow cytometer.

2.4.4 Neutralization of DV by plant-produced E60

DV2 was initially incubated with a serial dilution of pE60 (WT or GnGn) and mE60 (positive control). Uninfected Vero cells were incubated in each sample, later fixed and permeabilized. Plaques were quantified by focus reduction assay and biospot analysis. The neutralization assay demonstrated that pE60 (WT and GnGn) as well as mE60 were able to neutralize DV with full neutralization occurring when concentrations were above 1000 ng/mL (**Figure 18**).

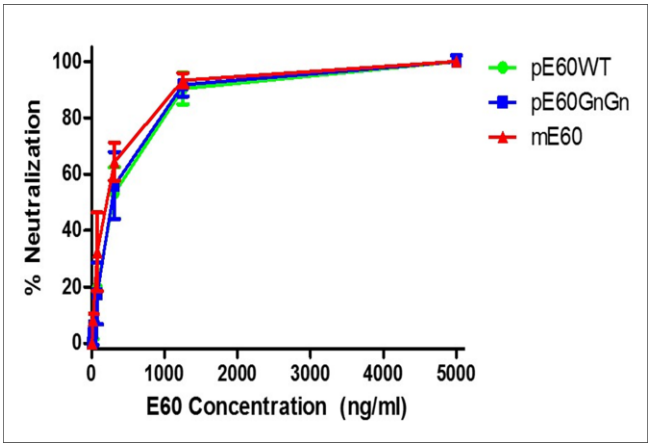


Figure 18: Neutralization of DV by pE60 variants. DV was incubated with serial dilutions of mE60 as positive control, pE60 (WT and GnGn) and used to infect Vero cells. Resulting plaques were analyzed by focus reduction assay and Biospot analysis.

3. Conclusions and future directions

Herein, we produced E60 chimeric human IgG1 mAb against all serotypes of DV in two different glycoforms (WT and GnGn) utilizing plant transient expression in *N. benthamiana*. Our results to date show that the feasibility of utilizing plant expression systems to produce great quantities (> 500ug/g FLW) of different glycoforms of pE60. In addition, both glycoforms of pE60 were able to assemble efficiently and demonstrated specific binding to the fusion loop on DII and while retaining neutralization activity seen in the mammalian cell-produced counterpart. Almost full neutralization of DV2 was achieved at concentration greater than 1000 ng/mL for both pE60 and mE60. These results indicate that plant-produced E60 is a promising candidate for developing efficacious therapeutics against DV. The next step would be to test the different glycoforms for ADCC, CDC, and ADE response in vitro and ultimately in vivo in a mouse model to demonstrate its enhanced safety over the mammalian cell-produced E60.

REFERENCES

Anne Tuiskunen Bäck, & Åke Lundkvist. (2013). Dengue viruses - an overview. Infection Ecology & Epidemiology, 3, 1-21.

Arnold, J. N., Wormald, M. R., Sim, R. B., Rudd, P. M., & Dwek, R. A. (2007). The impact of glycosylation on the biological function and structure of human immunoglobulins. Annual Review of Immunology, 25(1), 21-50. doi:10.1146/annurev.immunol.25.022106.141702

Balsitis, S. J., Harris, E., Williams, K. L., Lachica, R., Flores, D., Kyle, J. L., . . . Beatty, P. R. (2010). Lethal antibody enhancement of dengue disease in mice is prevented by fc modification. PLoS Pathogens, 6(2), e1000790. doi:10.1371/journal.ppat.1000790

Beltramello M, W.K., Simmons CP, Macagno A, Simonelli L, Quyen NT, Sukupolvi-Petty S, Navarro-Sanchez E, Young PR, de Silva AM, Rey FA, Varani L, Whitehead SS, Diamond MS, Harris E, Lanzavecchia A, Sallusto F. The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. Cell Host Microbe. 8, 271-283 (2010).

Both, L., Banyard, A. C., van Dolleweerd, C., Wright, E., Ma, J. K., & Fooks, A. R. (2013). Monoclonal antibodies for prophylactic and therapeutic use against viral infections.Vaccine, 31(12), 1553. doi:10.1016/j.vaccine.2013.01.025

Brien, J. D., Sukupolvi-Petty, S., Williams, K. L., Lam, C. K., Schmid, M. A., Johnson, S., . . . Diamond, M. S. (2013). Protection by immunoglobulin dual-affinity retargeting antibodies against dengue virus. *Journal of Virology, 87*(13), 7747-7753. doi:10.1128/JVI.00327-13

Brinton, M. A. (2014). Replication cycle and molecular biology of the west nile virus. Viruses, 6(1), 13-53. doi:10.3390/v6010013

Calisher, C. H., Karabatsos, N., Dalrymple, J. M., Shope, R. E., Porterfield, J. S., Westaway, E. G., & Brandt, W. E. (1989). Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. Journal of General Virology, 70(1), 37-43. doi:10.1099/0022-1317-70-1-37

Campbell, G. L., Marfin, A. A., Lanciotti, R. S., & Gubler, D. J. (2002). West nile virus. The Lancet Infectious Diseases, 2(9), 519-529. doi:10.1016/S1473-3099(02)00368-7

Chambers, T. J., Hahn, C. S., Galler, R., & Rice, C. M. (1990). Flavivirus genome organization, expression, and replication.Annual Review of Microbiology, 44(1), 649-688. doi:10.1146/annurev.mi.44.100190.003245

Chen Q. (2011a) Expression and Manufacture of Pharmaceutical Proteins in Genetically Engineered Horticultural Plants. In: Mou B and Scorza R (ed) Transgenic Horticultural Crops: Challenges and Opportunities, 1st edn., Vol. 1, pp. 83-113. CRC Press, Boca Raton.

Chen Q., He J., Phoolcharoen W. et al. (2011b) Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. Human Vaccines 7, 331-8.

Chen Q., Lai H., Hurtado J. et al. (2013) Agroinfiltration as an Effective and Scalable Strategy of Gene Delivery for Production of Pharmaceutical Proteins. Advanced Techniques in Biology and Medicine 1, 2.

Chen Q., Mason H. S., Mor T. et al. (2009) Subunit Vaccines Produced Using Plant Biotechnology. In: (ed) New Generation Vaccines, edn., Vol. pp. 306-14.

Cho, H., & Diamond, M. S. (2012). Immune responses to west nile virus infection in the central nervous system. Viruses,4(12), 3812-3830. doi:10.3390/v4123812

Costin, J. M., Fong, R. H., Yang, S., Lin, L., Robinson, J. E., Doranz, B. J., . . . Figueroa Sierra, M. (2013). Mechanistic study of broadly neutralizing human monoclonal antibodies against dengue virus that target the fusion loop. Journal of Virology,87(1), 52.

Dauphin, G., Zientara, S., Zeller, H., & Murgue, B. (2005). Corrigendum to "West nile: Worldwide current situation in animals and humans" [comparative immunology microbiology & infectious diseases 27 (2004) 343–355]. Comparative Immunology, Microbiology and Infectious Diseases, 28(3), 249-250. doi:10.1016/j.cimid.2004.11.001

del Angel, R. M., & Reyes-del Valle, J. (2013). Dengue vaccines: Strongly sought but not a reality just yet. PLoS Pathogens, 9(10), e1003551.

Dengue (2009). Elsevier Science & Technology.

Diamond, M. S. (2003). Evasion of innate and adaptive immunity by flaviviruses. Immunology and Cell Biology, 81(3), 196-206. doi:10.1046/j.1440-1711.2003.01157.x

Diamond, M. S. (2009a). Virus and host determinants of west nile virus pathogenesis. PLoS Pathogens, 5(6), e1000452. doi:10.1371/journal.ppat.1000452

Diamond, M. S. (2009b). Progress on the development of therapeutics against west nile virus. Antiviral Research, 83(3), 214-227. doi:10.1016/j.antiviral.2009.05.006

Dowd, K. A., Jost, C. A., Durbin, A. P., Whitehead, S. S., & Pierson, T. C. (2011). A dynamic landscape for antibody binding modulates antibody-mediated neutralization

of west nile virus. PLoS Pathogens, 7(6), e1002111. doi:10.1371/journal.ppat.1002111

Effler, P. V., Rigau-Perez, J. G., Hayes, J. M., Mills, K., Napier, M., Clark, G. G., . . . Hawaii Dengue Outbreak Investigation Team. (2005). Dengue fever, hawaii, 2001-2002. Emerging Infectious Diseases, 11(5), 742-749. doi:10.3201/eid1105.041063

Eleniste, P. P., Hofstetter, H., & Hofstetter, O. (2013). Expression and characterization of an enantioselective antigen-binding fragment directed against α-amino acids.Protein Expression and Purification, 91(1), 20-29. doi:10.1016/j.pep.2013.06.010

Estrada-Franco, J. G., Cortes, A. C., Ramírez, P. P., Tesh, R. B., Barrett, A. D. T., Weaver, S. C., . . . Ludwig, G. V. (2003). West nile virus in mexico: Evidence of widespread circulation since july 2002. Emerging Infectious Diseases, 9(12), 1604.

Farajollahi, A., Fonseca, D. M., Kramer, L. D., & Marm Kilpatrick, A. (2011). "Bird biting" mosquitoes and human disease: A review of the role of culex pipiens complex mosquitoes in epidemiology. Infection, Genetics and Evolution, 11(7), 1577-1585. doi:10.1016/j.meegid.2011.08.013

Faye L. and Gomord V. (2010) Success stories in molecular farming—a brief overview. Plant Biotechnology Journal 8, 525-8.

Figueiredo, L. T. M. (2012). Dengue in brazil. Revista Da Sociedade Brasileira De Medicina Tropical, 45(3), 285.

Gleba Y., Marillonnet S. and Klimyuk V. (2004) Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. Current Opinion in Plant Biology 7, 182-8.

Goodman, D. M. (2012). West nile virus. Jama, 308(10), 1052.

Gould, E. A., & Solomon, T. (2008). Pathogenic flaviviruses.Lancet, 371(9611), 500-509. doi:10.1016/S0140-6736(08)60238-X

Gromowski, G. D., Barrett, N. D., & Barrett, A. D. T. (2008). Characterization of dengue virus complex-specific neutralizing epitopes on envelope protein domain III of dengue 2 virus. The Journal of Virology, 82(17), 8828-8837. doi:10.1128/JVI.00606-08

Gubler, D. J. (1998). Dengue and dengue hemorrhagic fever.Clinical Microbiology Reviews, 11(3), 480-496.

Guy, B., Barrere, B., Malinowski, C., Saville, M., Teyssou, R., & Lang, J. (2011). From research to phase III: Preclinical, industrial and clinical development of the

sanofi pasteur tetravalent dengue vaccine. Vaccine, 29(42), 7229-7241. doi:10.1016/j.vaccine.2011.06.094

Guzmán, M. G., & Kourí, G. (2002). Dengue: An update. The Lancet Infectious Diseases, 2(1), 33-42. doi:10.1016/S1473-3099(01)00171-2

Guzman, M. G., Martínez, E., Nathan, M. B., Pelegrino, J. L., Simmons, C., Yoksan, S., . . . Margolis, H. S. (2010). Dengue: A continuing global threat. Nature Reviews. Microbiology, 8(12 Suppl), S7-S16. doi:10.1038/nrmicro2460

Guzman, M., #x0301, a G, & Kouri, G. (2003). Dengue and dengue hemorrhagic fever in the americas: Lessons and challenges. Journal of Clinical Virology, 27(1), 1-13. doi:10.1016/S1386-6532(03)00010-6

Halstead, S. B. (2007). Dengue. The Lancet, 370(9599), 1644-1652. doi:10.1016/S0140-6736(07)61687-0

Halstead, S.B. Pathogenesis of dengue: challenges to molecular biology. Science 239, 476-481 (1988).

Han, L. L., Tsai, T. F., Popovici, F., Alexander, J., J P, Laurentia, V., Tengelsen, L. A., . . . Campbell, G. L. (1999). Risk factors for west nile virus infection and meningoencephalitis, romania, 1996. The Journal of Infectious Diseases, 179(1), 230-233. doi:10.1086/314566

Harris L.J, Skaletsky E, & Wilson A.I.A. (1998). Crystallographic structure of an intact IgG1 monoclonal antibody. Journal of Molecular Biology, 275(5), 861-861.

Hayes, E. B., Komar, N., Nasci, R. S., Montgomery, S. P., O'Leary, D. R., & Campbell, G. L. (2005). Epidemiology and transmission dynamics of west nile virus disease. Emerging Infectious Diseases, 11(8), 1167-1173. doi:10.3201/eid1108.050289a

He J., Lai H., Brock C. et al. (2012) A Novel System for Rapid and Cost-Effective Production of Detection and Diagnostic Reagents of West Nile Virus in Plants. Journal of Biomedicine and Biotechnology 2012,

He, J., Lai, H., Engle, M., Gorlatov, S., Gruber, C., Steinkellner, H., . . . Chen, Q. (2014). Generation and analysis of novel plant-derived antibody-based therapeutic molecules against west nile virus. PloS One, 9(3), e93541.

Heinz, F. X., & Stiasny, K. (2012). Flaviviruses and flavivirus vaccines. Vaccine, 30(29), 4301. doi:10.1016/j.vaccine.2011.09.114

Huang Z., Phoolcharoen W., Lai H. et al. (2010) High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. Biotechnology and Bioengineering 106, 9-17.

Juan Gea-Banacloche, Richard T. Johnson, Anto Bagic, John A. Butman, Patrick R. Murray, & Amy Guillet Agrawal. (2004). West nile virus: Pathogenesis and therapeutic options. Annals of Internal Medicine, 140(7), 545.

Kaufmann, B., Nybakken, G. E., Chipman, P. R., Zhang, W., Diamond, M. S., Fremont, D. H., . . . Rossmann, M. G. (2006). West nile virus in complex with the fab fragment of a neutralizing monoclonal antibody. Proceedings of the National Academy of Sciences of the United States of America, 103(33), 12400-12404. doi:10.1073/pnas.0603488103

Kay, B., & Nam, V. S. (2005). New strategy against aedes aegypti in vietnam. The Lancet, 365(9459), 613-617. doi:10.1016/S0140-6736(05)17913-6

Kayser, V. et al. Glycosylation influences on the aggregation propensity of therapeutic monoclonal antibodies. Biotechnology Journal 6, 38-44 (2010).

Kissel, K., Hamm, S., Schulz, M., Vecchi, A., Garlanda, C., & Engelhardt, B. (1998). Immunohistochemical localization of the murine transferrin receptor (TfR) on blood-tissue barriers using a novel anti-TfR monoclonal antibody. Histochemistry and Cell Biology, 110(1), 63-72. doi:10.1007/s004180050266

Kliks SC, N.S., Nisalak A, Burke DS. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. AmJ Trop Med Hyg 38, 411-419 (1988).

Kramer, L. D., Li, J., & Shi, P. (2007). West nile virus. Lancet Neurology, 6(2), 171-181. doi:10.1016/S1474-4422(07)70030-3

Kramer, L. D., Styer, L. M., & Ebel, G. D. (2008). A global perspective on the epidemiology of west nile virus. Annual Review of Entomology, 53(1), 61-81. doi:10.1146/annurev.ento.53.103106.093258

Kuhn, R. J., Strauss, E. G., Baker, T. S., Strauss, J. H., Zhang, W., Rossmann, M. G., . . . Chipman, P. R. (2002). Structure of dengue virus: Implications for flavivirus organization, maturation, and fusion. Cell, 108(5), 717.

Lai H. and Chen Q. (2012) Bioprocessing of plant-derived virus-like particles of Norwalk virus capsid protein under current Good Manufacture Practice regulations. Plant Cell Reports 31, 573-84.

Lawther, B. K., Kumar, S., & Krovvidi, H. (2011). Blood-brain barrier. Continuing Education in Anaesthesia, Critical Care & Pain, 11(4), 128-132. doi:10.1093/bjaceaccp/mkr018

Lee, H. J., Engelhardt, B., Lesley, J., Bickel, U., & Pardridge, W. M. (2000). Targeting rat anti-mouse transferrin receptor monoclonal antibodies through blood-

brain barrier in mouse. The Journal of Pharmacology and Experimental Therapeutics,292(3), 1048

Lee, H., Butler, M., & Wu, S. (2012). Using recombinant DNA technology for the development of live-attenuated dengue vaccines. Enzyme and Microbial Technology, 51(2), 67-72. doi:10.1016/j.enzmictec.2012.05.005

Lee, S. B., & Esteban, M. (1994). The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. Virology, 199(2), 491-496. doi:10.1006/viro.1994.1151

Leuzinger K., Dent M., Hurtado J. et al. (2013) Efficient Agroinfiltration of Plants for High-level Transient Expression of Recombinant Proteins. Journal of Visualized Experiments e50521.

Lim, S. M., Koraka, P., Osterhaus, Albert D M E, & Martina, B. E. E. (2011). West nile virus: Immunity and pathogenesis. Viruses, 3(6), 811-828. doi:10.3390/v3060811

Lok, S., Roehrig, J. T., Diamond, M. S., Kuhn, R. J., Rossmann, M. G., Kostyuchenko, V., . . . Chipman, P. R. (2008). Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins. Nature Structural & Molecular Biology,15(3), 312-317. doi:10.1038/nsmb.1382

Manich, G., Cabezón, I., del Valle, J., Duran-Vilaregut, J., Camins, A., Pallàs, M., . . . Vilaplana, J. (2013). Study of the transcytosis of an anti-transferrin receptor antibody with a fab' cargo across the blood-brain barrier in mice. European Journal of Pharmaceutical Sciences : Official Journal of the European Federation for Pharmaceutical Sciences, 49(4), 556-564. doi:10.1016/j.ejps.2013.05.027

Morrey, J. D., Diamond, M. S., Siddharthan, V., Olsen, A. L., Roper, G. Y., Wang, H., . . . Nordstrom, J. L. (2006). Humanized monoclonal antibody against west nile virus envelope protein administered after neuronal infection protects against lethal encephalitis in hamsters. The Journal of Infectious Diseases,194(9), 1300-1308. doi:10.1086/508293

Mosquitoes (2009). Elsevier Science & Technology.

Murphy, B. R., & Whitehead, S. S. (2011). Immune response to dengue virus and prospects for a vaccine. Annual Review of Immunology, 29(1), 587-619. doi:10.1146/annurev-immunol-031210-101315

Negrouk V., Eisner G., Lee H.-i. et al. (2005) Highly efficient transient expression of functional recombinant antibodies in lettuce. Plant Science 169, 433-8.

Nicole P Lindsey, Jennifer A Lehman, J Erin Staples, Marc Fischer, & Centers for Disease Control and Prevention (CDC). (2013). West nile virus and other arboviral

diseases--united states, 2012. MMWR. Morbidity and Mortality Weekly Report,62(25), 513-517.

Normile, D. (2013). Tropical medicine. surprising new dengue virus throws a spanner in disease control efforts. Science (New York, N.Y.), 342(6157), 415.

Nybakken, G. E., Oliphant, T., Johnson, S., Burke, S., Diamond, M. S., & Fremont, D. H. (2005). Structural basis of west nile virus neutralization by a therapeutic antibody. Nature, 437(7059), 764-769. doi:10.1038/nature03956

Oliphant, T., Chung, K. M., Ebel, G. D., Kramer, L. D., Fremont, D. H., Diamond, M. S., . . . Marri, A. (2005). Development of a humanized monoclonal antibody with therapeutic potential against west nile virus. Nature Medicine, 11(5), 522-530. doi:10.1038/nm1240

Oliphant, T., Fremont, D. H., Pierson, T. C., Diamond, M. S., Nybakken, G. E., Engle, M., . . . Olshevsky, U. (2006). Antibody recognition and neutralization determinants on domains I and II of west nile virus envelope protein. Journal of Virology,80(24), 12149-12159. doi:10.1128/JVI.01732-06

Pastorino, B., Nougairède, A., Wurtz, N., Gould, E., & de Lamballerie, X. (2010). Role of host cell factors in flavivirus infection: Implications for pathogenesis and development of antiviral drugs. Antiviral Research, 87(3), 281-294. doi:10.1016/j.antiviral.2010.04.014

Petersen, L. R., Marfin, A. A., & Gubler, D. J. (2003). West nile virus. JAMA : The Journal of the American Medical Association,290(4), 524-528. doi:10.1001/jama.290.4.524

RE Bird, M Whitlow, KD Hardman, JW Jacobson, S Johnson, BM Kaufman, . . . GS Riordan. (1988). Single-chain antigen-binding proteins. Science, 242(4877), 423-426. doi:10.1126/science.3140379

Reed, J. C. (2000). Mechanisms of apoptosis. American Journal of Pathology, 157(5), 1415-1430. doi:10.1016/S0002-9440(10)64779-7

Rott, M., Caumo, K., Sauter, I., Eckert, J., da Rosa, L., & da Silva, O. (2010). Susceptibility of aedes aegypti. Parasitology Research, 107(1), 195.

Samuel, M. A., & Diamond, M. S. (2006). Pathogenesis of west nile virus infection: A balance between virulence, innate and adaptive immunity, and viral evasion. Journal of Virology,80(19), 9349-9360. doi:10.1128/JVI.01122-06

Shi, P. Y., Li, W., & Brinton, M. A. (1996). Cell proteins bind specifically to west nile virus minus-strand 3' stem-loop RNA.Journal of Virology, 70(9), 6278-6287.

Shrestha, B., Pinto, A. K., Green, S., Bosch, I., & Diamond, M. S. (2012). CD8+ T cells use TRAIL to restrict west nile virus pathogenesis by controlling infection in neurons. Journal of Virology, 86(17), 8937-8948. doi:10.1128/JVI.00673-12

Silverman, R. H. (2007). Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. Journal of Virology, 81(23), 12720-12729. doi:10.1128/JVI.01471-07

Simmons, C. P., Farrar, J. J., Nguyen, v. V. C., & Wills, B. (2012). Dengue. The New England Journal of Medicine,366(15), 1423.

Sips, G. J., Wilschut, J., & Smit, J. M. (2012). Neuroinvasive flavivirus infections. Reviews in Medical Virology, 22(2), 69-87. doi:10.1002/rmv.712

Sohi H. H., Jourabchi E. and Khodabandeh M. (2005) Transient expression of human growth hormone in potato (Solanum tuberosum), tobacco (Nicotiana tobacum) and lettuce (Lactuca sativa) leaves by agroinfiltration Iranian Journal of Biotechnology 3, 109-13.

Suthar, M. S., Diamond, M. S., & Gale, J., Michael. (2013). West nile virus infection and immunity. Nature Reviews. Microbiology, 11(2), 115-128. doi:10.1038/nrmicro2950

Szretter, K. J., Brien, J. D., Thackray, L. B., Virgin, H. W., Cresswell, P., & Diamond, M. S. (2011). The interferon-inducible gene viperin restricts west nile virus pathogenesis. Journal of Virology,85(22), 11557-11566. doi:10.1128/JVI.05519-11

Thompson, B. S., Moesker, B., Smit, J. M., Wilschut, J., Diamond, M. S., Fremont, D. H., & Rey, F. A. (2009). A therapeutic antibody against west nile virus neutralizes infection by blocking fusion within endosomes. PLoS Pathogens, 5(5) doi:10.1371/journal.ppat.1000453

Tolle, M. A. (2009). Mosquito-borne diseases. Current Problems in Pediatric and Adolescent Health Care, 39(4), 97-140. doi:10.1016/j.cppeds.2009.01.001

Ubol, S., & Halstead, S. B. (2010). How innate immune mechanisms contribute to antibody-enhanced viral infections.Clinical and Vaccine Immunology : CVI, 17(12), 1829-1835. doi:10.1128/CVI.00316-10

VanBlargan, L. A., Mukherjee, S., Dowd, K. A., Durbin, A. P., Whitehead, S. S., & Pierson, T. C. (2013). The type-specific neutralizing antibody response elicited by a dengue vaccine candidate is focused on two amino acids of the envelope protein. PLoS Pathogens, 9(12), e1003761.

Wallach, D., Varfolomeev, E. E., Malinin, N. L., Goltsev, Y. V., Kovalenko, A. V., & Boldin, M. P. (1999). Tumor necrosis factor receptor and fas signaling

mechanisms. Annual Review of Immunology, 17(1), 331-367. doi:10.1146/annurev.immunol.17.1.331

Wan, S., Lin, C., Wang, S., Chen, Y., Yeh, T., Liu, H., . . . Lin, Y. (2013). Current progress in dengue vaccines. Journal of Biomedical Science, 20(1), 37-37. doi:10.1186/1423-0127-20-37

Wang, W., Singh, S., Zeng, D. L., King, K., & Nema, S. (2007). Antibody structure, instability, and formulation. Journal of Pharmaceutical Sciences, 96(1), 1-26. doi:10.1002/jps.20727

Webster, D. P., Farrar, J., & Rowland-Jones, S. (2009). Progress towards a dengue vaccine. Lancet Infect Dis, 9(11), 678-687. doi:10.1016/S1473-3099(09)70254-3

Weinberger, M., Kitzes, R., Siegman-Igra, Y., Giladi, M., Pick, N., Mendelson, E., . . . Mishal, J. (2001). West nile fever outbreak, israel, 2000: Epidemiologic aspects. Emerging Infectious Diseases, 7(4), 686-691.

Welte, T., Wang, T., Eden, W., Aronson, J., Gong, B., Rachamallu, A., . . . O'Brien, R. L. (2011). Vγ4+ T cells regulate host immune response to west nile virus infection. FEMS Immunology & Medical Microbiology, 63(2), 183-192. doi:10.1111/j.1574-695X.2011.00840.x

Whitehorn, J., & Simmons, C. P. (2011). The pathogenesis of dengue. Vaccine, 29(42), 7221-7228. doi:10.1016/j.vaccine.2011.07.022

WHO. Dengue: guidelines for diagnosis, treatment, prevention and control _ New ed. Geneva: World Health Organization; 2009.

Williams, K. L., Sukupolvi-Petty, S., Beltramello, M., Johnson, S., Sallusto, F., Lanzavecchia, A., . . . Fernandez-Sesma, A. (2013). Therapeutic efficacy of antibodies lacking FcγR against lethal dengue virus infection is due to neutralizing potency and blocking of enhancing antibodies. PLoS Pathogens, 9(2) doi:10.1371/journal.ppat.1003157

Wroblewski T., Tomczak A. and Michelmore R. (2005) Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. Plant Biotechnology Journal 3, 259-73.

Ying, T., Chen, W., Gong, R., Feng, Y., & Dimitrov, D. S. (2012). Soluble monomeric IgG1 fc. The Journal of Biological Chemistry, 287(23), 19399-19408. doi:10.1074/jbc.M112.368647

Zhang, Y., Rossmann, M. G., Corver, J., Chipman, P. R., Zhang, W., Pletnev, S. V., . . . Kuhn, R. J. (2003). Structures of immature flavivirus particles. The EMBO Journal, 22(11), 2604-2613. doi:10.1093/emboj/cdg270

Zheng, K., Bantog, C., & Bayer, R. (2011). The impact of glycosylation on monoclonal antibody conformation and stability. Mabs, 3(6), 568-576. doi:10.4161/mabs.3.6.17922

APPENDIX A

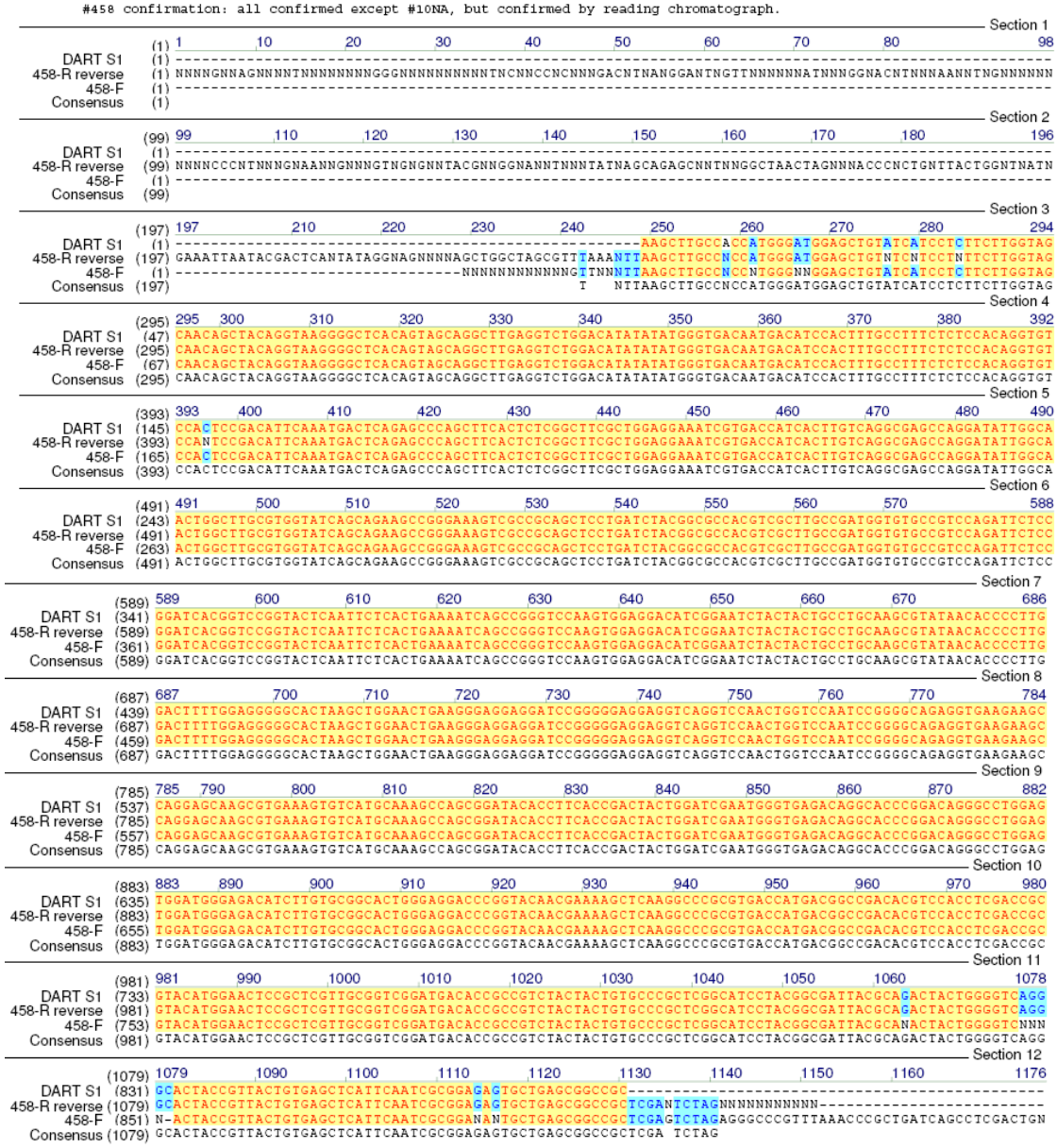
TABLE 1: DIABODY GENE CONSTRUCTS

Gene Construct	Strain number	Expected Size when cut with *Not*I/*Hind*III (Base Pair (bp))
VL8D3-VHE16-FNRGEC (Hygro)	458	881 and 5529
VLE16-VH8D3-VEPKSC (Hygro)	459	878 and 5529
VL8D3-VHE16-FNRGEC (Zeo)	460	881 and 4947
VLE16-VH8D3-VEPKSC (Zeo)	461	878 and 4947
VL8D3-VHE16-VEPKSC (Hygro)	467	881 and 5529
VLE16-VH8D3-FNRGEC (Hygro)	468	878 and 5529
VL8D3-VHE16-VEPKSC (Zeo)	469	881 and 4947
VLE16-VH8D3-FNRGEC (Zeo)	470	878 and 4947
pcDNA3.1/Hygro (+)	232	5529
pcDNA3.1-zeo (+)	263	4947

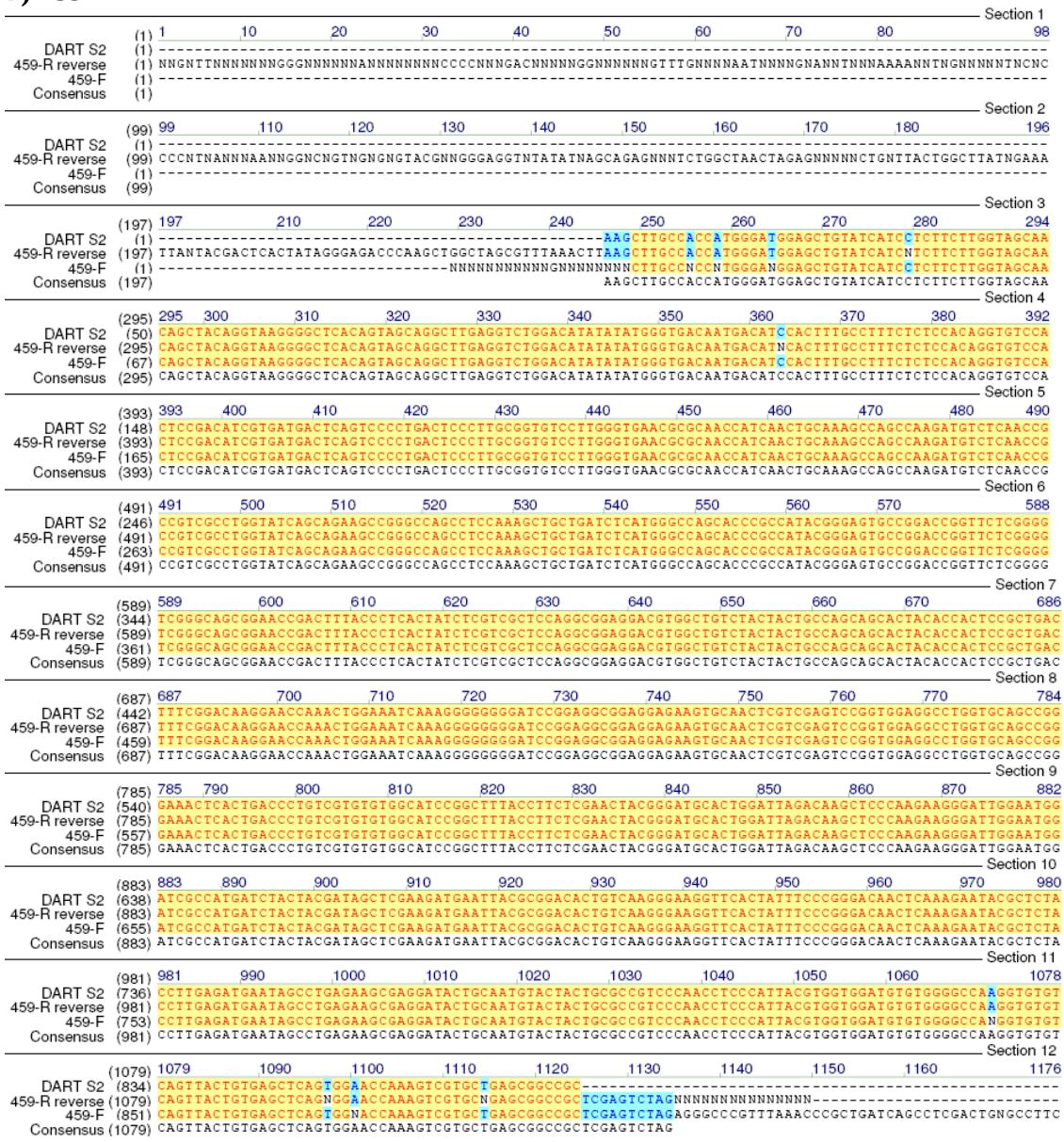
APPENDIX B

FIGURE 5: SEQUENCE COMPARISON OF E16-8D3 DIABODY CONSTRUCTS

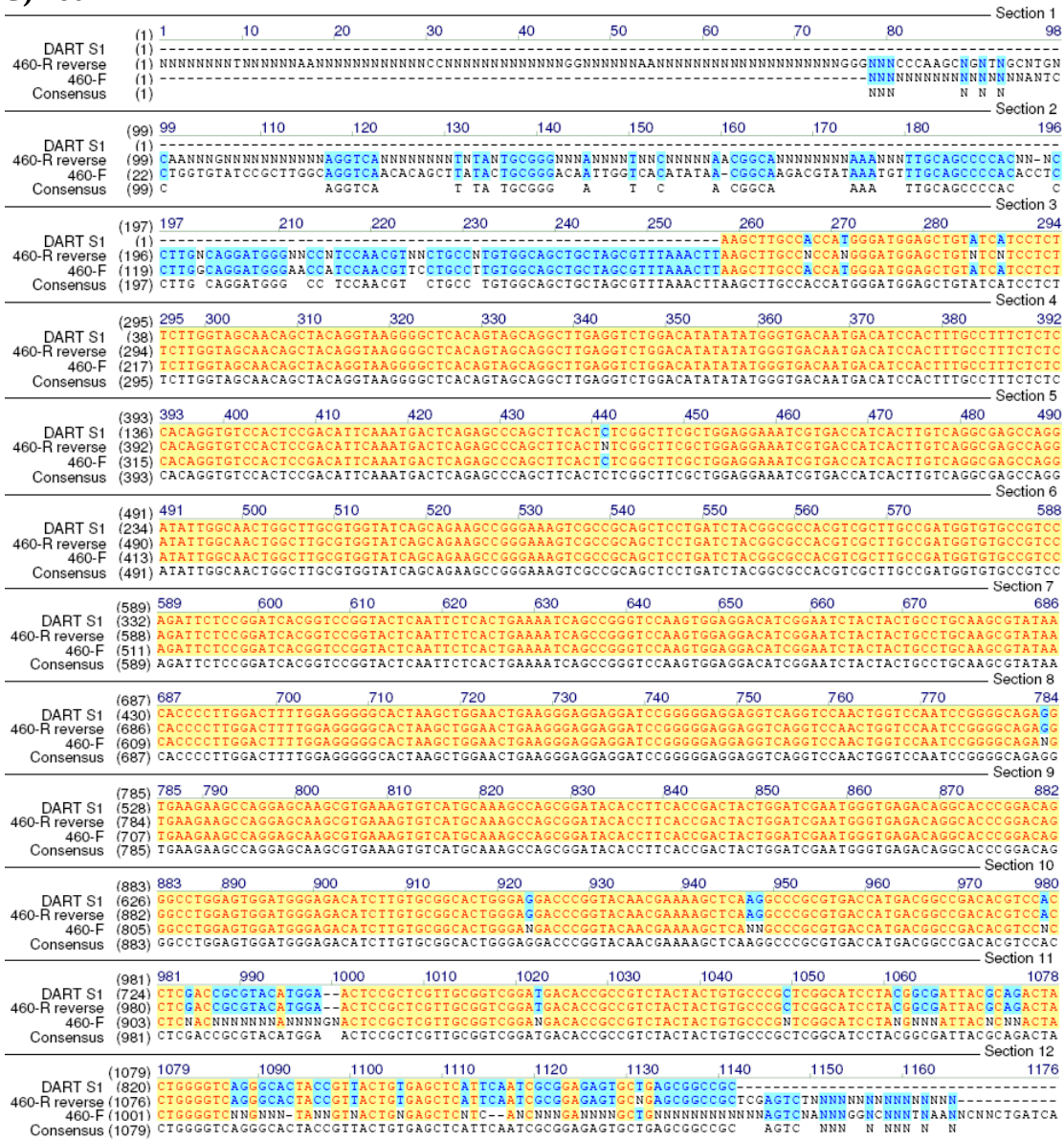
A) 458


#458 confirmation: all confirmed except #10NA, but confirmed by reading chromatograph.
Section 1
Section 2
Section 3
Section 4
Section 5
Section 6
Section 7
Section 8
Section 9
Section 10
Section 11
Section 12
DART S1
458-R reverse
458-F
Consensus

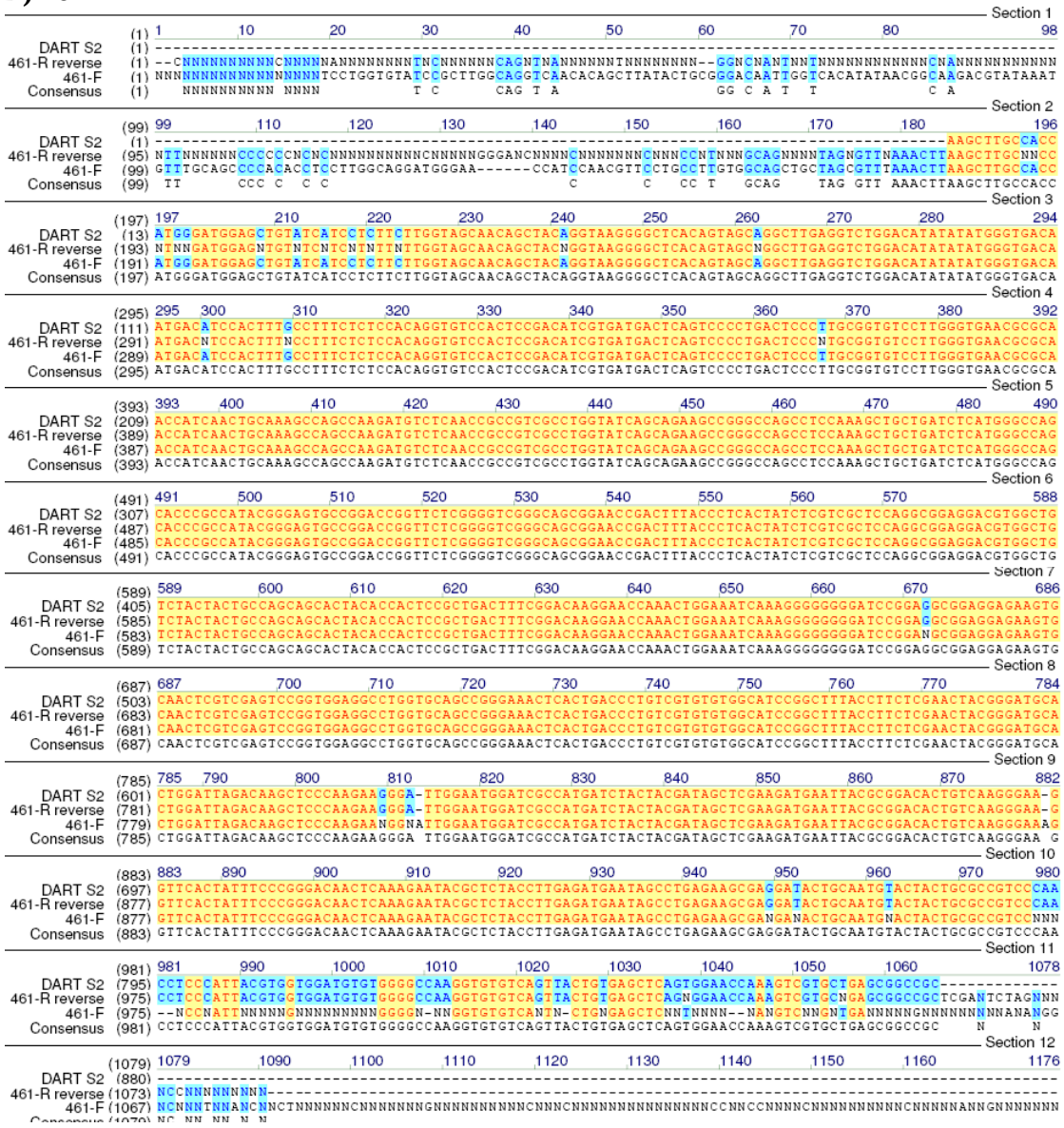
B) 459


Section 1
DART S2 (1) 1 10 20 30 40 50 60 70 80 98
459-R reverse (1) NNGNTTNNNNNNNGGGNNNNNNANNNNNNNNCCCCNNNGACNNNNNGGNNNNNNGTTTGNNNNAATNNNNGNANNTNNNAAAANNTNGNNNNNTNCNC
459-F (1)
Consensus (1)
Section 2
(99) 99 110 120 130 140 150 160 170 180 196
DART S2 (1)
459-R reverse (99) CCCNTNANNNAANNGGNCNGTNGNGNGTACGNNGGGAGGTNTATATNAGCAGAGNNNTCTGGCTAACTAGAGNNNNNCTGNTTACTGGCTTATNGAAA
459-F (1)
Consensus (99)
Section 3
(197) 197 210 220 230 240 250 260 270 280 294
DART S2 (1) AAGCTTGCCACCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAA
459-R reverse (197) TTANTACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTGCCACCATGGGATGGAGCTGTATCATCNTCTTCTTGGTAGCAA
459-F (1) NNNNNNNNNNNGNNNNNNNNCTTGCCNCCNTGGGANGGAGCTGTATCATCCTCTTCTTGGTAGCAA
Consensus (197) AAGCTTGCCACCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAA
Section 4
(295) 295 300 310 320 330 340 350 360 370 380 392
DART S2 (50) CAGCTACAGGTAAGGGGCTCACAGTAGCAGGCTTGAGGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCA
459-R reverse (295) CAGCTACAGGTAAGGGGCTCACAGTAGCAGGCTTGAGGTCTGGACATATATATGGGTGACAATGACATNCACTTTGCCTTTCTCTCCACAGGTGTCCA
459-F (67) CAGCTACAGGTAAGGGGCTCACAGTAGCAGGCTTGAGGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCA
Consensus (295) CAGCTACAGGTAAGGGGCTCACAGTAGCAGGCTTGAGGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCA
Section 5
(393) 393 400 410 420 430 440 450 460 470 480 490
DART S2 (148) CTCCGACATCGTGATGACTCAGTCCCCTGACTCCCTTGCGGTGTCCTTGGGTGAACGCGCAACCATCAACTGCAAAGCCAGCCAAGATGTCTCAACCG
459-R reverse (393) CTCCGACATCGTGATGACTCAGTCCCCTGACTCCCTTGCGGTGTCCTTGGGTGAACGCGCAACCATCAACTGCAAAGCCAGCCAAGATGTCTCAACCG
459-F (165) CTCCGACATCGTGATGACTCAGTCCCCTGACTCCCTTGCGGTGTCCTTGGGTGAACGCGCAACCATCAACTGCAAAGCCAGCCAAGATGTCTCAACCG
Consensus (393) CTCCGACATCGTGATGACTCAGTCCCCTGACTCCCTTGCGGTGTCCTTGGGTGAACGCGCAACCATCAACTGCAAAGCCAGCCAAGATGTCTCAACCG
Section 6
(491) 491 500 510 520 530 540 550 560 570 588
DART S2 (246) CCGTCGCCTGGTATCAGCAGAAGCCGGGCCAGCCTCCAAAGCTGCTGATCTCATGGGCCAGCACCCGCCATACGGGAGTGCCGGACCGGTTCTCGGGG
459-R reverse (491) CCGTCGCCTGGTATCAGCAGAAGCCGGGCCAGCCTCCAAAGCTGCTGATCTCATGGGCCAGCACCCGCCATACGGGAGTGCCGGACCGGTTCTCGGGG
459-F (263) CCGTCGCCTGGTATCAGCAGAAGCCGGGCCAGCCTCCAAAGCTGCTGATCTCATGGGCCAGCACCCGCCATACGGGAGTGCCGGACCGGTTCTCGGGG
Consensus (491) CCGTCGCCTGGTATCAGCAGAAGCCGGGCCAGCCTCCAAAGCTGCTGATCTCATGGGCCAGCACCCGCCATACGGGAGTGCCGGACCGGTTCTCGGGG
Section 7
(589) 589 600 610 620 630 640 650 660 670 686
DART S2 (344) TCGGGCAGCGGAACCGACTTTACCCTCACTATCTCGTCGCTCCAGGCGGAGGACGTGGCTGTCTACTACTGCCAGCAGCACTACACCACTCCGCTGAC
459-R reverse (589) TCGGGCAGCGGAACCGACTTTACCCTCACTATCTCGTCGCTCCAGGCGGAGGACGTGGCTGTCTACTACTGCCAGCAGCACTACACCACTCCGCTGAC
459-F (361) TCGGGCAGCGGAACCGACTTTACCCTCACTATCTCGTCGCTCCAGGCGGAGGACGTGGCTGTCTACTACTGCCAGCAGCACTACACCACTCCGCTGAC
Consensus (589) TCGGGCAGCGGAACCGACTTTACCCTCACTATCTCGTCGCTCCAGGCGGAGGACGTGGCTGTCTACTACTGCCAGCAGCACTACACCACTCCGCTGAC
Section 8
(687) 687 700 710 720 730 740 750 760 770 784
DART S2 (442) TTTCGGACAAGGAACCAAACTGGAAATCAAAGGGGGGGGGATCCGGAGGCGGAGGAGAAGTGCAACTCGTCGAGTCCGGTGGAGGCCTGGTGCAGCCGG
459-R reverse (687) TTTCGGACAAGGAACCAAACTGGAAATCAAAGGGGGGGGGATCCGGAGGCGGAGGAGAAGTGCAACTCGTCGAGTCCGGTGGAGGCCTGGTGCAGCCGG
459-F (459) TTTCGGACAAGGAACCAAACTGGAAATCAAAGGGGGGGGGATCCGGAGGCGGAGGAGAAGTGCAACTCGTCGAGTCCGGTGGAGGCCTGGTGCAGCCGG
Consensus (687) TTTCGGACAAGGAACCAAACTGGAAATCAAAGGGGGGGGATCCGGAGGCGGAGGAGAAGTGCAACTCGTCGAGTCCGGTGGAGGCCTGGTGCAGCCGG
Section 9
(785) 785 790 800 810 820 830 840 850 860 870 882
DART S2 (540) GAAACTCACTGACCCTGTCGTGTGTGGCATCCGGCTTTACCTTCTCGAACTACGGGATGCACTGGATTAGACAAGCTCCCAAGAAGGGATTGGAATGG
459-R reverse (785) GAAACTCACTGACCCTGTCGTGTGTGGCATCCGGCTTTACCTTCTCGAACTACGGGATGCACTGGATTAGACAAGCTCCCAAGAAGGGATTGGAATGG
459-F (557) GAAACTCACTGACCCTGTCGTGTGTGGCATCCGGCTTTACCTTCTCGAACTACGGGATGCACTGGATTAGACAAGCTCCCAAGAAGGGATTGGAATGG
Consensus (785) GAAACTCACTGACCCTGTCGTGTGTGGCATCCGGCTTTACCTTCTCGAACTACGGGATGCACTGGATTAGACAAGCTCCCAAGAAGGGATTGGAATGG
Section 10
(883) 883 890 900 910 920 930 940 950 960 970 980
DART S2 (638) ATCGCCATGATCTACTACGATAGCTCGAAGATGAATTACGCGGACACTGTCAAGGGAAGGTTCACTATTTCCCGGGACAACTCAAAGAATACGCTCTA
459-R reverse (883) ATCGCCATGATCTACTACGATAGCTCGAAGATGAATTACGCGGACACTGTCAAGGGAAGGTTCACTATTTCCCGGGACAACTCAAAGAATACGCTCTA
459-F (655) ATCGCCATGATCTACTACGATAGCTCGAAGATGAATTACGCGGACACTGTCAAGGGAAGGTTCACTATTTCCCGGGACAACTCAAAGAATACGCTCTA
Consensus (883) ATCGCCATGATCTACTACGATAGCTCGAAGATGAATTACGCGGACACTGTCAAGGGAAGGTTCACTATTTCCCGGGACAACTCAAAGAATACGCTCTA
Section 11
(981) 981 990 1000 1010 1020 1030 1040 1050 1060 1078
DART S2 (736) CCTTGAGATGAATAGCCTGAGAAGCGAGGATACTGCAATGTACTACTGCGCCGTCCCAACCTCCCATTACGTGGTGGATGTGTGGGGCCAAGGTGTGT
459-R reverse (981) CCTTGAGATGAATAGCCTGAGAAGCGAGGATACTGCAATGTACTACTGCGCCGTCCCAACCTCCCATTACGTGGTGGATGTGTGGGGCCAAGGTGTGT
459-F (753) CCTTGAGATGAATAGCCTGAGAAGCGAGGATACTGCAATGTACTACTGCGCCGTCCCAACCTCCCATTACGTGGTGGATGTGTGGGGCCANGGTGTGT
Consensus (981) CCTTGAGATGAATAGCCTGAGAAGCGAGGATACTGCAATGTACTACTGCGCCGTCCCAACCTCCCATTACGTGGTGGATGTGTGGGGCCAAGGTGTGT
Section 12
(1079) 1079 1090 1100 1110 1120 1130 1140 1150 1160 1176
DART S2 (834) CAGTTACTGTGAGCTCAGTGGAACCAAAGTCGTGCTGAGCGGCCGC----------
459-R reverse (1079) CAGTTACTGTGAGCTCAGNGGAACCAAAGTCGTGCNGAGCGGCCGCTCGAGTCTAGNNNNNNNNNNNNNNN----------
459-F (851) CAGTTACTGTGAGCTCAGTGGNACCAAAGTCGTGCTGAGCGGCCGCTCGAGTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGNGCCTTC
Consensus (1079) CAGTTACTGTGAGCTCAGTGGAACCAAAGTCGTGCTGAGCGGCCGCTCGAGTCTAG

C) 460

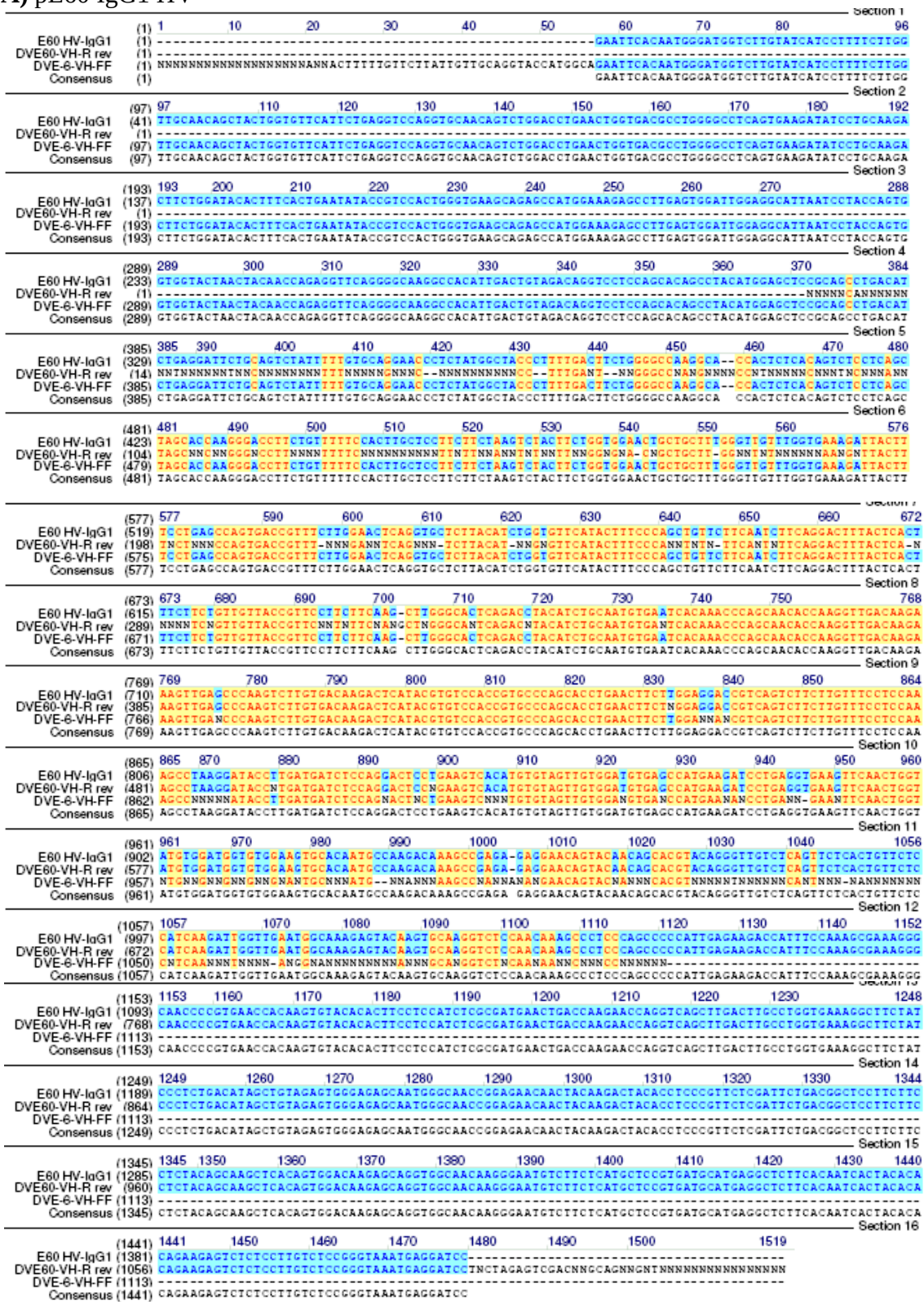

Section 1
DART S1
460-R reverse
460-F
Consensus
Section 2
Section 3
Section 4
Section 5
Section 6
Section 7
Section 8
Section 9
Section 10
Section 11
Section 12

D) 461


Section 1
Section 2
Section 3
Section 4
Section 5
Section 6
Section 7
Section 8
Section 9
Section 10
Section 11
Section 12
DART S2
461-R reverse
461-F
Consensus

APPENDIX C
FIGURE 13: SEQUENCE COMPARISON OF E16-8D3 DIABODY CONSTRUCTS

A) pE60 IgG1 HV



B) pE60 IgG1 LC