Generation and mimotope identification of aglycosylated neutralizing human monoclonal antibody against 4 serotypes of dengue virus without ADE activity (#18165)

First submission

Please read the **Important notes** below, the **Review guidance** on page 2 and our **Standout reviewing tips** on page 3. When ready **submit online**. The manuscript starts on page 4.

Important notes

Editor and deadline

Mario Alberto Flores-Valdez / 16 Jun 2017

Files	5 Figure file(s) 2 Table file(s) 4 Other file(s) Please visit the overview page to **download and review** the files not included in this review PDF.
Declarations	**Cell lines were used.**

Review guidelines

Please read in full before you begin

How to review

When ready **submit your review online**. The review form is divided into 5 sections. Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

You can also annotate this PDF and upload it as part of your review

To finish, enter your editorial recommendation (accept, revise or reject) and submit.

BASIC REPORTING

- Clear, unambiguous, professional English language used throughout.
- Intro & background to show context. Literature well referenced & relevant.
- Structure conforms to **PeerJ standards**, discipline norm, or improved for clarity.
- Figures are relevant, high quality, well labelled & described.
- Raw data supplied (see **PeerJ policy**).

EXPERIMENTAL DESIGN

- Original primary research within **Scope of the journal**.
- Research question well defined, relevant & meaningful. It is stated how the research fills an identified knowledge gap.
- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- Impact and novelty not assessed. Negative/inconclusive results accepted. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- Data is robust, statistically sound, & controlled.
- Conclusions are well stated, linked to original research question & limited to supporting results.
- Speculation is welcome, but should be identified as such.

The above is the editorial criteria summary. To view in full visit **https://peerj.com/about/editorial-criteria/**

7 Standout reviewing tips

The best reviewers use these techniques

Tip	*Example*
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
Give specific suggestions on how to improve the manuscript	*Your introduction needs more detail. I suggest that you improve the description at lines 57- 86 to provide more justification for your study (specifically, you should expand upon the knowledge gap being filled).*
Comment on language and grammar issues	*The English language should be improved to ensure that your international audience can clearly understand your text. I suggest that you have a native English speaking colleague review your manuscript. Some examples where the language could be improved include lines 23, 77, 121, 128 – the current phrasing makes comprehension difficult.*
Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Give specific suggestions on how to improve the manuscript	*Line 56: Note that experimental data on sprawling animals needs to be updated. Line 66: Please consider exchanging "modern" with "cursorial".*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Generation and mimotope identification of aglycosylated neutralizing human monoclonal antibody against 4 serotypes of dengue virus without ADE activity

Subenya Injampa [1,2], **Nataya Muenngern** [1], **Chonlatip Pipattanaboon** [1], **Surachet Benjathummarak** [1], **Khwanchit Boonha** [1], **Waranya Wongwit** [2], **Hathairad Hananantachai** [2], **Pongrama Ramasoota** [1,2], **Pannamthip Pitaksajjakul** Corresp. [1,2]

[1] Center of Excellence for Antibody Reserach, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

[2] Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Corresponding Author: Pannamthip Pitaksajjakul
Email address: pannamthip.pit@mahidol.ac.th

Background. Dengue disease is a leading cause of illness and death in the tropics and subtropics. Most severe cases occur among patients secondarily infected with a different dengue virus (DENV) serotype from the first infection, resulting in antibody-dependent enhancement activity (ADE). Our previous study generated the neutralizing human monoclonal antibody, D23-1B3B9 (B3B9), targeted to 1st domain II of E protein, which showed strong neutralizing activity (NT) to all four DENV serotypes. However, at sub-neutralizing concentrations, it showed ADE activity *in vitro*. In addition, for understanding of antibody-antigen interaction, determination of an epitope residue of this antibody is further required. Methods. In this study, phage display random peptide libraries were used to identify the common B-cell epitopes that recognized by cross neutralizing human monoclonal antibody clone B3B9. Further, to generate a similar antibody that avoids ADE, we constructed a new expression plasmid using the existing IgG heavy chain plasmid as a template for Fc modification at position N297Q by site-directed mutagenesis. The resulting plasmid was then co-transfected with a light chain plasmid to produce full recombinant IgG (rIgG) in mammalian cells (N297Q-B3B9). This rIgG was characterized for neutralizing and enhancing activity by using different FcɣR bearing cells. To produce sufficient quantities of B3B9 rIgG for further characterization, CHO-K1 cells stably secreting N297Q-B3B9 rIgG were then established. Results. The epitope of human monoclonal antibody clone B3B9 was located in the N-terminal fusion loop of E protein of DENV (107LXXXG111) based on the obtained consensus sequences. With Fc modification, the generated N297Q-B3B9 rIgG showed cross-neutralizing activity to all four DENV serotypes, similar to wild type rIgG. In both FcγRI- and RII-bearing THP-1 cells and FcγRII-bearing K562 cells, N297Q-B3B9 rIgG lacked ADE activity against all DENV serotypes at sub-neutralizing concentrations. Fortunately, the N297Q-B3B9 rIgG secreted from these stable cells showed NT and ADE

activities similar to those of the N297Q-B3B9 rIgG obtained from transient expression. The stable CHO-K1 cells can be further developed as high producer stable cells and used for further characterization as a promising dengue therapeutic candidate. Discussions. To our knowledge, this is the first aglycosylated human monoclonal antibody rIgG, targeted to fusion loop of EDII, which showed cross-neutralizing activity to 4 serotypes of DENV, but not caused any viral enhancement activity *in vitro*. A protective activity in animal model is further required to evaluate its application as immune therapy.

Generation and mimotope identification of agylcosylated neutralizing human monoclonal antibody against 4 serotypes of dengue virus without ADE activity

Subenya Injampa[a,b], Nataya Muenngern[b], Chonlatip Pipattanaboon[b], Surachet Benjathummarak[b], Khwanchit Boonha[b], Waranya Wongwit[a], Hathairad Hananantachai[a], Pongrama Ramasoota[a,b], Pannamthip Pitaksajjakul[a,b,*]

[a] Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Thailand

[b] Center of Excellence for Antibody Research, Faculty of Tropical Medicine, Mahidol University, Thailand

*Corresponding author at: Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, 420/6 Ratchathewi, Bangkok, Thailand, 10400

E-mail address: pannamthip.pit@mahidol.ac.th (P. Pitaksajjakul).

Abstract

Background. Dengue disease is a leading cause of illness and death in the tropics and subtropics. Most severe cases occur among patients secondarily infected with a different dengue virus (DENV) serotype from the first infection, resulting in antibody-dependent enhancement activity (ADE). Our previous study generated the neutralizing human monoclonal antibody, D23-1B3B9 (B3B9), targeted to 1st domain II of E protein, which showed strong neutralizing activity (NT) to all four DENV serotypes. However, at sub-neutralizing concentrations, it showed ADE activity *in vitro*. In addition, for understanding of antibody-antigen interaction, determination of an epitope residue of this antibody is further required.

Methods. In this study, phage display random peptide libraries were used to identify the common B-cell epitopes that recognized by cross neutralizing human monoclonal antibody clone B3B9. Further, to generate a similar antibody that avoids ADE, we constructed a new expression plasmid using the existing IgG heavy chain plasmid as a template for Fc modification at position N297Q by site-directed mutagenesis. The resulting plasmid was then co-transfected with a light chain plasmid to produce full recombinant IgG (rIgG) in mammalian cells (N297Q-B3B9). This rIgG was characterized for neutralizing and enhancing activity by using different FcγR bearing cells. To produce sufficient quantities of B3B9 rIgG for further characterization, CHO-K1 cells stably secreting N297Q-B3B9 rIgG were then established.

Results. The epitope of human monoclonal antibody clone B3B9 was located in the N-terminal fusion loop of E protein of DENV (107LXXXG111) based on the obtained consensus sequences. With Fc modification, the generated N297Q-B3B9 rIgG showed cross-neutralizing activity to all four DENV serotypes, similar to wild type rIgG. In both FcγRI- and RII-bearing THP-1 cells and FcγRII-bearing K562 cells, N297Q-B3B9 rIgG lacked ADE activity against all DENV serotypes

at sub-neutralizing concentrations. Fortunately, the N297Q-B3B9 rIgG secreted from these stable cells showed NT and ADE activities similar to those of the N297Q-B3B9 rIgG obtained from transient expression. The stable CHO-K1 cells can be further developed as high producer stable cells and used for further characterization as a promising dengue therapeutic candidate.

Discussions. To our knowledge, this is the first aglycosylated human monoclonal antibody rIgG, targeted to fusion loop of EDII, which showed cross-neutralizing activity to 4 serotypes of DENV, but not caused any viral enhancement activity *in vitro*. A protective activity in animal model is further required to evaluate its application as immune therapy.

Keywords: Therapeutic antibody, Dengue virus, Neutralization, Human monoclonal antibody, Epitope mapping

Introduction

Dengue disease, transmitted by mosquito-borne infection, is a leading cause of illness and death in the tropics and subtropics. More than one-third of the world population lives in areas at risk of dengue virus (DENV) infection (Natasha Evelyn and Mikkel, 2013). DENV is the member of the *Flavivirus* genus in the *Flaviviridae* family. There are four antigenically different serotypes of DENV (DENV-1 to DENV-4) (Kuhn et al., 2002). Primary infection with one DENV serotype induces the production of homotypic neutralizing antibodies, which provide lifelong immunity against the infecting serotype and typically cause asymptomatic or self-limited dengue fever (DF) as well as some degree of cross-protective immunity against the other three DENV serotypes. These cross-reactive antibodies persist for a period of several months to a few years. In secondary infection with heterologous DENV serotypes, patients usually have an

increased risk of developing severe disease, including life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). This is because the production of cross-reactive but non-neutralizing antibodies can lead to higher viremia resulting from antibody-dependent enhancement activity (Schmidt, 2010). Currently, there is no available antiviral drug specific for all four DENV serotypes. As such, therapeutic antibodies able to neutralize all four DENV serotypes, particularly fully human monoclonal antibodies (HuMAbs), are considered to be the main option for passive immune therapy.

Previously, we generated HuMAb clone D23-1B3B9 (B3B9) with strong *in vitro* neutralizing activity (NT) against DENV-1 to DENV-4. This HuMAb targeted to domain II of envelope proteins residue 52-132, analyzed by western blot using truncated E protein (Setthapramote et al., 2012; Sasaki et al., 2013). However, the information of epitope residues need to be clarified. In this study, a phage display random peptide libraries were used to identify the common B-cell epitopes that recognized by this cross-neutralizing anti-dengue human monoclonal antibody.

Moreover, investigating the viral infection enhancing activity of this HuMAb clone B3B9 on Fc-gamma receptor (FcγR)-bearing cells revealed an increasing in DENV infection at sub-neutralizing concentrations, which limit its application as a therapeutic candidate (Sasaki et al., 2013). Here, to modify that antibody for therapeutic use as an antiviral HuMAb, we aimed to diminish its enhancing activity. We constructed plasmids expressing antibody heavy chain (HC) and light chain (LC) genes and modified the Fc region of the HC constant domain 2 at position N297Q by site-directed mutagenesis. The modified HC plasmid was co-transfected with the LC plasmid into HEK293T mammalian cells to produce full recombinant IgG (rIgG). To evaluate

the Fc-modified rIgG as a potential therapeutic candidate for dengue treatment, its NT and ADE activity were determined *in vitro* and compared with those of wild type rIgG.

Materials and Methods

Cell lines and DENV strains

HEK293T cells were maintained in Dulbecco's modified Eagle medium (Gibco, Grand Island, New York, USA.) with 10% fetal bovine serum (FBS). For NT test, Vero cells were cultured in minimal essential medium (MEM) (GE Healthcare UK Ltd., Buckinghamshire, UK) with 10% fetal bovine serum. THP-1 and K562 cells, which were used in ADE assays, were cultured in RPMI 1640 medium (Gibco) with 10% FBS. The DENV strains used in this study were the Mochizuki strain of DENV1, the 16681 strain of DENV2, the H87 strain of DENV3, and the H241 strain of DENV4. All DENVs were propagated in C6/36 cells, which were maintained in Leibovitz's L-15 medium (Gibco) supplemented with 10% FBS and 0.3% of BACTO Tryptose Phosphate Broth (TPB) (sigma-aldrich, Missouri, USA). CHO-K1 cells were maintained in MEM medium supplemented with 10% FBS and 1% non-essential amino acid (Gibco).

Human monoclonal antibody

Human monoclonal antibody clone B3B9 that show cross-neutralizing activity to 4 serotypes of DENV was used in this study for mapping the epitope using random 7 and 12 amino acid peptide phage display libraries.

Epitope mapping by phage display of random peptide libraries

Phage affinity selection (Biopanning)

Purified B3B9 HuMAb was used in phage display panning experiment to characterize their binding epitopes. Panning was performed by using Ph.D.-12 and Ph.D.-C7C Phage Display Peptide Library Kit (New England Bio Labs Inc., UK) as previously described, with some modifications (Tewawong et al., 2012). Briefly, 50 µl of protein A/G magnetic beads were blocked with BSA by incubated at room temperature for 1 hr, and washed. Phages (5×10^{10} plaque forming units (PFU)) were incubated at room temperature for 30 minutes with purified B3B9 HuMAb to a final volume of 200 µl with Tris Buffered Saline with Tween (TBST). Phage - HuMAb mixture was transferred to the tube containing the washed magnetic beads and incubated for 20 minutes at room temperature. After incubation, nonbinding phages that unable to be captured on magnetic beads, were washed with washing buffer (0.05% TBST). The bound phages were eluted with l ml glycine elution buffer (0.2 M Glycine-HCl, 1 mg/ml BSA, pH 2.2). Then, the bound phages were amplified by direct infection to *Escherichia coli* (*E. coli*) ER2738. The amplified phages were purified by precipitation with 20% PEG 8000/2.5 M NaCl and used in the next cycle. Three rounds of selection were performed. Specificity of selected phage clones were confirmed by phage ELISA. Briefly, 96-well microtiter plates were coated with 100 µl of HuMAb, 2 wells for each sample, at 4°C for overnight. Then, the wells were washed for 2 times with 0.05% Phosphate Buffered Saline with Tween (PBST). After that, wells that coated with HuMAb including well for BSA control were filled with blocking buffer. Amplified phages of each clone were also being coated at this step in another well for phage expression control. After incubation, plates were washed as above for 5 times. At this step, *E.coli* lysate of control M13 phages (no fusion peptide) amplification were added to one of HuMAb-coated well and amplified phages (with fusion peptide) were added to the other HuMAb-coated well as well as BSA control well. The wells of phage expression control were blocked during this step. The

plates were incubated and washed as above. Consequently, anti-M13 antibody-HRP conjugated; dilute 1:5,000 in blocking buffer were added. All incubations were performed in humidity chamber at 37°C, for 1 hr. After washing, the reaction were developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma-aldrich), and terminated the reaction by adding 100 µl of 2.0 M H_2SO_4. The absorbance was measured at 450 nm using ELISA reader (TECAN). Plasmids that isolated from positive clone of ELISA were sequenced with -96 gIII sequencing primer 5'-TGA GCG GAT AAC AAT TTC AC-3'. The inserted random 7 and 12 amino acids were identified and analysed. The matched peptides with dengue viral genome were analyzed by using BioEdit program.

Phage affinity binding by inhibition ELISA

After sequences analysis, the candidate phage from each type of consensus peptide specifically bind to B3B9 HuMAb was further confirmed their binding activity with target antibody by phage inhibition ELISA. Firstly, 2-fold serial dilutions of phage were prepared and bind to the HuMAb by ELISA to determine the reciprocal phage dilution of each clone of amplified phages. The bound phage was detected using anti-M13 polyclonal antibody, and developed signal with TMB substrate. For inhibition ELISA, B3B9 HuMAb was prepared with 2-fold serial dilution started at 20 µg/ml. Then, the diluted antibody was incubated with equal volume of phage at specified dilution at room temperature for 1 hr. After incubation, the mixtures were transferred to HuMAb pre-coated plate, and incubated for another 1 hr. The plate was then washed. Free phages, left from binding in solution phase, that bind to coated HuMAb on the plate were detected by anti-M13-HRP, and signal was developed with TMB substrate. The absorbance values were measured at 450 nm using an ELISA reader (TECAN). Absorbance

values of each concentration (A) of antibody in solution phase were divided by absorbance values of no antibody (control) (A0), resulting in normalized values (A/A0).

Generation of aglycosylated human monoclonal antibody clone B3B9

Plasmids for rIgG production

Variable HC and LC sequences of B3B9 HuMAb were previously isolated from hybridoma cells. HC and LC expression plasmids containing variable and constant regions were constructed as previously described (Pitaksajjakul et al., 2014). The HC plasmid was used as a template for site-directed mutagenesis with the In-Fusion Cloning System (In-Fusion® HD Cloning Plus; Clontech Laboratories Inc., Shiga, Japan). Primers were designed according to the manufacturer's instructions to mutate the amino acid at position 297 from asparagine to glutamine. This system combines the action of the In-Fusion HD enzyme with inverse polymerase chain reaction (PCR), which generates linearized DNA from a plasmid template. The PCR reaction was composed of the CloneAmp Hifi PCR premix, 300 nM each of forward and reverse primer, 5 ng of plasmid, and distilled water to final volume of 25 µl. The amplification was performed with 35 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 5 s. The inverse PCR products were gel-purified using a PureLink® Quick Gel Extraction Kit (Invitrogen, California, USA) following the manufacturer's protocol. The linearized plasmids obtained from inverse PCR were then fused by the In-Fusion enzyme, by following the manufacturer's instructions. The reaction was performed at 50 °C for 15 mins. The in-fusion reaction was chemically transformed into Stella chemical competent cells supplied by the kit. Individual clones were randomly selected for DNA sequencing.

DNA sequencing and plasmid preparation

The sequences of the mutated HC plasmids were confirmed by DNA sequencing. Plasmids that contained the target mutation were amplified in *E. coli* and isolated using a Purelink™ plasmid Midiprep kit (Invitrogen) from 100 ml culture for further transfection in mammalian cells.

Transient expression of N297Q-B3B9 rIgG in HEK293T cells

Plasmids expressing N297Q-B3B9 HC and LC were transfected to HEK293T cells to produce whole rIgG with the N297Q mutation as previously described (Pitaksajjakul et al., 2014). The culture medium containing secreted N297Q-B3B9 rIgG was collected and used in immunofluorescence assays (IFAs) to determine the DENV-binding activity. The N297Q-B3B9 rIgG was purified using a protein A affinity column. The purity of purified antibody was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

IFA for DENV-binding activity

IFAs were used to determine the binding and specificity of N297Q-B3B9 rIgG for all four DENV serotypes. IFA plates were prepared by infecting Vero cells with DENV at a multiplicity of infection of 0.01. The plates were incubated for 3 days before being fixed and permeabilized with 3.7% paraformaldehyde and 0.1% Triton X-100, respectively. Culture fluid from transfected cells was added, and the plates were incubated at 37 °C for 1 h. AlexaFluor 488-conjugated anti-human IgG (1:1,000 dilution) (Invitrogen) was added as secondary antibody. The result was observed under fluorescence microscope (IX71, Olympus). For this assay, PBS was used as a negative control.

199

200 HuMAb isotyping

201 The IgG isotype of the N297Q-B3B9 rIgG was determined by PCR as previously
202 described (Omokoko et al., 2014) using complementary DNA isolated from HuMAb clone B3B9
203 hybridoma as a template. The gene-specific primers used to amplify IgG1, -2, -3, and -4 are
204 listed in Supplementary Table 1. IgG1 and IgG3 were amplified using the same forward primer.
205 An identical reverse primer was used to amplify all IgG isotypes. These primers were amplified
206 at the hinge region between the Fab and Fc parts that produce different sized PCR products. The
207 expected sizes of the PCR products were 211, 207, 346, and 210 bp for IgG1, IgG2, IgG3, and
208 IgG4, respectively. The PCR reaction was composed of 0.5 µg of cDNA, 1 mM Tris-HCl (pH
209 8.0), 5 mM KCl, 1.25 mM deoxyribonucleotide triphosphate (dNTP), 1.25 units of ExTaq DNA
210 polymerase (TAKARA, Shiga, Japan), and 5 mM of each primer, with distilled water to a final
211 volume of 25 µl. The amplification of each primer pairs was performed in separate reaction with
212 35 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s. The PCR products were separated
213 by agarose gel electrophoresis and visualized by staining with SYBR Safe DNA Gel stain
214 (Invitrogen).

215

216 Foci reduction neutralization test (FRNT)

217 FRNT assay was performed as previously described (Pitaksajjakul et al., 2014). DENV
218 were mixed with different concentrations of purified HuMAbs (B3B9 or N297Q-B3B9 rIgG) and
219 incubated at 37 °C for 1 h. Each mixture was added to Vero cells in 96-well cell culture plates
220 and incubated for 2 h. Then, the plates were overlayed with 2% carboxymethyl cellulose (CMC)
221 in MEM medium with 2% FBS and incubated for 2 days for DENV4 and for 3 days for DENV1,

2, and 3. After that, the plates were fixed with 3.7% paraformaldehyde/PBS and 0.1% Triton X-100/PBS. Immunostaining was performed by an incubation with anti-DENV human antibodies, followed by Alexa-conjugated anti-human IgG (H+L) (1:1,000 dilution). Foci numbers were counted under a fluorescence microscope (IX71, Olympus). The percent reduction was calculated by comparing the foci number for each antibody concentration with the number of foci obtained from a virus–PBS mixture (negative control).

Antibody dependent enhancement (ADE) assay on K562 cells

The ADE activity of the N297Q-B3B9 rIgG was also assessed on FcγRIIa-bearing K562 cells (Konishi et al, 2010). Antibodies at serial four-fold dilutions and viruses were mixed in 10% FBS RPMI medium in 96-well poly-L-lysine-coated plates (Corning Inc., New York, USA) and incubated at 37 °C. After 2 h, 50 µl of 2 $\times 10^6$ cells/ml K562 cells were added. The cell–HuMAb–virus mixture was co-cultured at 37 °C under 5% CO_2 for 2 days. The cells were then fixed with an acetone/methanol fixing solution at −20 °C. Immunostaining was performed by adding an anti-DENV monoclonal antibody and incubating at 4 °C overnight. Then, horseradish peroxidase-conjugated anti-human IgG (H+L) diluted in 0.05% Tween and 1% FBS in PBS was added, and the samples were incubated at 37 °C for 1 h. The signal was developed with a DAB substrate solution (KPL, Maryland, USA), and the infected cells were counted. The balancing of neutralizing and enhancing activity was determined based on the number of DENV-infected cells obtained from the well without antibody.

Antibody dependent enhancement (ADE) assay on THP-1 cells

DENV at a multiplicity of infection of 0.1 was incubated with serially diluted antibodies in serum-free RPMI medium at 37 °C for 1 h. Then, 150 µl of 5×10^5 cells/ml THP-1 cells, which express both FcγRI and II on their surfaces, was added, and the samples were incubated at 37 °C under 5% CO_2. After 2 h, RPMI medium with 4% FBS was added. The cell–HuMAb–DENV solution was incubated for 3 days, after which RNA was extracted from the infected cells using TRIzol® reagent (Invitrogen). The viral RNA was quantified by a one-step Realtime PCR using glyceraldehyde 3-phosphate dehydrogenase (GADPH) as an internal control (Sasaki et al., 2013). The result is presented as the fold enhancement of virus copies compared with the virus–PBS mixture (control).

Generation of stable, antibody-secreting CHO-K1 cells

The HC-expressing constructed plasmid (pQCXIP-CH) contained a puromycin-resistant gene and the LC-expressing constructed plasmid (pQCXIH-CL) contained a hygromycin-resistant gene. After determining the optimal concentrations of the corresponding two antibiotics, stable antibody-secreting CHO-K1 cells were selected using puromycin and hygromycin at 8 µg/ml and 800 µg/ml, respectively. For stable cell generation, briefly, CHO-K1 cells were seeded in a 6-well cell culture plate at the day before transfection to obtain a cell density of 90–95% on the next day. The medium was replaced with Opti-MEM I reduced serum medium. Plasmid DNA was mixed with transfection reagent (Lipofectamine 2000) and added to the cells, which were then incubated for 5 h. After that, the medium was replaced with culture medium (10% FBS, 1% NEAA MEM). The cells were incubated at 37 °C with 5% CO_2 for 24 h. After that, the medium was replaced with culture medium containing two antibiotics at the concentrations specified above. The medium was changed every 3–4 days until 60% of live cells

were observed. The transfected cells were used for cell cloning by limiting dilution on 96 well-cell culture plates and incubated for 10–14 days. The positive clones that were able to secrete the target anti-DENV rIgG were selected by IFA using the culture supernatant of each well containing a single stable clone. The cells were scaled up for further characterization. The level of IgG contained in the culture fluid of each positive clone was roughly determined by an IgG quantitation enzyme-linked immunosorbent assay (Bethyl Laboratories, Inc., Texas, USA.). The functionalities of the purified N297Q rIgG secreted from the stable cells were confirmed by IFAs, NT and ADE assays.

Results

Epitope mapping by phage display of random peptide libraries

Biopanning of a phage displayed C7C and 12 random peptide library was performed using the affinity selection of purified B3B9 HuMAb. After biopanning, the number of output phages obtained from Ph.D. C7C and Ph.D.12 increased from 6.9 x 10^5 to 9.8 x 10^8 PFU and 6.9 x 10^5 to 7.7 x 10^8 PFU respectively, which suggested that specific enrichment had occurred (Fig 1A). Plasmids of immunopositive phage clones that show binding activity with HuMAb by ELISA were isolated for DNA sequencing. Inserted oligonucleotide sequences of phage DNAs were translated to peptide sequences. Peptide sequences were aligned using BioEdit program to analyse the epitopes and the binding motif of HuMAb. Phage clones were shown to display a consensus sequences LXXXG (Fig 1B). After comparing this peptide with the amino acid sequences of E proteins of DENV1–4, it was found that all these LXXXG motif was matched to 107LFGKG111 within the highly conserved N-terminal fusion loop peptide of the E protein domain II (Fig 1C). To investigate the solution-phase binding of each candidate random phage

peptide with target B3B9 HuMAb, eight candidates phage clones which matched to the motif of 107LXXXG111 were selected for this study (Fig 1B, Table 1). The adjusted concentration of phage lysates was From phage inhibition ELISA, it was found that the binding of all phage clones were inhibited by HuMAb with dose-dependent manner, as shown in Fig 1D.

Generation of agylcosylated HuMAb clone B3B9

Plasmid construction and expression

The substitution of amino acid at position 297 from Asparagine to glutamine was successfully created. After transfection those HC and LC plasmid to HEK293T cell, the successful production of wild type and mutant (N297Q) rIgG was verified by the optimal binding activity observed via IFA. The N297Q-B3B9 rIgG displayed cross-reactivity to all four DENV serotypes similarly to the wild type rIgG (Fig. 2).

IgG isotyping

Reverse transcription PCR was performed to determine the N297Q-B3B9 rIgG isotype. Using B3B9 hybridoma complementary DNA as template, the PCR product with approximately 211 bp of IgG1 was observed (data not shown)

Neutralizing activity by FRNT

To determine the function of rIgG, we assessed the NT of N297Q-B3B9 rIgG and B3B9 rIgG in Vero cells. N297Q-B3B9 rIgG displayed almost the same level of NT as the B3B9 rIgG for all DENV serotypes. The NT levels of various concentrations of these two antibodies against

all four DENV serotypes are shown in Fig. 3A. Among these four serotypes, B3B9 and N297Q-B3B9 rIgG showed identical NT50s to DENV-2, 3, and 4 (0.125 µg/ml for DENV2 and 2 µg/ml for both DENV3 and DENV4). However, the NT50 concentration against DENV1 of N297Q-B3B9 rIgG was slightly higher than that of B3B9 rIgG (Fig.3B).

Antibody dependence enhancement K562 cell

To study the balancing of NT and ADE in Fc receptor bearing K562 cell, our B3B9 rIgG could neutralized DENV2 and 3, but not DENV1 and 4. Moreover, this antibody induced a virus enhancement at concentrations 400-0.0015 µg/ml, 0.39–0.006 µg/ml, 100–0.0015 µg/ml, and 400-0.00038 µg/ml for DENV1, 2, 3, and 4, respectively (Fig. 4A). In contrary, N297Q-B3B9 rIgG showed NT for all four DENV serotypes and showed no enhancing activity in any of the tested antibody concentrations.

Antibody dependence enhancement THP-1 cell

In THP-1 cells, mutant IgG (N297Q-B3B9) that cannot bind to FcγR showed a complete reduction of ADE activity in all tested antibody concentrations. In contrast, wild type rIgG induced a 9541-, 43-, 3061-, and 2020-fold enhancement in the virus copy number compared with the control for DENV1, 2, 3, and 4, respectively (Fig. 4B).

Generation and characterization of CHO-K1 stable cell lines producing N297Q-B3B9 rIgG

From 125 stable clones that we screened, one showed the highest IgG secretion level (9,587 µg/ml). This clone was continually cultured to collect culture fluid for purification. Preliminary study against DENV2 showed that all tested concentrations of the modified rIgG

secreted from the stable cell line showed similar viral NT (Fig. 5A) and no ADE activity (Fig. 5B)

Discussion

Due to the complexity of DENV infection and pathogenesis (Marasco and Sui, 2007), the ideal therapeutic antibodies should be fully human-derived and capable of inhibiting all four serotypes to reduce the risk of ADE causing more severe symptoms (Chan et al., 2013). We previously generated cross-neutralizing HuMAb B3B9 (Setthapramote et al., 2012). The identification of B cell epitope of this antibody is crucial to understand the antibody/epitope interaction at a molecular level. Recently, several epitope mapping of MAb (murine and human) specific to dengue virus have been studied (Shrestha et al., 2010; Sukupolvi Petty S et al., 2010, Schieffelin et al., 2010; Beltramello et al., 2010; de Alwis et al., 2012). It was found that MAbs generated from mice are mostly serotype-specific that targeted to DIII of envelope protein (Shrestha et al., 2010). However, most of the HuMAbs were targeted to DI-II of envelope proteins which is more cross-reactive (Beltramello et al., 2010; de Alwis et al., 2012). Correlated with our previous studies, the selected cross-neutralizing HuMAb B3B9 also targeted to DII of envelope proteins residue 52-132, analyzed by western blot using truncated E protein (Sasaki et al., 2013). However, the detail of epitope residue needs to be clarified.

To determine the critical residue using phage display random peptide library, we mapped the epitopes of our HuMAb to LXXXG which correspond to 107LFGKG111 located in the conserved N-terminal fusion loop of EDII. This is a major target epitope of human antibodies for NT and ADE activity. Although this HuMAb showed strong NT to all DENV serotypes, at sub-

neutralizing concentrations, it promoted ADE (Sasaki et al., 2013). To overcome that problem, here, we then generated rIgG with an engineered Fc.

The Fc N-linked carbohydrate (Asn-297) is the oligosaccharide attachment residue in the CH2 domain. This site is important for maintaining antibody structure, and its modification can change the binding affinity between the Fc region and the FcγR that are present on B-cells, natural killer cells, granulocytes, and monocytes (Subedi and Barb, 2015). First, we evaluate the generated N297Q-B3B9 rIgG for its activity in Vero cell and found that the engineering antibody showed proper neutralizing activity comparable to B3B9 rIgG, except DENV1, with lower NT activity of N297Q-B3B9 comparing with B3B9 rIgG. This might occurred from its low efficiency of B3B9 rIgG to neutralize DENV1 (Sasaki et al., 2013), and may effect to the varied NT50 concentration between B3B9 and N297Q-B3B9 rIgG.

Next, since Vero cell is used only for evaluated neutralizing activity (Setthapramote et al., 2012; Sasaki et al., 2013), because it is absent of FcγR. Therefore, using K562 cells that express FcγR can assesses the balancing of NT and ADE (Konishi et al., 2010). In this simplified ADE assay, the virus infection enhancement is represented as the number of infected cells from each antibody concentration compared with those from a control performed in the absence of antibody. In this cell type, we found that B3B9 rIgG that showed cross-neutralizing activity to 4 DENV serotypes in Vero cell, only neutralized DENV 2 and 3, but not DENV1 and 4. In contrary, N297Q-B3B9 rIgG showed cross-neutralizing activity to 4 serotypes of DENV. This is the advantage of using FcγR bearing cell for NT study, that can be used to preliminary determine the *in vivo* status (Konishi et al., 2010).

However, since K562 cell only contains FcγRIIa, we further determined the ADE activity in THP-1 cells that contained both FcγRI and FcγRII. In accordance with ADE study in K562

cell, our N297Q-B3B9 rIgG showed clearly reduction of viral enhancement activity in THP-1 cell.

Different from study of Ramadhany et al., 2016, eventhough N297A HuMAb maintained the same NT activity as the parental HuMAb, unfortunately, this HuMAb still induced low levels of virus infection enhancement (Ramadhany et al., 2015). One reason might be resulted from the type of mutated amino acid. The substitution of Asparagine with Glutamine (conservative mutation) reduces the effects of functional properties because the side chains of these two amino acids differ by only one methylene group (Tao and Morrison, 1989).

Balsitis et al., 2010 reported that a N297Q mutation of mouse and chimeric human-mouse IgG that efficiency reduced ADE *in vitro* could decreased the mortality of DENV-infected mice (Balsitis et al., 2010). Moreover, Williams et al., 2013 described the ability of mouse MAb targeted to fusion loop at EDII which showed neutralizing efficiency as therapeutic activity in antibody enhancing lethal disease. Moreover, N297Q mouse MAb can competed enhancing antibody in polyvalent serum in *in vitro* study (Williams et al., 2013). These reasons support the possibility of N297Q-B3B9 rIgG, which target to fusion loop of EDII, to be use as therapeutic antibody in the future.

For further characterization of N297Q-B3B9 rIgG as a dengue therapeutic candidate, large quantities will be required, so we used an antibiotic system to generate a CHO-K1 cell line that stably expresses N297Q-B3B9 rIgG. The development of stable and high-producing cell lines for therapeutic protein production can be further applied biopharmaceutically.

Together, our results suggest that N297Q-B3B9 rIgG is a human monoclonal antibody that can neutralize all four serotypes of DENV without viral enhancing activity. As a fully human-derived monoclonal antibody, it avoids the problem of a human anti-mouse antibody

response and after a thorough characterization, this mutant B3B9 rIgG should be considered as an option for dengue therapeutic treatment.

Since N-glycan asparagine residue (N297) position effect to half-life of antibody (Chan et al., 2013) and other antibody-mediated effector immune functions, like antibody-dependent cellular cytotoxicity and the complement pathway for protective activity (Hayes et al., 2016). Hence, thoroughly characterization of the modified N297Q rIgG were further required to elucidate its humeral and cellular immune response. Thus, future studies should consider these effects of N297Q-B3B9 rIgG and protectivity of our N297Q-B3B9 HuMAb in animal model as dengue therapeutic candidate.

Acknowledgements

The authors would like to thank Dr. Atsushi Yamanaka for his continuous advisement and encouragement.

Funding

This work was supported by the Faculty of Tropical Medicine, Mahidol University and the National Research Council of Thailand. We also thanks The Deutscher Akademischer Austauschdienst (DAAD, German Academic Exchange Service) fund for the student scholar.

References

Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, Mehlhop E, Johnson S, Diamond MS, Beatty PR, Harris E. 2010. Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. *PLoS. Pathogen* 6: e1000790.

427 Beltramello M, Williams KL, Simmons CP, Macagno A, Simonelli L, Quyen NT, Sukupolvi-
428 Petty S, Navarro-Sanchez E, Young PR, de Silva AM, Rey FA, Varani L, Whitehead SS,
429 Diamond MS, Harris E, Lanzavecchia A, Sallusto F. 2010. The human immune response to
430 Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and
431 enhancing activity. *Cell Host Microbe* 8: 271-283.
432 Chan KR, Ong EZ, Ooi EE. 2013. Therapeutic antibodies as a treatment option for dengue fever.
433 *Expert Review of Anti-Infective Therapy* 11: 1147–1157.
434 De Alwis R, Smith SA, Olivarez NP, Messer WB, Huynh JP, Wahala WM, et al. 2012.
435 Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions.
436 *Proc Natl. Acad Sci U.S.A* 109: 7439-7444.
437 Hayes JM, Wormald MR, Rudd PM, Davey GP. 2016. Fc gamma receptors: glycobiology and
438 therapeutic prospects. *Journal of Inflammatory Research*. 9: 209-219.
439 Konishi E, Tabuchi Y, Yamanaka A. 2010. A simple assay system for infection-enhancing and
440 neutralizing antibodies to dengue type 2 virus using layers of semi-adherent K562 cells. *Journal*
441 *of Virological Methods* 163: 360–367.
442 Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, Jones CT, Mukhopadhyay
443 S, Chipman PR, Strauss EG, Baker TS, Strauss JH. 2002. Structure of dengue virus: implications
444 for flavivirus organization, maturation, and fusion. *Cell* 108:717–725.
445 Marasco WA, Sui J. 2007. The growth and potential of human antiviral monoclonal antibody
446 therapeutics, Nat. *Biotechnology* 25: 1421–1434.
447 Natasha Evelyn AM, Mikkel BQ, Annelies WS. 2013. Epidemiology of dengue: past, present
448 and future prospects. *Clinical Epidemiology* 5:299–309.

449 Omokoko MD, Pambudi S, Phanthanawiboon S, Masrinoul P, Setthapramote C, Sasaki T,
450 Kuhara M, Ramasoota P, Yamashita A, Hirai I, Ikuta K, Kurosu T. 2014. A highly conserved
451 region between amino acids 221 and 266 of dengue virus non-structural protein 1 is a major
452 epitope region in infected patients, *Am. J. Trop. Med. Hyg* 91:146–155.
453 Pitaksajjakul P, Benjathummarak S, Pipattanaboon C, Wongwit W, Okabayashi T, Kuhara M,
454 Misaki R, Fujiyama K, Ramasoota P. 2014. Antibody germline characterization of cross-
455 neutralizing human IgGs against 4 serotypes of dengue virus, *Biochemical Biophysical Research*
456 *Communication* 446: 475–480.
457 Ramadhany R, Hirai I, Sasaki T, Ono K, Ramasoota P, Ikuta K, Kurosu T. 2015. Antibody with
458 an engineered Fc region as a therapeutic agent against dengue virus infection. *Antiviral Research*
459 124: 61–68.
460 Sasaki T, Setthapramote C, Kurosu T, Nishimura M, Asai A, Omokoko MD, Pipattanaboon C,
461 Pitaksajjakul P, Limkittikul K, Subchareon A, Chaichana P, Okabayashi T, Hirai I,
462 Leaungwutiwong P, Misaki R, Fujiyama K, Ono K, Okuno Y, Ramasoota P, Ikuta K. 2013.
463 Dengue virus neutralization and antibody-dependent enhancement activities of human
464 monoclonal antibodies derived from dengue patients at acute phase of secondary infection,
465 *Antiviral Research* 98: 423–431.
466 Schieffelin JS, Costin JM, Nicholson CO, Oregeron NM, Fontaine KA, Isern S, et al. 2010.
467 Neutralizing and non-neutralizing monoclonal antibodies against dengue virus E protein derived
468 from a naturally infected patients. *Virol J* 7:28.
469 Schmidt AC. 2010. Response to dengue fever - the good, the bad, and the ugly? *New England*
470 *Journal of Medici*ne 363: 484–487.

471 Setthapramote C, Sasaki T, Puiprom O, Limkittikul K, Pitaksajjakul P, Pipattanaboon C,
472 Sasayama M, Leungwutiwong P, Phumratanaprapin W, Chamnachanan S, Kusolsuk T,
473 Jittmittraphap A, Asai A, Arias JF, Hirai I, Kuhara M, Okuno Y, Kurosu T, Ramasoota P, Ikuta
474 K. 2012. Human monoclonal antibodies to neutralize all dengue virus serotypes using
475 lymphocytes from patients at acute phase of the secondary infection, *Biochemical Biophysical*
476 *Research Communication* 423: 867–872.
477 Shrestha B, Brien JD, Sukupolvi-Petty S, Austin SK, Edeling MA, Kim T, et al. 2010. The
478 development of therapeutic antibodies that neutralize homologous and heterologous genotypes of
479 dengue virus type 1. *PLoS Pathog* 6: e1000823.
480 Subedi GP, Barb AW. 2015. The structural role of antibody N-glycosylation in receptor
481 interactions. *Structure* 23: 1573–1583.
482 Sukupolvi-Petty S, Austin SK, Engle M, Brien JD, Dowd KA, Williams KL, et al. 2010.
483 Structure and function analysis of therapeutic monoclonal antibodies against dengue virus type 2.
484 *J Virol* 84(18): 9227-9239.
485 Tao MH, Morrison SL. 1989. Studies of aglycosylated chimeric mouse-human IgG. Role of
486 carbohydrate in the structure and effector functions mediated by the human IgG constant region,
487 *Journal of Immunology* 143: 1595–1601.
488 Tewawong N, Pitaksajjkul P, Dekumyoy P, Ekpo P, Ramasoota P. 2012. Mimotope
489 identification using phage displayed random peptide libraries against monoclonal antibodies
490 specific to house dust mite. *Southeast Asian J Trop Med Public Health* 43(3): 614-623.
491 Williams KL, Sukupolvi-Petty S, Beltramello M, Johnson S, Sallusto F, Lanzavecchia A. 2013.
492 Therapeutic efficacy of antibodies lacking Fcγ receptor binding against lethal dengue virus

infection is due to neutralizing potency and blocking of enhancing antibodies. *PLoS Pathog* 9(2): e1003157.

Figure Legend

Figure 1. Epitope mapping by phage display of random peptide libraries. (A) Affinity selection of phage-display. Ph.D.-12 and Ph.D.-C7C Phage Display Peptide Libraries were used in biopanning step. The constant units of phage ($5x10^{10}$ PFU) was used for three rounds of biopanning. Increasing of percent yields of output phages represents the specific enrichment. (B) Alignment of phage-displayed peptide sequences selected by HuMAbs. Phage clones were shown to display a consensus sequences LXXXG (show in the box) (C) Comparison of the amino acid sequences of E proteins DENV1–4. In the box, DENV1–4 shared the same amino acids at positions 107 (L), 108 (F), 109 (G), 110 (K), and 111 (G). (D) Phage inhibition ELISA of 8 phage clones which matched the motif of 107LXXXG111. Phage lysate of selected clones bound with HuMAb in solution phase and the free phage clones were detected by ELISA. The absorbance values were measured at 450 nm using an ELISA reader (TECAN). Absorbance values of each concentration (A) were divided by absorbance values of no antibody (control) (A_0), resulting in normalized values (A/A0).

Figure 2. Immunofluorescence assay of B3B9 and N297Q-B3B9 rIgG against four DENV serotypes. Vero cells were infected with DENV1, 2, 3, or 4. The ability of the HuMAbs (B3B9 and N297Q-B3B9 rIgG) in the culture fluids of HEK293T cells transiently expressing these antibodies to bind to different serotypes of DENV (DENV1–4) was assessed by performing IFAs. PBS was used as a negative control (Control). Representative images are shown.

515 **Figure 3**. Neutralizing activity of N297Q-B3B9 and B3B9 rIgG antibody against four DENV
516 serotypes. (A) NT levels of N297Q-B3B9 rIgG and B3B9 rIgG in Vero cells were assessed by
517 foci reduction neutralization tests. The foci of infected cells were counted and compared with the
518 no antibody control, and the results were calculated as the percent reduction in focus forming
519 units. The number of foci was calculated as the average of triplicate experiments. (The error bars
520 show standard deviation of the experiments). (B) NT50 concentration of N297Q-B3B9 and
521 B3B9 rIgG antibody against DENV serotypes.

522 **Figure 4**. ADE assays in THP-1 and K562 cells. (A) ADE assay on THP-1 cells. The antibody
523 concentrations were serially diluted ten-fold and are represented on the X-axis. The fold
524 enhancement in the virus copy number of the sample with each antibody was compared with that
525 of the no antibody control and is represented on the Y-axis. The plotted values were obtained
526 from the average of duplicates from two repeated experiments. (B) Enhancement activity of the
527 four DENV serotypes in FcγRII-bearing K562 cells. The number of infected cells (log_{10}) from
528 each antibody concentration compared with that of the control without antibody is shown. At
529 20× magnification, the numbers of infected cells were derived from the average of the counted
530 infected cells from three frames, in duplicate, multiplied by a surface area amplification factor to
531 obtain the total number of cells in each well. Dotted lines indicate cut-off values for
532 differentiating neutralizing and enhancing activity from baseline of no antibody control. (The
533 error bars show standard deviation of the repeated experiments).

534 **Figure 5**. The NT and ADE activity against DENV2 of N297Q-B3B9 rIgG derived from a
535 stable and transient CHO-K1 cell line. A line of CHO-K1 cells stably expressing N297Q-B3B9
536 rIgG was established. (A) The NT of N297Q-B3B9 rIgG against DENV2. The X-axis represents
537 the concentration of antibodies, and the Y-axis represents the percent reduction in focus-forming

538 units compared with the foci number of the control sample that lacked antibody. The number of
539 foci was calculated as the average of triplicate experiments. (B) An ADE assay of N297Q-B3B9
540 rIgG against DENV2 was performed in K562 cells. The infected cells were counted at each
541 antibody concentration and compared with the control (DENV2-infected cells without antibody).
542 (The error bars show standard deviation of the repeated experiments)
543 **Table 1.** The consensus peptide sequences of eight selected phage clones. These clones matched
544 to the motif 107LXXXG111 of DENV genome.
545
546
547

Figure 1

Figure 1. Epitope mapping by phage display of random peptide libraries.

(A) Affinity selection of phage-display. Ph.D.-12 and Ph.D.-C7C Phage Display Peptide Libraries were used in biopanning step. The constant units of phage (5x1010 PFU) was used for three rounds of biopanning. Increasing of percent yields of output phages represents the specific enrichment. (B) Alignment of phage-displayed peptide sequences selected by HuMAbs. Phage clones were shown to display a consensus sequences LXXXG (show in the box) (C) Comparison of the amino acid sequences of E proteins DENV1–4. In the box, DENV1–4 shared the same amino acids at positions 107 (L), 108 (F), 109 (G), 110 (K), and 111 (G). (D) Phage inhibition ELISA of 8 phage clones which matched to the motif of 107LXXXG111. Phage lysate of selected clones bound with HuMAb in solution phase and the free phage clones were detected by ELISA. The absorbance values were measured at 450 nm using an ELISA reader (TECAN). Absorbance values of each concentration (A) were divided by absorbance values of no antibody (control) (A0), resulting in normalized values (A/A0).

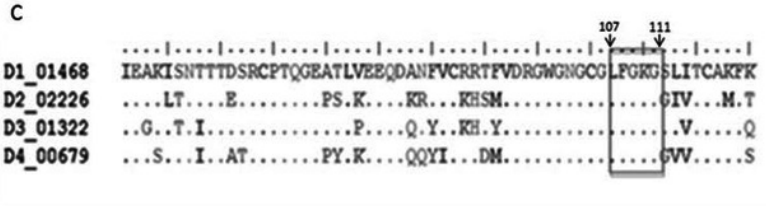
A

For Ph.D. C7C	Input phage (PFU)	Output phage (PFU)	Yield (%)
Round 1	$5x10^{10}$	$6.9x10^{5}$	0.0014
Round 2	$5x10^{10}$	$1.01x10^{8}$	0.202
Round 3	$5x10^{10}$	$9.8x10^{8}$	1.96
For Ph.D. 12			
Round 1	$5x10^{10}$	$6.9x10^{5}$	0.0014
Round 2	$5x10^{10}$	$2.4x10^{8}$	0.48
Round 3	$5x10^{10}$	$7.7x10^{8}$	1.54

B

C7C_2-2	H	G	F	Y	D	L	E	C	G	G					
C7C_2-6	H	G	F	Y	D	L	E	C	G	G					
C7C_3-1	H	G	F	Y	D	L	E	C	G	G					
C7C_3-2	H	G	F	Y	D	L	E	C	G	G					
C7C_3-7	H	G	F	Y	D	L	E	C	G	G					
PhD12_2-1			S	S	W	L	Q	S	Y	G	L	T	T	H	
PhD12_2-3			S	S	W	L	Q	S	Y	G	L	T	T	H	
PhD12_2-4			S	S	W	L	Q	S	Y	G	L	T	T	H	
PhD12_3-4			S	S	W	L	Q	S	Y	G	L	T	T	H	
PhD12_3-10			S	S	W	L	Q	S	Y	G	L	T	T	H	
PhD12_2-2			S	N	F	L	S	A	Y	G	W	N	V	G	
PhD12_2-7		S	P	G	W	L	T	S	Y	G	W	T	T		
PhD12_3-1			N	S	F	L	G	A	Y	G	W	L	E	A	
PhD12_3-6			N	S	F	L	G	A	Y	G	W	L	E	A	
PhD12_3-7	S	Y	N	P	F	L	A	S	Y	G	W	W			
PhD12_3-9	H	N	R	T	F	L	D	A	Y	G	L	D			
PhD12_3-12				G	F	L	E	R	Y	G	L	A	S	V	Q
PhD12_2-5	V	N	W	W	S	Q	Q	E	L	L	L	K			
PhD12_2-9	W	S	G	F	Y	D	L	P	·	T	T	R			
PhD12_2-11	V	S	S	S	R	V	F	H	S	·	W	W			
PhD12_3-5	W	S	G	F	Y	D	L	P	·	T	T	R			
PhD12_3-8	Y	V	R	T	D	F	L	S	A	Y	E	R			

C



D

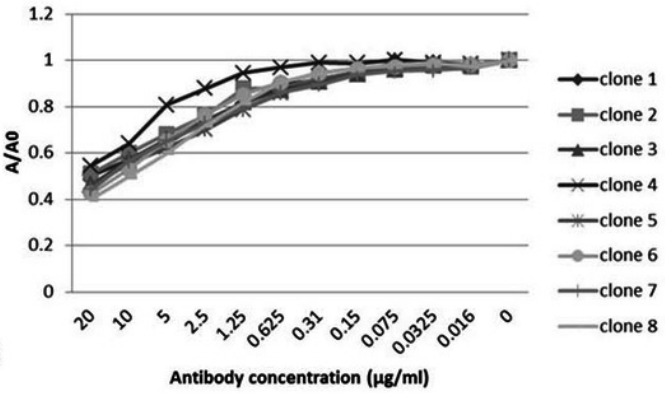


Figure 2

Figure 2. Immunofluorescence assay of B3B9 and N297Q-B3B9 rIgG against four DENV serotypes.

Vero cells were infected with DENV1, 2, 3, or 4. The ability of the HuMAbs (B3B9 and N297Q-B3B9 rIgG) in the culture fluids of HEK293T cells transiently expressing these antibodies to bind to different serotypes of DENV (DENV1–4) was assessed by performing IFAs. PBS was used as a negative control (Control). Representative images are shown.

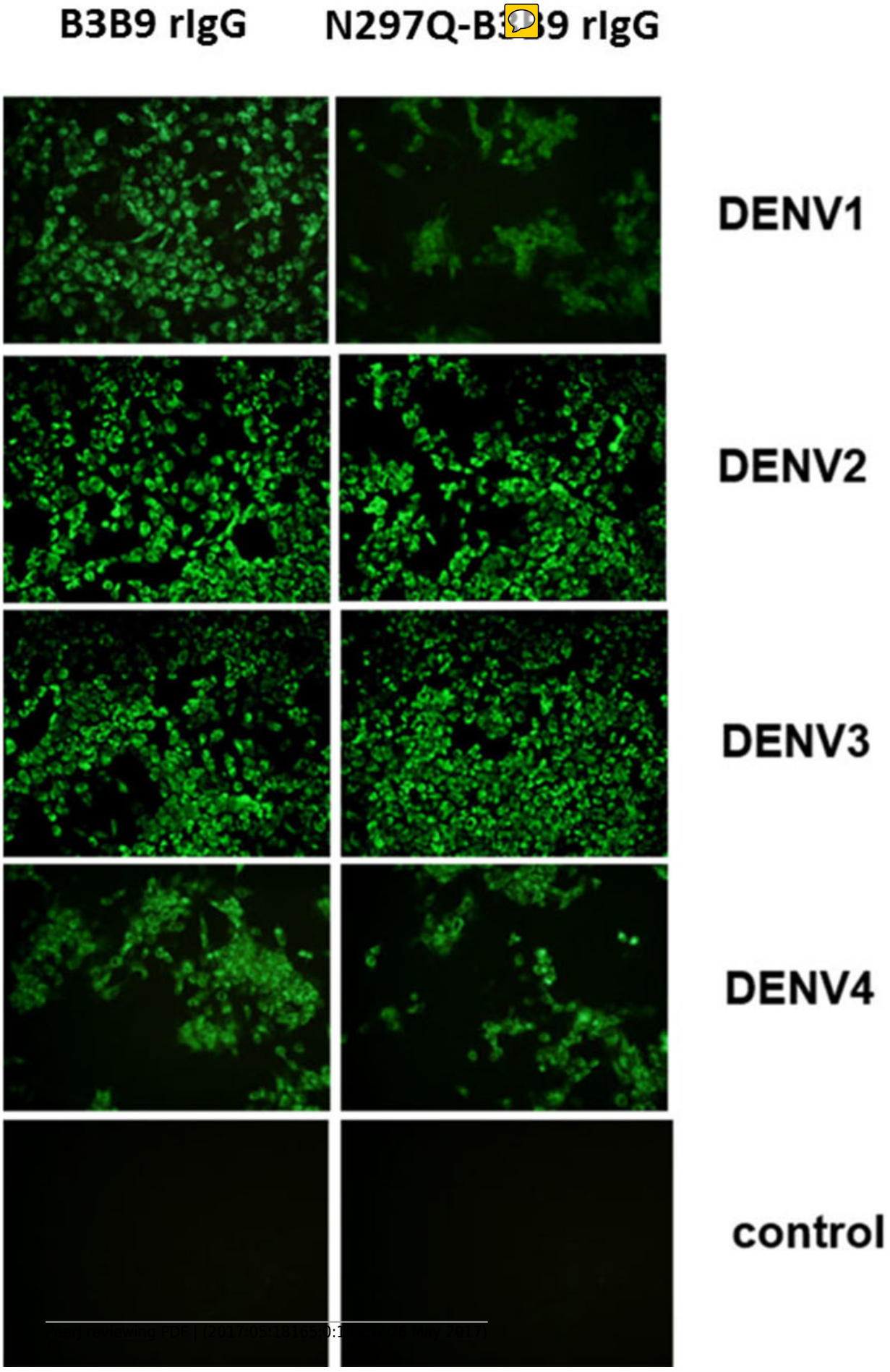
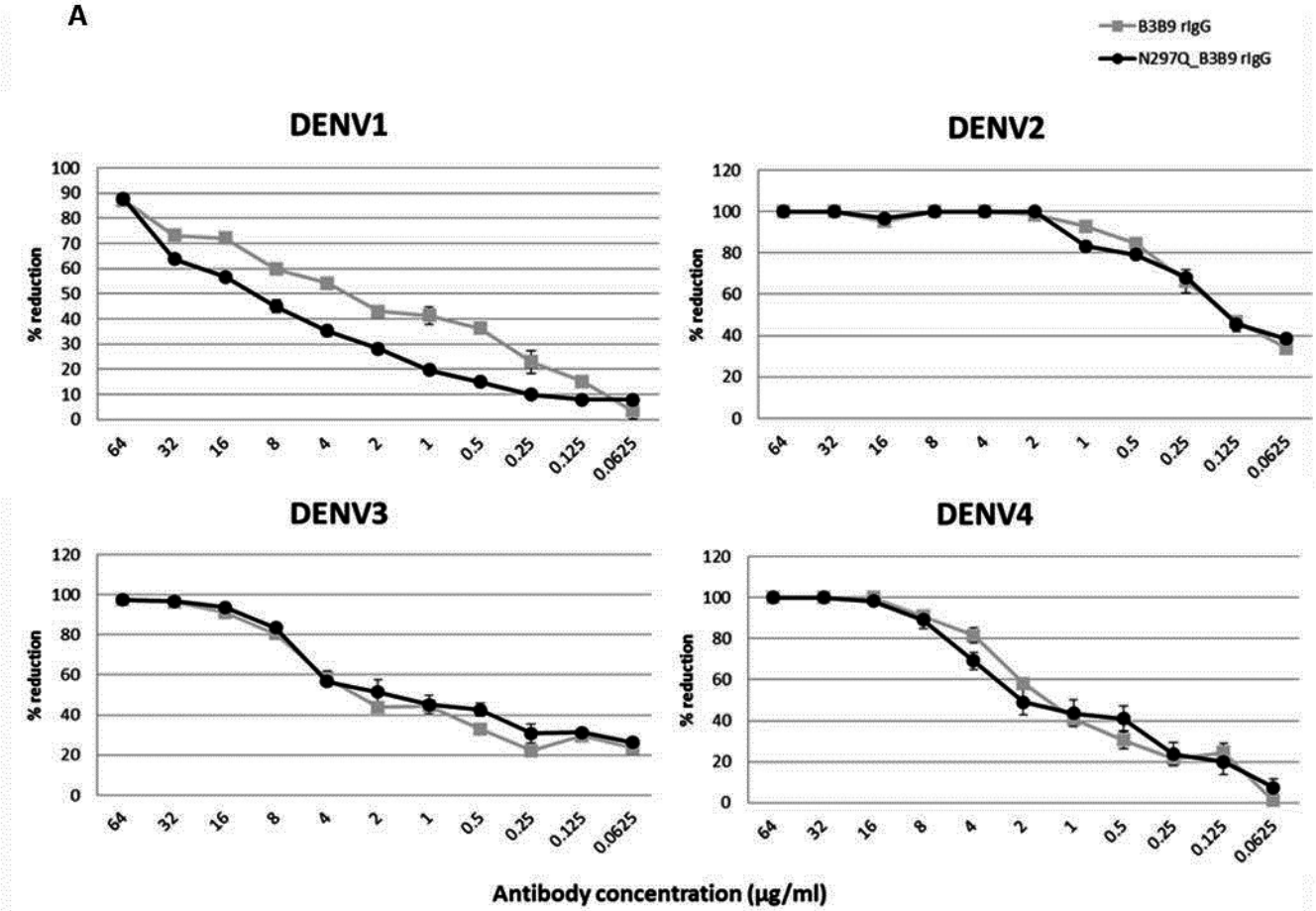

B3B9 rIgG
N297Q-B3B9 rIgG
DENV1
DENV2
DENV3
DENV4
control

Figure 3

Neutralizing activity of N297Q-B3B9 and B3B9 rIgG antibody against four DENV serotypes.

(A) NT levels of N297Q-B3B9 rIgG and B3B9 rIgG in Vero cells were assessed by foci reduction neutralization tests. The foci of infected cells were counted and compared with the no antibody control, and the results were calculated as the percent reduction in focus forming units. The number of foci was calculated as the average of triplicate experiments. (The error bars show standard deviation of the experiments). (B) NT50 concentration of N297Q-B3B9 and B3B9 rIgG antibody against DENV serotypes.

A



B

Antibody	Neutralizing activity (NT50) (µg/ml)			
	DENV1	DENV2	DENV3	DENV4
rIgG	3	0.125	2	2
N297Q rIgG	12	0.125	2	2

Figure 4

Figure 4. ADE assays in THP-1 and K562 cells. (A) ADE assay on THP-1 cells.

(A) ADE assay on THP-1 cells. The antibody concentrations were serially diluted ten-fold and are represented on the X-axis. The fold enhancement in the virus copy number of the sample with each antibody was compared with that of the no antibody control and is represented on the Y-axis. The plotted values were obtained from the average of duplicates from two repeated experiments. (B) Enhancement activity of the four DENV serotypes in FcγRII-bearing K562 cells. The number of infected cells (log10) from each antibody concentration compared with that of the control without antibody is shown. At 20□ magnification, the numbers of infected cells were derived from the average of the counted infected cells from three frames, in duplicate, multiplied by a surface area amplification factor to obtain the total number of cells in each well. Dotted lines indicate cut-off values for differentiating neutralizing and enhancing activity from baseline of no antibody control. (The error bars show standard deviation of the repeated experiments).

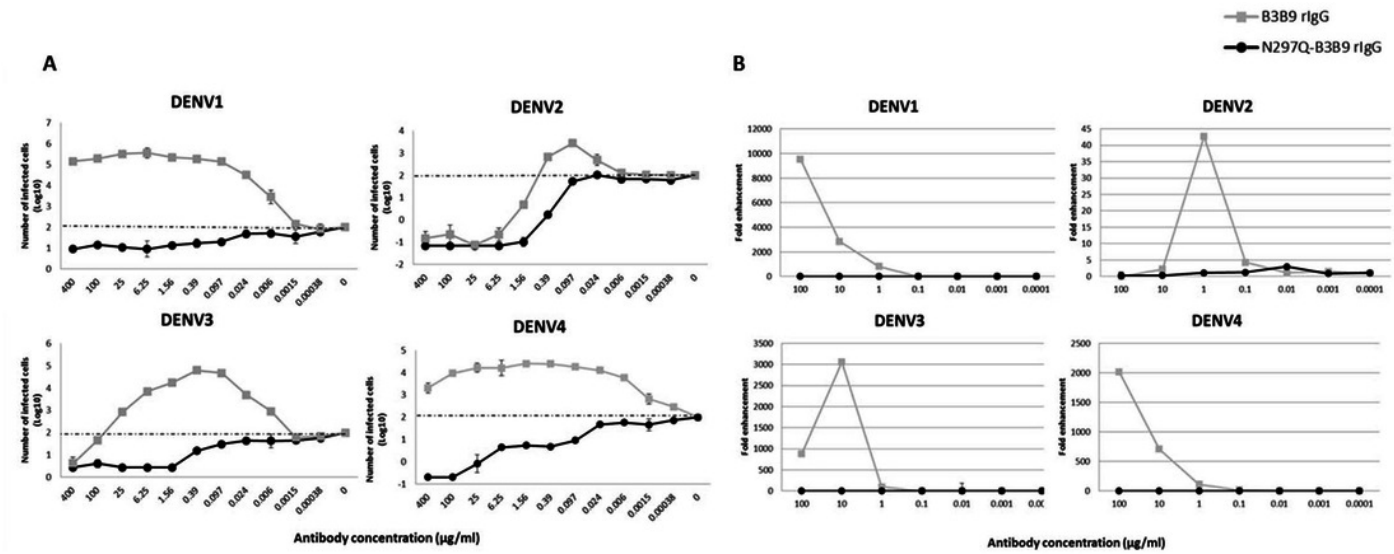


Figure 5

Figure 5. The NT and ADE activity against DENV2 of N297Q-B3B9 rIgG derived from a stable and transient CHO-K1 cell line.

(A) The NT of N297Q-B3B9 rIgG against DENV2. The X-axis represents the concentration of antibodies, and the Y-axis represents the percent reduction in focus-forming units compared with the foci number of the control sample that lacked antibody. The number of foci was calculated as the average of triplicate experiments. (B) An ADE assay of N297Q-B3B9 rIgG against DENV2 was performed in K562 cells. The infected cells were counted at each antibody concentration and compared with the control (DENV2-infected cells without antibody). (The error bars show standard deviation of the repeated experiments)

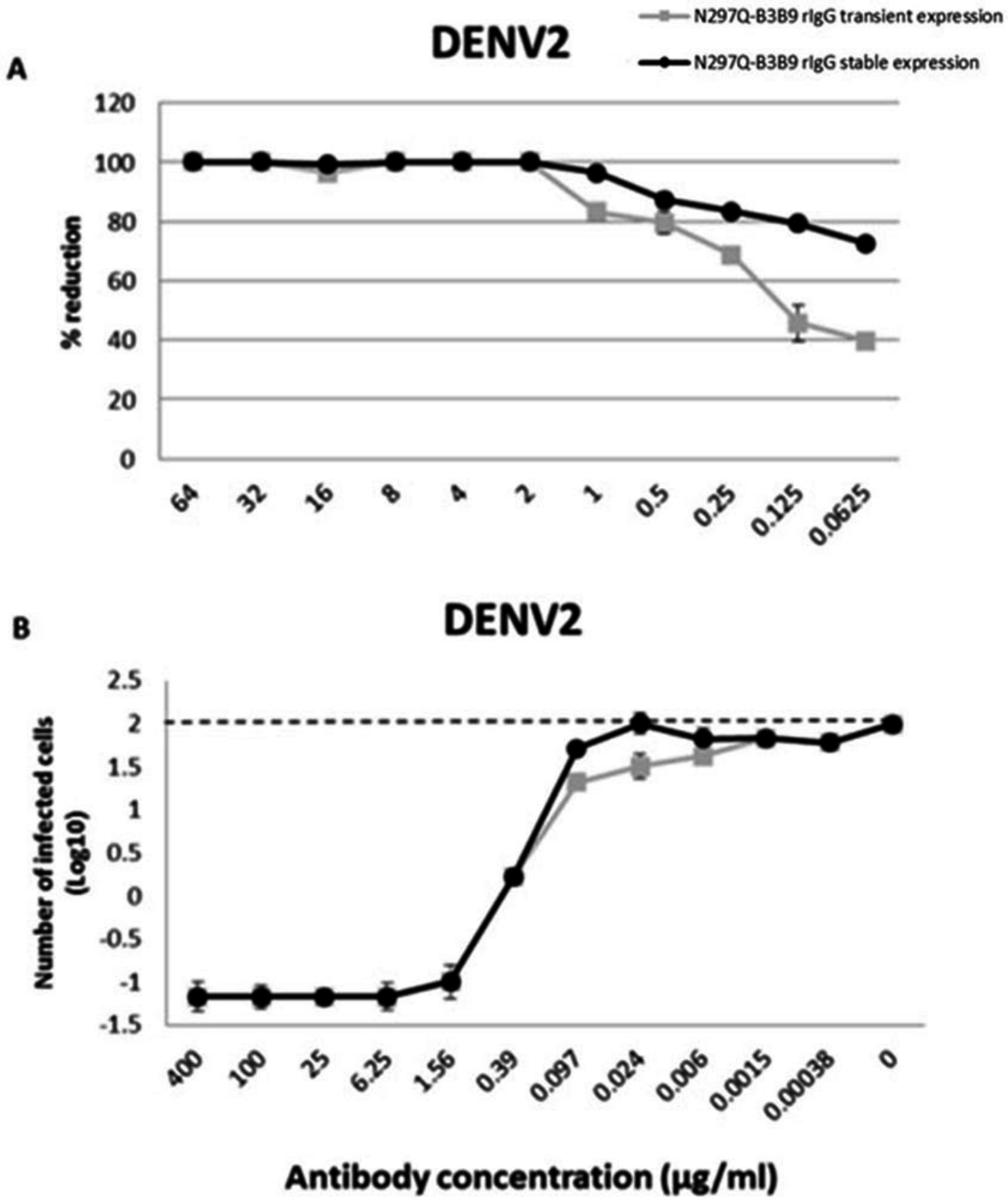

A
DENV2
N297Q-B3B9 rIgG transient expression
N297Q-B3B9 rIgG stable expression
% reduction
120
100
80
60
40
20
0
64
32
16
8
4
2
1
0.5
0.25
0.125
0.0625
B
DENV2
Number of infected cells (Log10)
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
400
100
25
6.25
1.56
0.39
0.097
0.024
0.006
0.0015
0.00038
0
Antibody concentration (µg/ml)

Table 1(on next page)

Table 1. The consensus peptide sequences of eight selected phage clones.

These clones matched to the motif 107LXXXG111 of DENV genome.

Clone number	Consensus Sequences
1	LECGG
2	LQSYG
3	LSAYG
4	LTSYG
5	LGAYG
6	LASYG
7	LDAYG
8	LERYG

Table 2(on next page)

Primers used for IgG isotype identification

Primers used for IgG isotype identification

Supplementary Table 1. Primers used for IgG isotype identification

Primers	Sequences
Human IgG1&3 Fw	5′ GTGACAAAACTCACACATG 3′
Human IgG2 Fw	5′ CAAATGTTGTGTCGAGTGC 3′
Human IgG4 Fw	5′ CAAATATGGTCCCCCATGC 3′
Human IgG Rv	5′ TTTGTCTTGGCATTATGCAC 3′

FW is forward primer, Rv is reverse primer