Development of Engineered Bacteria for Agglutination-Based Dengue Detection

WPI

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Abstract

Annually, 2.5 billion individuals risk contracting the dengue virus and a lack access to a simple diagnostic test for this disease. We aimed to design a diagnostic test for dengue infection by genetically engineering *E. coli* to express surface dengue envelope proteins. The resulting bacteria should agglutinate with dengue antibodies to form bacterial films, indicating an immune response to dengue virus in a patient serum sample. Here we demonstrate a successful proof-of-concept using *E. coli* expressing GFP on the surface, and are able to detect anti-GFP antibodies to a sensitivity of 0.005 ug/mL. Our system lays the groundwork for the development of a cost effective dengue screening tool, which will enable diagnosis on a broad scale and improve survival.

Acknowledgements

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Introduction

Dengue fever is a viral infection that can lead to high fever, headache, fatigue, nausea, and joint pain (CDC, 2012). As many as 400 million people contract dengue annually, and over 2.5 billion people are at risk worldwide (CDC, 2014). Dengue is present in 100 tropical and subtropical countries across all continents, including popular tourist destinations (CDC, 2016). Symptoms present during early infection are not unique, so the disease is easily confused with other diseases such as malaria, West Nile, Zika, and influenza. The most serious diseases caused by dengue viruses are dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which can cause ascites, pleural effusions, shock, and death if prompt medical attention is not given (CDC, 2014). Treatment options are limited, especially as the disease progresses, but supportive care with acetaminophen analgesics, rehydration therapy, and rest reduce morbidity and mortality (CDC, 2012). Preventative measures include the application of mosquito repellant and limiting mosquito breeding sources, as dengue is predominantly spread through mosquito bites (CDC, 2014). It is impossible to predict whether a patient's disease will progress to a severe status. Therefore, prompt diagnosis of dengue is crucial to treatment outcome.

Dengue fever is caused by the dengue virus (DENV) (Guzman, et al., 2010). The genome of DENV, a flavivirus, is 11 kilobases long with a single open reading frame (ORF), as seen in Figure 1.

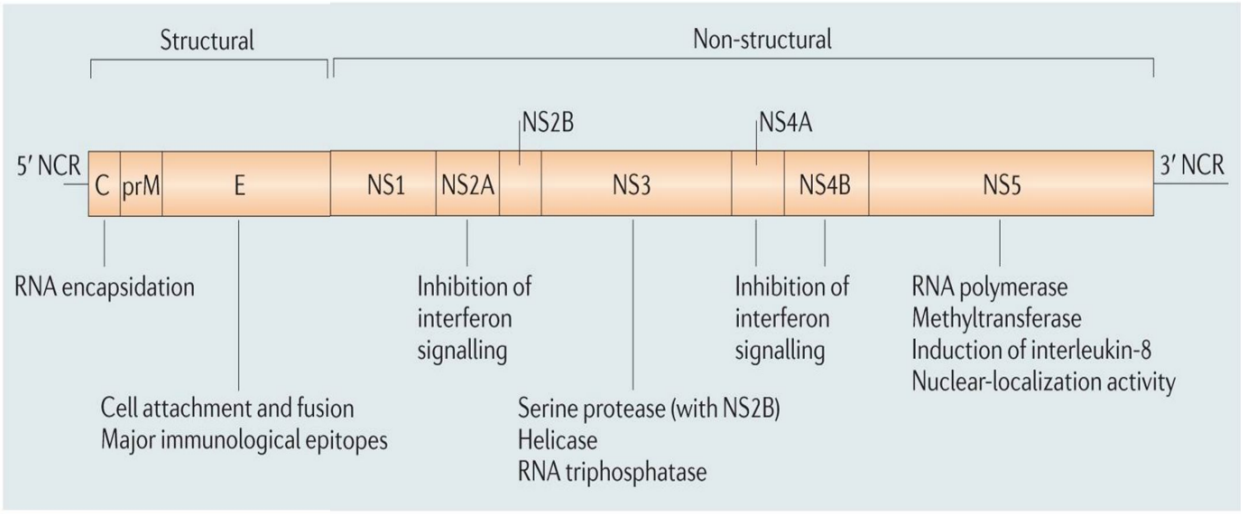


Figure 1: Dengue Virus Open Reading Frame (Vasilakis et al.)

There are four distinct serotypes of DENV: DENV-1, DENV-2, DENV-3, and DENV-4. The serotype genomes are approximately 65% identical, with a corresponding 30%-35% difference in amino acids (Dejnirattisai et al., 2015; Guzman et al., 2010). Structurally, DENV has one polyprotein comprised of three structural and seven non-structural proteins. The structural proteins are the capsid, membrane-associated, and envelope proteins. The envelope protein contains the domains that are predominantly involved in antibody binding. Studies have shown that antibodies binding Domain III (DIII) of the envelope protein most effectively neutralize DENV (Beltramello et al., 2010). Domain III is located in section E of the ORF shown in Figure 1 and antibody binding is depicted in Figure 2 below.

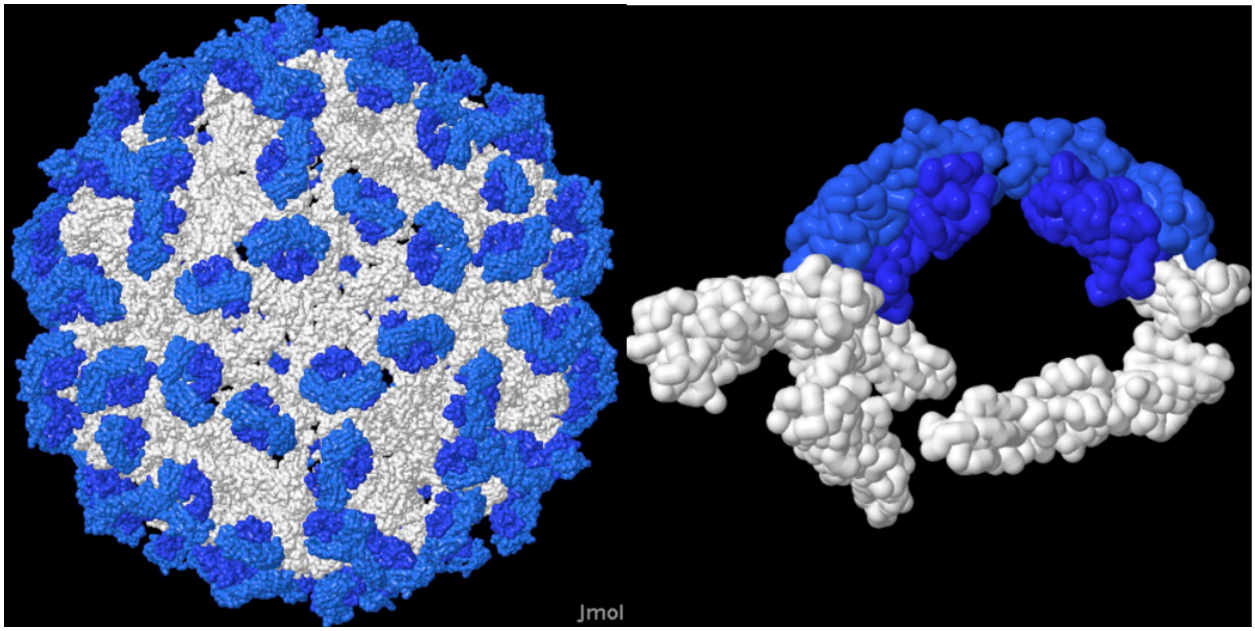


Figure 2. Right: Dengue capsid (white) with bound antibodies (blue) Left: Trimer of DENV (white) with two bound fragment antigen-binding regions of an antibody (blue) (Vasilakis et al.)

DENV initially infects mononuclear phagocytic cells, then spreads to other cell types. Infection with multiple strains of DENV can lead to a phenomenon called antibody-dependent enhancement (ADE). ADE occurs when an antibody against the first infecting

serotype is ineffective at neutralizing a second serotype and instead enhances the uptake of the second infecting serotype into immune cells. Antibodies responsible for ADE bind to Domain I (DI) and Domain II (DII) of the envelope protein of the secondary infecting serotype, causing Fc receptor-mediated uptake into white blood cells. These antibodies are called cross-antibodies, and can lead to more severe symptoms during secondary infection (Beltramello et al., 2010).

Current methods used to diagnose dengue include virus isolation, antigen detection, and enzyme-linked immunosorbent assays (ELISA). Use of these methods is not universal, however, because they are not always cost- and time-effective (World Health Organization, 2009). A locally administered test that can identify DENV in a host within hours would increase chances of patient survival, as the sooner a patient is diagnosed and can receive treatment, the better their prognosis because monitoring of symptoms can begin (Wiwanitkit, 2010).

Agglutination, or "the clumping together of bacteria or cells, resulting often from their reaction with corresponding immune or modified serum," is a method that can be used to determine if an antigen is present in a sample (Douglas et al., 1995). Direct agglutination tests require relatively few reagents and typically do not require refrigeration, keeping testing costs and energy consumption low. These tests also require little expertise to interpret, allowing the tests to be carried out by personnel without extensive medical training. However, the qualitative nature of the assays can lead to misinterpretations of results. Additionally, the simplicity of the test comes with higher sensitivity but lower specificity.

Despite these drawbacks, these tests have been used for diagnosis in multiple other instances. For example, an ELISA test and a microscopic agglutination test (MAT) to test for human leptospirosis were compared. In this instance, acute-phase MAT was found to have higher specificity than the ELISA test (Niloofa et al., 1998). Perhaps the most common use of a direct agglutination test is for blood typing. In a blood typing agglutination test, blood samples are mixed with A and B type antibodies to determine the blood type. Another well-known agglutination test is the Widal test for typhoid fever, a test that is simple and easy to use, particularly in endemic areas, but raises concerns about the qualitative nature of the interpretation of the results, and the quality of antigens used (Olopoenia, 2000).

The 2014 Worcester Polytechnic Institute International Genetically Engineered Machine (iGEM) competition team attempted to develop a direct agglutination assay to diagnose caprine arthritis encephalitis virus (CAEV) in goat populations (Team:WPI-Worcester, 2014.). The iGEM protocol transformed competent *E. coli* cells with a vector containing a hybrid cell surface protein designed to cause the cells to agglutinate in the presence of CAEV antibodies (Team:WPI-Worcester, 2014). The hybrid protein blended *Bacillus anthracis* anchored surface protein BclA with a major antigen of CAEV, the capsid protein p28, an idea originally based on work by Park et al. (Team:WPI-Worcester, 2014; Park et al., 2013). The transformed *E. coli* expressed the BclA-CAEV hybrid on their surface. If blood or serum from an animal that had been exposed to CAEV was mixed with the transformed *E. coli*, the BclA-CAEV should bind to CAEV antibodies in the sample and agglutinate, forming a visible precipitate. The iGEM team was unable to achieve agglutination with anti-CAEV antibodies, but developed a proof of principle experiment using YFP that demonstrated it is possible to achieve agglutination using engineered cell surface proteins (Team:WPI-Worcester, 2014).

In theory, the agglutination-based assay in engineered *E. coli* conceived by the iGEM team could be applied to any disease that results in robust antibody production in the affected host (Team:WPI-Worcester, 2014). To apply this method to dengue fever, we have designed a hybrid protein of BclA and the envelope protein of DENV-2, to be expressed on the cell surface in *E. coli*. Optimally, this hybrid will agglutinate in the presence of a sample containing DENV-2 antibodies, yielding a simple, efficient direct agglutination assay for dengue infection. Our goals include:

- Reproducing and optimizing the 2014 iGEM proof of principle experiment with surface-expressed BclA-YFP
- Cloning the novel recombinant BclA-DENV
- Confirming induction and surface expression of the BclA-DENV protein
- Optimizing the agglutination assay using Dengue antibodies and bacteria expressing BclA-DENV

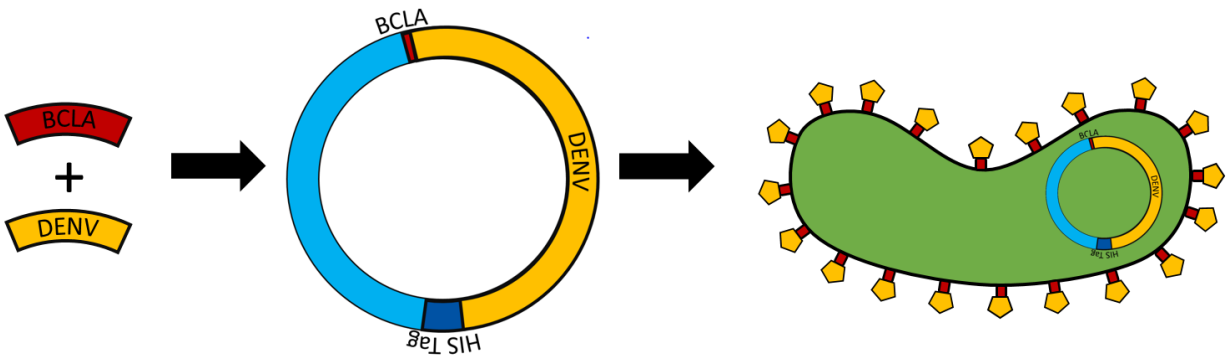


Figure 3. Project Goal Diagram

By creating a diagnostic agglutination test for dengue fever, thousands across the globe can more easily receive the treatment they need in a timely manner.

Through our project,, the iGEM YFP-BclA proof of principle assay was reproduced and optimized successfully, with greater sensitivity than the original proof of principle. A novel, inducible BclA-DENV2 recombinant plasmid was then created and replicated using a Gibson Cloning procedure, and transformed into *E. coli*. Cloning accuracy was confirmed by sequencing the plasmid, and a BclA-DENV2 agglutination assay was conducted by referencing the optimized iGEM proof of principle. After anticipated results of the BclA-DENV2 agglutination assay were not obtained, an SDS-PAGE gel was attempted in order to confirm the induction of BclA-DENV2.

Materials and Methods

Transformation of Competent Cells

Transformation of BclA-YFP (2014 WPI iGEM team, Worcester, MA), GFP (BBA_I20270, parts.igem.org), and RFP (BBA_J04450, parts.igem.org) into DH5α cells (New England Biolabs, Ipswich, MA) (Protocols Competent Cells, n.d.) was performed using standard heat shock techniques (Transformation Protocol, n.d.).

Confirming BclA-YFP and GFP Expression

BclA-YFP and GFP cells were grown overnight at 37°C in LB medium with chloramphenicol. Cells were observed with a Vertical A1 AXIO Zeiss fluorescent microscope (Gotlingen, Germany), using appropriate conditions for fluorescent excitation. Images were exposed at 667 mR/hr; contrast was increased to 100, saturation to -100, and sharpness to 150.

Agglutination Assay

Cells of were grown overnight at 37°C in LB medium with antibiotic (chloramphenicol, kanamycin, or ampicillin). When induction was necessary, cultures were diluted 0.5:5 in fresh LB, 1 mM of IPTG was added and incubation continued at 30°C (t = 3h). Dilutions were performed to reach OD_{600} of 0.05 (Genesys 20, 4001/4 Thermo Fisher Scientific, USA). PBS + 5% BSA (50 uL) was added to every well of a 96 well plate. A 1:2 serial dilution was performed using the antibody across a row with a starting volume of 10 ug/mL. Cell cultures (50 uL) were added to the appropriate well. The plate was visually inspected the next morning. Samples used in this assay include: GFP, inducible PET28a GFP, BclA-YFP (2014 WPI iGEM, Worcester, MA), and BclA-DENV2. Antibodies used include: Anti- Dengue Virus E glycoprotein antibody (Ab80914, Abcam, Cambridge, MA), GFP Tag Monoclonal Antibody (MA5-15256, ThermoFisher Scientific, Rockford, IL), and eGFP Tag Polyclonal Antibody (CAB4211, ThermoFisher Scientific, Rockford, IL.

Creating the BclA-DENV2 Construct

The construct was designed using open source software Benchling (benchling.com). The N terminus of BclA (AJ516945.1) DENV2 (ABY82135.1) and a His tag were used. The BclA-DENV2 construct was created inside of a PET21a vector (LQ567820.1). PET21a was cut at the BamHI and HindIII restriction sites, and the BclA-DENV2 construct was added to the PET21a vector. The entire construct sequence is available in Appendix A, and a diagram depicting the finished construct can be found in Figure 4.

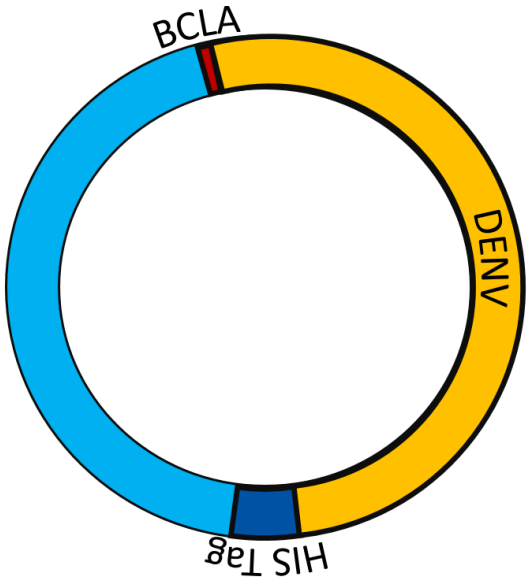


Figure 4. Finished construct

Initial Cloning of Vector

The BclA-DENV2 insert (sequence in Appendix A) was created by gene synthesis (IDT DNA, Coraville, IA). The insert was then amplified using PCR. The BclA-DENV2 construct was resuspended in 50 uL of AE buffer. Stock of each primer (100 uM) was diluted tenfold with AE buffer. Each primer, (sequences available in Appendix B) designed with the construct in Benchling (IDT DNA, Coraville, IA) (1.25 uL of each) was added to 10 ng BclA-DENV2 from gene synthesis (IDT DNA, Coraville, IA) and 25 uL of One Taq® Quick-Load® 2x MM with STD Buffer (New England Biolabs, Ipswich, MA) PCR mix. The mixture was brought to a final volume of 50 uL; PCR was performed on the mixture with the parameters outlined in Table 1.

Table 1. PCR Parameters for amplification of BclA-DENV2

95°C	2 min	
95°C	30 sec	
55°C	45 sec	30 x
72°C	2 min	
72°C	10 min	
10°C	Infinite hold	

Restriction Digest

A restriction digest was performed to cut the vector at BamHI and HindIII. The restriction endonucleases (2 uL each, 10,000 U/mL)(New England Biolabs, Ipswich, MA) were added to 6 uL of 10X CutSmart Buffer (New England Biolabs, Ipswich, MA) and 4.1 ug of plasmid at 37˚C for 1h.

Mini Prep of the Empty Vector

The empty vector DNA was prepared using the Pure Yield ™ Plasmid Midiprep System (Promega, Madison, WI) per the manufacturer's instructions.

Test Digest of the Mini Prep of the Empty Vector

To perform the test digest of the first mini prep, 5 uL of the mini prep, 1 uL of 10X CutSmart buffer (New England Biolabs, Ipswich, MA), 1 uL of Xhol (New England Biolabs, Ipswich, MA) restriction enzyme, and 3 uL of water were incubated for 1 hour at 37°C. Following incubation, the mixture was run on a standard agarose gel with 2 uL of load buffer and 2 uL of SYBR (Cambrex, Rockland, ME) using standard techniques. The length of the band produced by running the gel was compared to the researched length to determine the success of the restriction digest.

Gibson Cloning

The reactions for Gibson Cloning were set up according to Table 2.

Table 2. Reactions for Gibson Cloning

Gibson Cloning				
	PCR Product (Construct)	Vector	Cloning Mix	Water
1 neg control	0uL	1uL	10uL	9uL
2 max	4uL	1uL	10uL	5uL
3	2uL	1uL	10uL	7uL
4	1uL	1uL	10uL	8uL

After the samples were assembled, they were incubated in the thermocycler (MyCycler Thermal Cycler, BioRad, Hercules, CA) for 15 minutes at 50°C. Cells were then transformed into competent cells by standard techniques.

Mini Preps after Gibson Cloning

Mini preps were again performed as described.

Confirmation of Clone Success and Sequence Analysis

A standard agarose gel was run with 10 uL of each completed mini prep, with 2 uL of 6X purple gel loading dye (New England Biolabs, Ipswich, MA) and 2 uL of SYBR (Cambrex, Rockland, ME) to determine which clones contained the insert. The B1 mini prep with the concentration above 30 ng/uL was sent for sequencing. An alignment was performed on Benchling (www.benchling.com) to confirm sequence fidelity.

Confirmation of Induction of GFP and BclA-DENV2

Induced and uninduced GFP were visualized under the fluorescent microscope (AXIO, Vertical A1, Zeiss Microscopy, Gotlingen, Germany). An SDS-PAGE Mini-PROTEAN TGX Precast gel (Biorad, Hercules, CA) was performed, and the resulting gel was stained

coomassie –G250 stain (0.2% solution in water) (Lawrence, 2009) and InVision His-tag In-gel Stain (Introgen Life Technologies, Carlsbad, CA). To perform the gel, 50 uL of the diluted overnight cultures, including those with IPTG, used in the development of the agglutination assay were centrifuged for 1 minute at 11,000 x g. A stain was created by adding 20 uL of DTT to 200 uL of SDS stain 2X sample buffer. The stain (50 uL) was added to each centrifuged tube of cells. Samples were then boiled for 10 minutes. Each sample (20 uL), and 5 uL of PageRule Prestained protein ladder (Thermo Fisher Scientific, Rockford, IL) was added into two wells of the SDS-PAGE gel which was run at 120V for 1.5 hours. Half the gel was stained with coomassie stain, and half was stained with His-tag stain. For the coomassie stain, the gel was washed for 10 minutes in water before being stained for 15 minutes with the blue coomassie stain. The gel was then washed in water for 15 minute increments, until stain removal was complete. The gel was imaged using the coomassie settings in the Biorad Universal Hood II gel imager (Universal Hood II, Biorad, Hercules, CA). To visualize the His-tag stain, the gel was rinsed in fixing solution for one hour, then washed twice in water for 10 minutes before an overnight rinse in the InVision His-tag In-gel Stain (Introgen Life Technologies, Carlsbad, CA). The following day, the gel was washed twice in 20 mM phosphate solution for 10 minutes, and visualized immediately using the Ethidium Bromide settings in the Biorad Universal Hood II gel imager (Universal Hood II, Biorad, Hercules, CA).

Results

Confirmation of YFP and GFP Expression

To begin, we confirmed the expression of GFP and BclA-YFP from the 2014 iGEM team plasmids, and observed a notable difference between the externally expressed GFP and the internally expressed BclA-YFP. Fluorescent microscopy images of both samples were taken at 400X magnification and differences in brightness and saturation were observed. The internal GFP sample (Figure 5a) shows uniform expression throughout the bacterial cell. Meanwhile, the external BclA-YFP sample (Figure 5b) shows clear polarity with fluorescence at either end of the rod as well as a dark area in the center of the cell. These patterns are consistent with an internally expressed fluorescent protein (the uniform brightness in Figure 5a) and an externally expressed fluorescent protein (the polarity in Figure 5b).

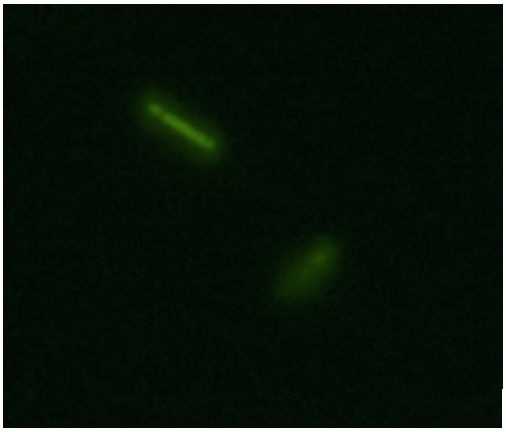
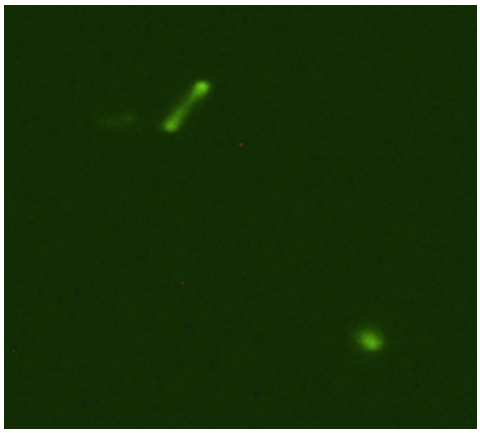
a.  b. 

Figure 5: The GFP (a) and BclA-YFP (b) samples under 400X magnification

Proof of Principle Agglutination Assay

We then confirmed the proof of principle experiment demonstrating that specific agglutination can be achieved with surface-expressed BclA-YFP. Agglutination causes the formation of a biofilm facilitated by the crosslinking of the cells by the antibody, whereas cells that do not agglutinate precipitate to a small pellet in the bottom center of the well. This concept is illustrated in Figure 6a.

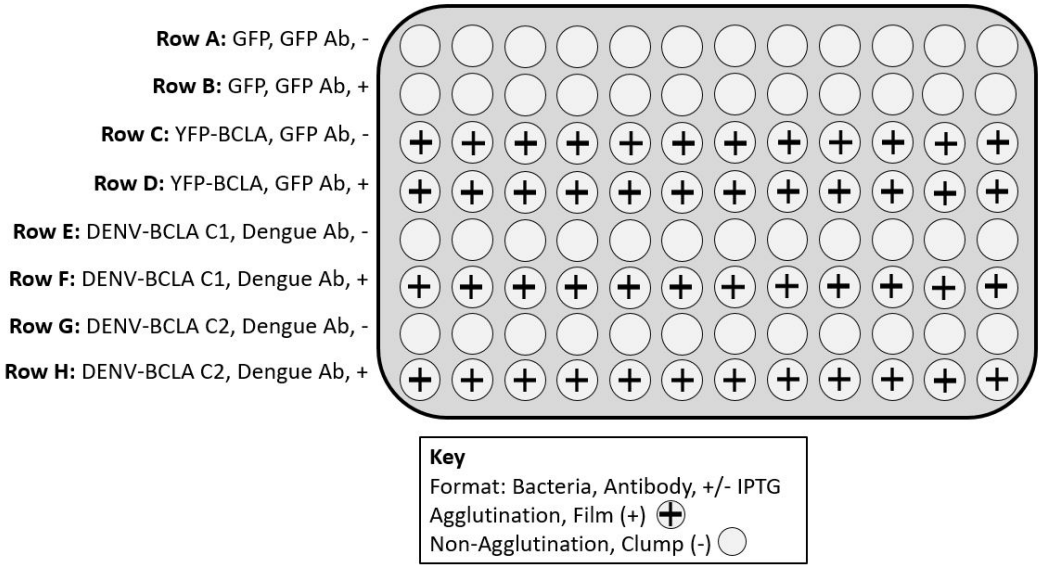


Figure 6a: The anticipated results of the agglutination assay

E. coli cells expressing an empty control vector (containing only the dual transcriptional terminator, DT), BclA-YFP, or GFP, were applied to the agglutination assay using both polyclonal and monoclonal GFP antibodies, as in Figure 6b. Of the three bacterial samples, only the wells containing BclA-YFP, found in row A and row D, showed agglutination. Figure 6b shows the results of the proof of principle agglutination assay, with only the rows containing BclA-YFP showing the agglutination film, while other rows form bacterial pellets, indicating that no agglutination occurs. . It was determined that the assay was sensitive until an antibody concentration of 0.005 ug/mL.

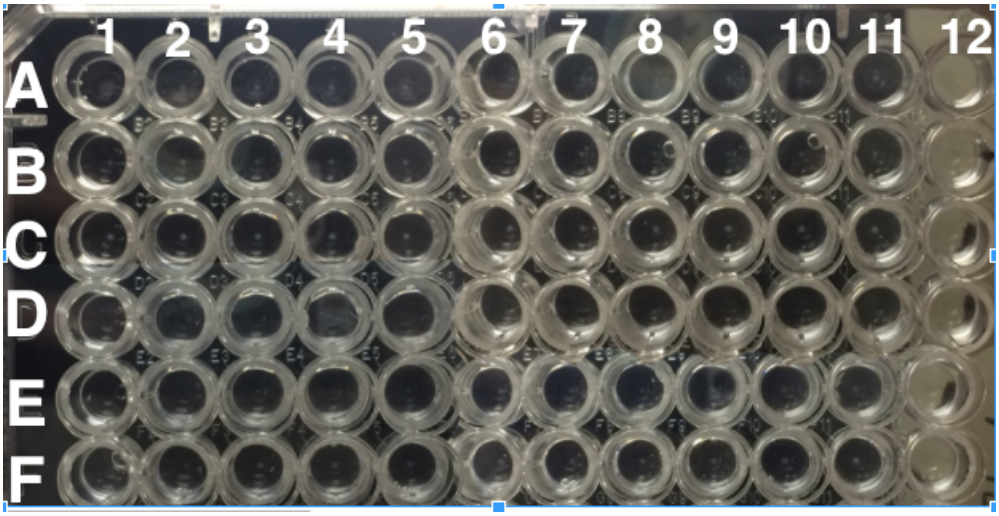


Figure 6b: The plate containing the proof of principle agglutination assay.

Cloning the Construct

After agglutination was successful, the BclA-DENV2 construct was amplified via PCR for Gibson cloning. The construct was then cloned into the PET21a vector using varying amounts of construct, (0 uL, 4 uL, 2 uL, and 1 uL). The vector was cut using the BamHI and HindIII restriction sites. After cloning, the DNA was transformed into two types of competent cells, DH5α and 10-Beta to ensure transformation success. These transformations were plated on Ampicillin resistant plates as shown in Figures 7a and 7b.

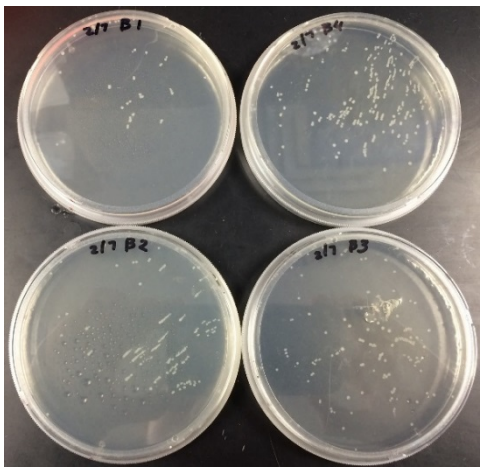
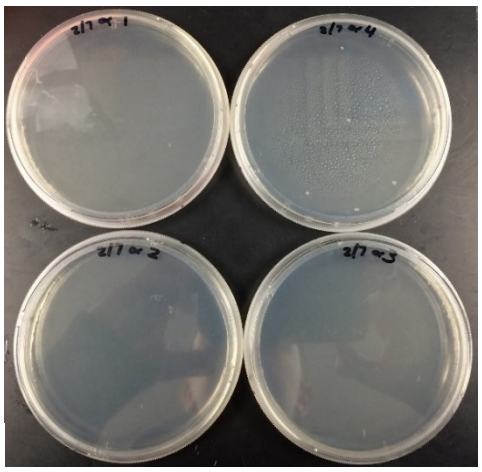
a.  b. 

Figure 7: The plates (10-Beta, a, DH5α, b) containing the colonies after Gibson cloning and transformation.

The number of colonies on each plate was counted and is summarized in Table 3. The 10-Beta strain of *E. coli* produced more colonies than the DH5α strain. The plate that grew colonies in the DH5α cells was plate 4, with 7 colonies. 10-Beta cells experienced a higher transformation efficiency. All plates experienced colony formation, even the negative control. However the negative control plate had significantly fewer colonies (41) compared to the rest of the plates with 10-Beta cells indicating that the colonies were real transformants.

Table 3: The amount of colonies counted on each plate

Plate Number	Construct Volume (uL)	DH5α (# colonies)	10-Beta (# colonies)
1	0	0	41
2	4	0	150
3	2	0	152
4	1	7	317

Following the transformation and colony maturation, minipreps of three colonies from each cell types were performed. Yields ranged from 7.6 ng/uL to 42.0 ng/uL. Ultimately, these concentrations were extremely low compared to what we expected.

Confirmation of Cloning Success

To ensure cloning success, minipreps of three colonies from each cell types were performed. Yields ranged from 7.6 ng/uL to 42.0 ng/uL. Ultimately, these concentrations were extremely low compared to what we expected. These three minipreps were run on a gel along with an empty vector sample cut with XhoI as shown in figure 8.

Lanes 3-5 (A1, A2, and A3) were samples taken from the DH5α colonies, while lanes 6-8 (B1, B2, and B3) were samples from the 10-Beta cells. Lane 2 was the digested vector and lane 1 was the ladder. Only samples B1 and B2 show an increase in DNA length compared to the length of the vector, indicating that these two samples successfully incorporated the insert into the vector. Based upon this result, clones B1 and B2 were chosen to be sequenced. However, the concentration of B2 (7.6 ng/uL) was too low for sequencing. Therefore, only clone B1 was sent for sequencing.

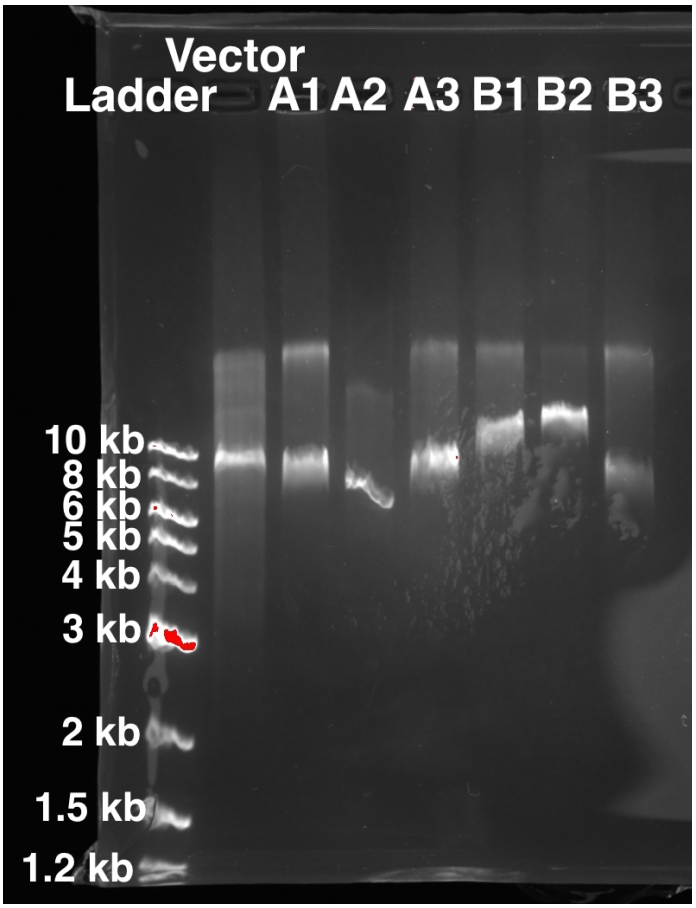


Figure 8: The gel run with the six cloning samples, the empty PET21a vector, and a ladder for comparison

When the sequence of B1 was returned, it was compared to the PET21a vector and BclA-DENV2 construct. There were two mutations to note of: one at base 5732, where a cytosine (C) was substituted for a thymine (T). This created a silent mutation, and did not change the amino acid from histidine. The other noteworthy mutation occurred at base 5622, where a C was placed instead of a T. Unfortunately, this created a non-conservative point mutation, as the codon CTT codes for lysine while codon TTT codes for phenylalanine. This change is displayed in Figure 9. It was unknown if these mutations would hinder the protein folding of the dengue virus envelope protein.

Figure 9: The alignment performed in Benchling showing the mutation at amino acid 137.

Agglutination Assay

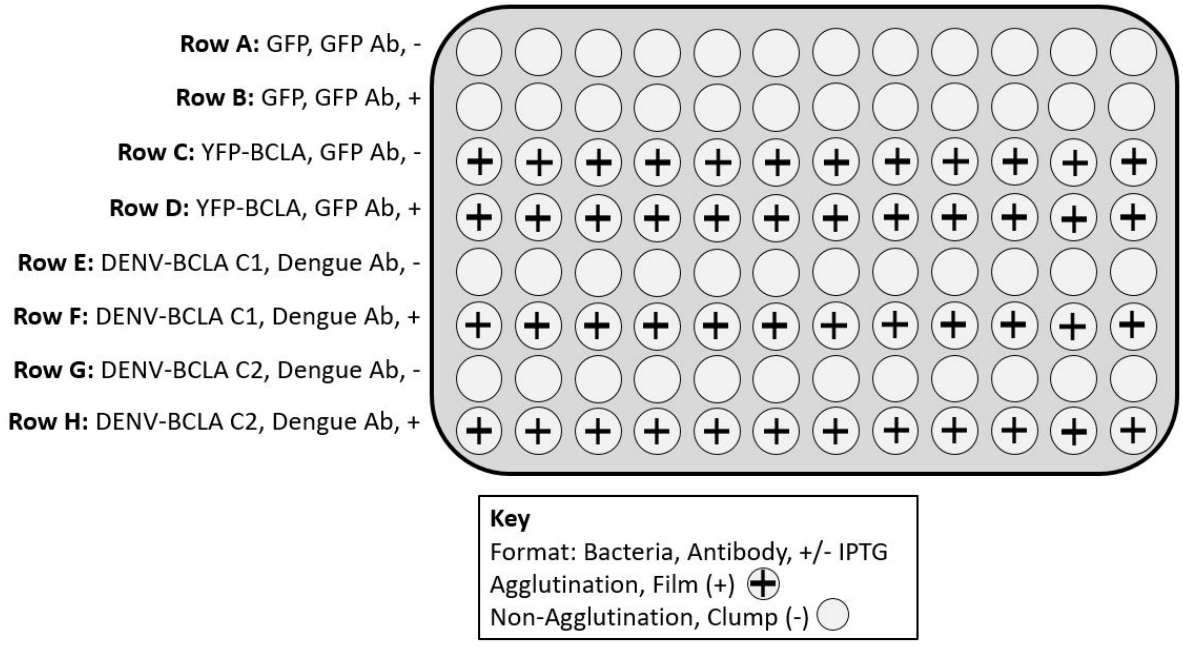


Figure 10: The expected results of the DENV agglutination assay

Following the confirmation of our construct, we then attempted an agglutination assay using inducible GFP on PET28a, BclA-YFP on PET21a, and two BclA-DENV2 on PET21a, with GFP and Dengue antibodies. The anticipated results are summarized in figure 10. However, there was no agglutination present; every well had a bacterial pellet at the bottom.

Confirmation of GFP and BclA-DENV Induction

To troubleshoot the failure of the agglutination assay, we first confirmed the induction of both GFP and BclA-DENV2 with IPTG. After the induction protocol was followed, samples of induced GFP (left) and uninduced GFP (right) were viewed under the fluorescent microscope as shown in figure 11.

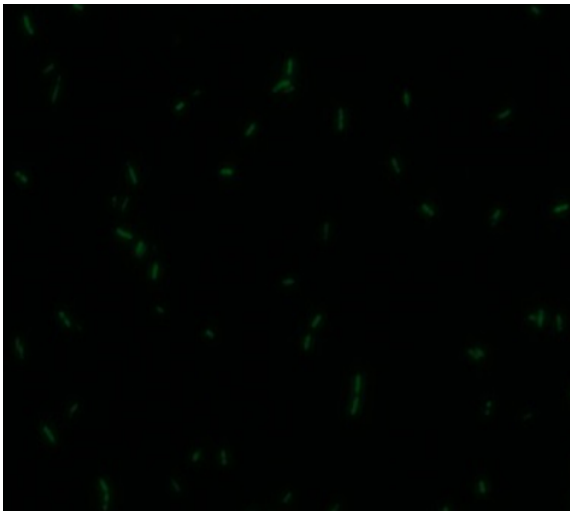
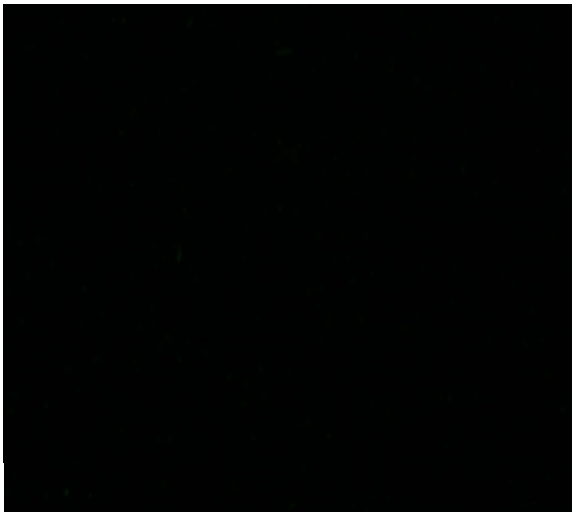
a.  b. 

Figure 11: Induced GFP expression (a), and uninduced GFP expression (b).

The induced sample shows clear fluorescence while the uninduced sample shows no fluorescence, indicating that the induction protocol was satisfactory in inducing GFP. To further confirm the success of the induction process, four samples (uninduced GFP, induced GFP, uninduced BcLA-DENV2, and induced BclA-DENV2) were analyzed for expression by SDS-PAGE and stained with either Coomassie stain or a Histine-tag stain as shown in figure 12.

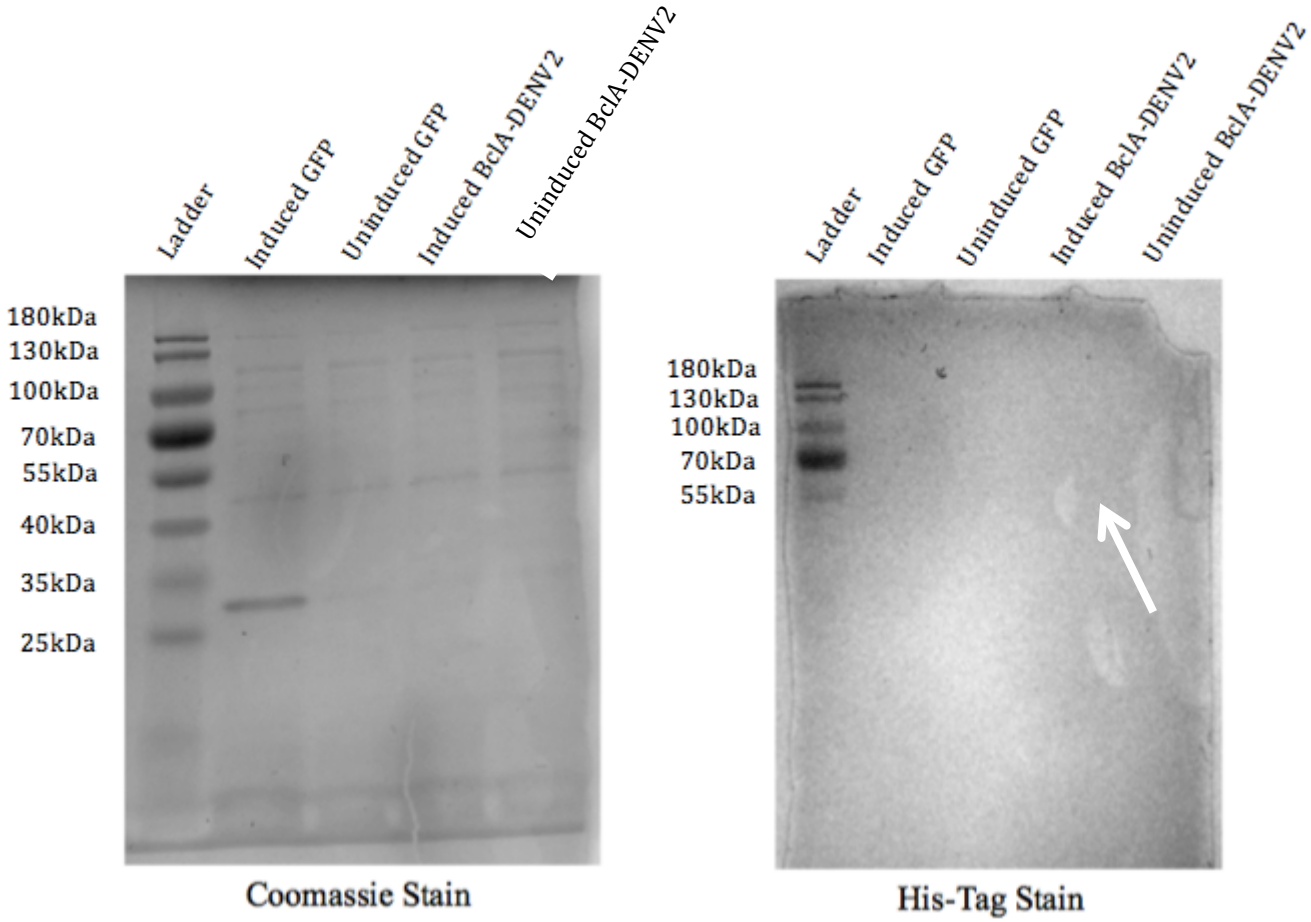


Figure 12: The two gels stained with Coomassie Stain and a Histine-Tag Stain.

On the Coomassie stain, the large band that appeared at approximately 27 kDa in the second lane (induced GFP) is the expected band for GFP, meaning that GFP induction was successful. There was no band for the induced BclA-DENV2 present. Furthermore, there were no bands on the histine-tag stain. If induction of the BclA-DENV2 was successful, one would expect to see a band at approximately 56.7 kDa, as shown by the arrow in the His-Tag Stain in Figure 12..

Discussion

The agglutination assay developed in this experiment depends on IgG antibodies interacting with dengue antigens presented on the surface of *E. coli* via a recombinant BclA construct. Human serum contains a total concentration of IgG immunoglobulin of 7.5-22 mg/mL (Sigma-Aldrich, n.d.). Determining the concentration of a single, disease-specific IgG in serum is difficult to determine and usually not relevant to research or therapeutic purposes, and is therefore infrequently calculated. While IgG levels have not been calculated for dengue, levels of specific IgGs in response to pneumococcal pneumonia infection in children is in the 0.1-1.0 μg/mL range (Esposito et. al, 2003). The sensitivity of the agglutination assay developed in this experiment is 0.005 μg/mL, well below the observed IgG concentration post-pneumococcal pneumonia infection. Assuming that the IgG response to dengue infection is similar, the assay is anticipated to have sensitivity well within a clinically relevant range.

A non-conservative mutation occurred at base 5622, where a thymine was substituted for a cystine and resulted in a lysine in the place of a phenylalanine. Phenylalanine is a hydrophobic, neutrally charged amino acid, while lysine has a reactive, positively charged ε-amino group (Dayhoff, n.d.). While both amino acids are derivatives of alanine, their differences in charge and function in proteins could have resulted in misfolding of the dengue antigen, which in turn could impact binding of the anti-dengue IgG. This conformational change could result in decreased or nonexistent assay functionality.

Future steps to advance this project include confirming measurable levels of surface-bound BclA-DENV2 when induced using IPTG. Confirmation of successful induction could be confirmed using SDS-PAGE and staining with Coomassie and His-Tag stains. Expected results of a successful SDS-PAGE are included in the Results Section. Once adequate BclA-DENV2 induction has occurred, the agglutination assay should yield the expected results.

After confirmation of the dengue agglutination assay, the assay could be tested using serum from patients exposed to dengue, and the sensitivity of the assay using serum affirmed. Experiments investigating the effectiveness of the agglutination assay across all

four dengue serotypes and determining if the agglutination assay could distinguish between recent and past dengue infection would need to be conducted. Due to the tropical and subtropical climate of dengue-impacted areas, heat stability of assay reagents would also need to be investigated. Substitutions of reagents for increased heat stability, shelf life, or cost-effectiveness would need to be considered, and a cost-analysis of assay production be conducted. Instructions for running and reading the assay would need to be developed, and storage guidelines published. At this early stage in the development process, the marketed test kit would include round-bottom plates and quantities of all necessary reagents, including cultures of the BclA-DENV2 transformed cells, for performing a set number of tests.

This project aimed to create a novel, agglutination-based test for dengue by synthesizing a recombinant protein displaying dengue envelope antigens on the surface of *E. coli.* When exposed to dengue IgG antibodies, which would be present in an individual infected with dengue within approximately 10 days of primary dengue infection, the *E. coli* would agglutinate, forming a visible film in a 96-well, round bottom plate (Laboratory Guidance and Diagnostic Testing, n.d.). This visual test for dengue could be administered on-site, foregoing the need to send multiple samples off to a lab for expensive testing. With this information, doctors could administer proper care to those infected and, hopefully, prevent dangerous progressions of dengue fever. In order to accomplish this, our team reaffirmed a proof of principle experiment that was the focus of the 2014 WPI IGEM team, engineered a novel recombinant BclA-DENV protein, and transformed it into a suitable *E. coli* strain. Induction of this BclA-DENV recombinant was attempted, but not confirmed. Our final step would be to optimize the agglutination assay for the dengue agglutination assay in order to ensure a diagnostically specific, reproducible assay for the detection of dengue infection.

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Appendix A

The sequence of the created construct. Uppercase letters indicate the BclA protein, while lowercase letters indicate the DENV envelope protein.

tggcgaatgggacgcgccctgtagcggcgcattaagcgcggcgggtgtggtggttacgcgcagcgtgaccgctacacttg
ccagcgccctagcgcccgctcctttcgctttcttcccttcctttctcgccacgttcgccggctttccccgtcaagctcta
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GCCCTACGCAAGGGGAGCCATCTCTTAAAGAAGAACAAGATAAACGCTTCGTATGCAAGCACTCAATGGTGGACCGTGGT
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AAACACAGCACGGAACCATTGTTATCCGCGTACAATACGAAGGAGATGGTAGCCCTTGTAAGATTCCCTTCGAGATCATG
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ataactagcataaccccttggggcctctaaacgggtcttgaggggtttttgctgaaaggaggaactatatccggat

Appendix B

The sequences of the ordered primers for PCR amplification of the construct.
Forward Primer:
ATGACTGGTGGACAGCAAATG

Reverse Primer:
GTGCTCGAGTGCGGCCGC