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DEVELOPMENT OF A TWO-HYBRID SYSTEM FOR RAPIDLY DETECTING FLAVIVIRAL INFECTIONS

A Major Qualifying Project Report

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ABSTRACT

Studies have shown that an innate immune response often occurs during viral infection of mammalian cells when two particular protein components, TLR-3 and TRIF, bind together after recognition of foreign RNA inside a cell. The TLR-3/TRIF interaction was used to design a fluorescence-based two-hybrid system for rapidly detecting Flaviviral infections. A plasmid encoding TRIF was engineered to express half of a Venus (YFP) reporter protein, while TA cloning was used to confirm the clonability of the TLR-3 gene. Future experiments include cloning the TLR-3 gene into the Venus vector, and then stably transfecting the fusion proteins into Vero cells. Upon addition of an experimental sample containing viral RNA to this cell-line, the modified TLR-3 and TRIF proteins should dimerize, therefore allowing the YFP to assemble, causing the cell to fluoresce.

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ACKNOWLEDGEMENTS

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BACKGROUND

Introduction

Current detection methods for the diagnosis of viral pathogens include RT-PCR, ELISAs, immunoblots, virus neutralization tests, hemagglutination-inhibition, complement fixation, and immunofluorescence microscopy, etc. These tests are often crucial for diagnosis, but they are time consuming, expensive, and sometimes unreliable. In order to attempt to circumvent these problems, this project investigated the use of a rapid bimolecular fluorescence complementation assay in transfected mammalian cells to detect viral pathogens by measuring the interaction of key host signal transduction pathway proteins known to interact during flavivirus infections.

Flaviviruses

The family Flaviviridae contains as many as 70 viral pathogens, including viruses causing diseases such as Dengue Fever, Dengue Hemorrhagic Fever, Yellow Fever, Japanese Encephalitis, and West Nile. Flaviviruses are transmitted through arthropod vectors, mainly mosquitoes or ticks. It is thought that these viruses were derived from a common ancestor between ten and twenty thousand years ago, and they are still rapidly evolving to fill new ecological niches (Solomon and Mallewa, 2001). Flaviviruses are divided into groups using factors such as vector or clinical syndromes. Symptoms can include fever, joint pain and rashes. For the more severe viral hemorrhagic fevers the symptoms can also include plasma leakage, enlarged livers with elevated liver enzymes, platelets decrease, and decreased fluid content of the blood. There are currently no effective antiviral drugs against these viruses, and very few

vaccines. The exception is Yellow Fever, for which an effective vaccine has been available since the 1930's, as well as Japanese Encephalitis and tick-borne encephalitis (Cardosa, 1998).

The Flaviviral genome (**Figure-1**) consists of approximately eleven kilobases of single stranded positive sense RNA. The RNA genome is wrapped in a nucleocapsid protein within an enveloped membrane, and contains three structural and seven nonstructural (NS) proteins.

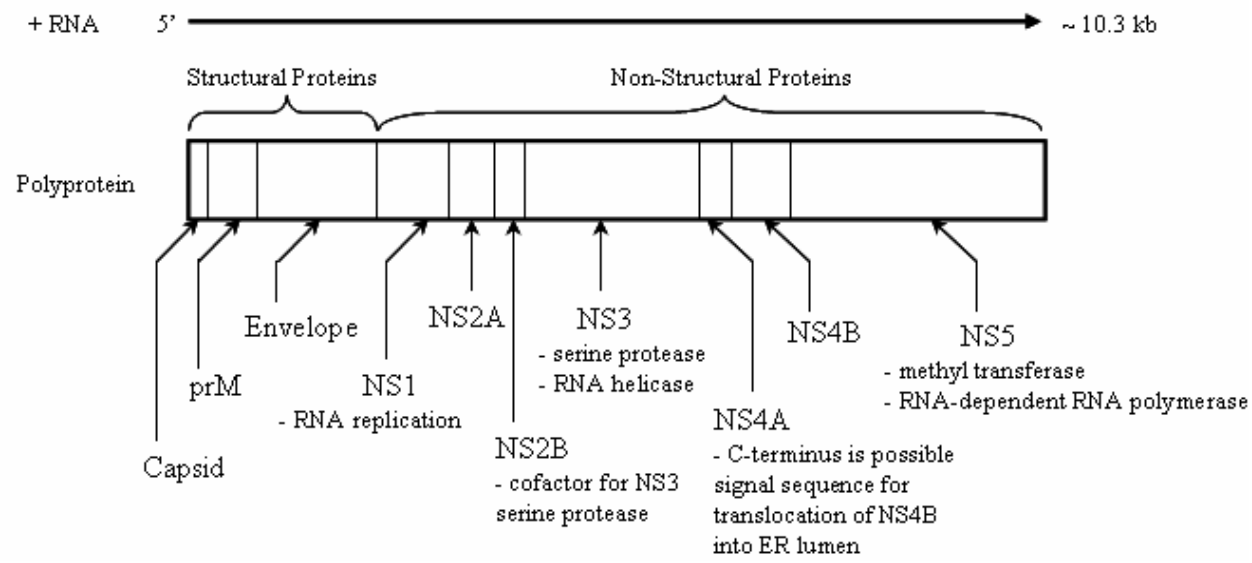

Figure-1: Diagram of the Flaviviral Genome. Figure shows the structural proteins of Capsid, prM, and Envelope with the seven non-structural proteins. The seven structural proteins include a description of their function. Taken from FlaviTrack, 2010.

The structural proteins, C, prM (M), and E are important members of the mature virus (**Figure-2**). C (capsid) is thought to be involved in packaging, M (membrane) in export, and protein E (envelop) is thought to be important for viral entry into host cells, as well as being a major target of the humoral immune response. The non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 are involved in various functions during virus genome replication and assembly, and include a protease responsible for cleaving certain positions of the polyprotein

to create mature proteins. They also include an RNA-directed RNA polymerase responsible for genome amplification (Chambers et al., 1990).

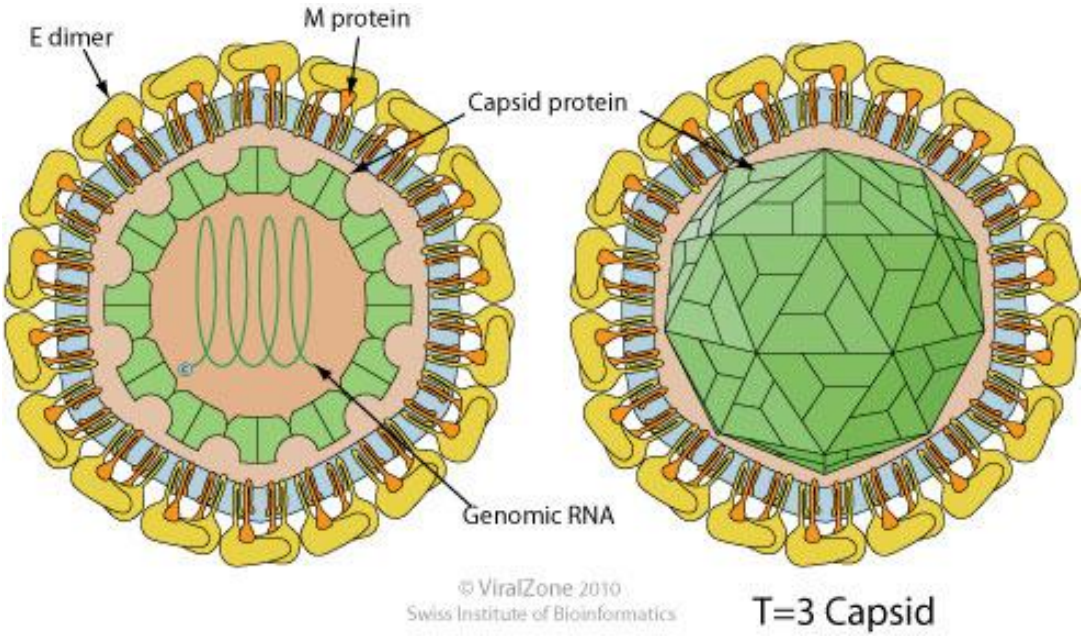

Figure-2: Diagram of a Flavivirus Virion. The flaviviral virion is pictured above showing the main structural parts.
http://education.expasy.org/images/Flaviviridae_virion.jpg

Flavivirus Infection and Replication

Flaviviruses are mainly transmitted by arthropod vectors, mostly mosquitoes or ticks. The viruses enter the body via an insect bite, then attaches to cells. The virus enters the cells and begins the replication process, which is noticeable by the sudden onset of severe headache, fever, muscle and joint pain. **Figure 3** depicts the replication cycle of the flavivirus. Flaviviruses bind host receptors using the E glycoprotein and are absorbed into vesicles by the host cell to begin the cycle of replication. The virus membrane then fuses with the vesicle membrane, allowing the

viral RNA genome to be released into the cytoplasm. The positive sense genomic single stranded RNA (ssRNA) is then translated into a polyprotein. This polyprotein is cleaved into all three structural and seven non-structural proteins. Genome replication occurs at the surface of the endoplasmic reticulum. Using the genomic RNA as a template, negative sense complementary ssRNA is synthesized, which in turn is used to synthesize new genomic RNA. Assembly of the virus is completed at the endoplasmic reticulum. The virion buds at the E.R., is transported to the Golgi Apparatus, and finally buds from the cell membrane, releasing mature virus. This process is repeated until the host is inundated with viral pathogens.

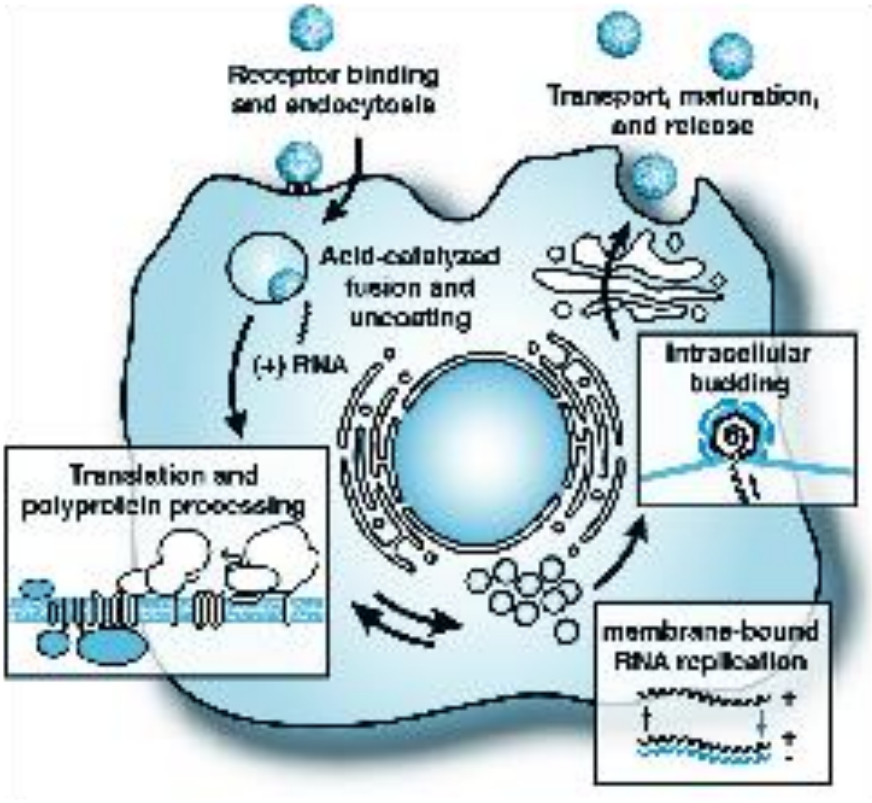

Figure-3: Replication Cycle of a Flavivirus. Retrieved from Fields Virology.

Viral-Induced Host Signal Transduction Pathways

During viral infections, the host antiviral innate immune response is activated upon recognition of viral components by the host pattern-recognition receptors. These receptors, sometimes called pattern-recognition receptors (PRRs), recognize viral molecules such as genomic DNA and RNA or double-stranded RNA (dsRNA) (Iwasaki and Medzhitov, 2004). PRRs are often located on dendritic cells, which when bound to ligands, initiate an immune response resulting in the production of cytokines, particularly type I interferons (both IFN-β and IFN-α). One example of a PRR is a Toll-Like Receptor (TLR) (**Figure-4**).

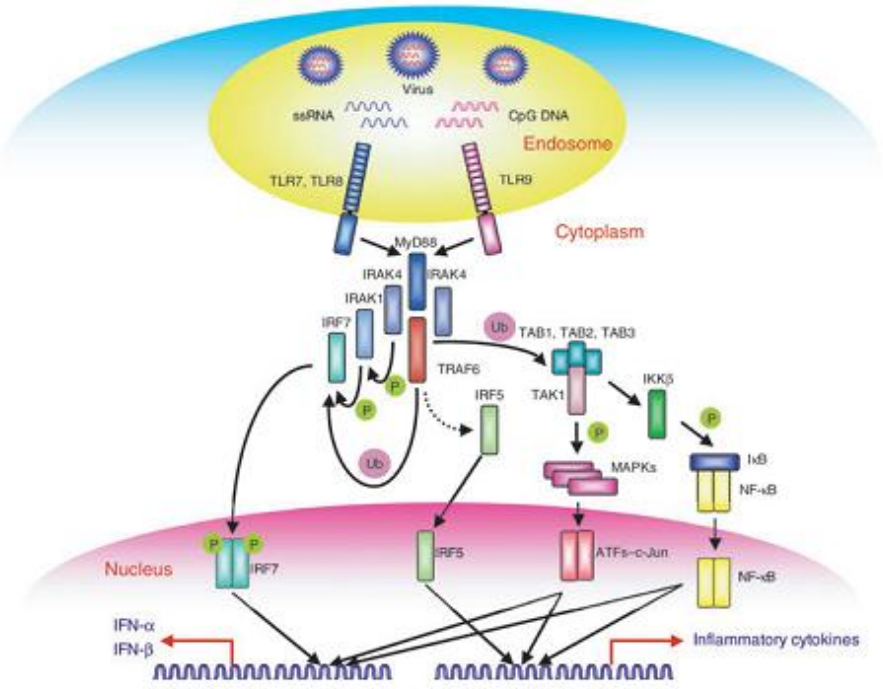

Figure-4: Diagram of Toll-Like Receptors 7, 8, and 9 of the Innate Immune System. This diagram shows the position of three different toll-like receptors present in the pathway regulating expression of type I interferon. TLR-7 and TLR-8 (blue) recognize foreign ssRNA, while TLR-9 (purple) recognizes foreign CpG DNA. The TLRs then recruit MyD88 (dark blue) which continues through the pathway (Kawai and Akira 2006).

TLRs distinguish viruses' conserved molecular motifs called "pathogen-associated molecular patterns" (PAMPs). Three important TLRs found in mammalian cells are TLR-7, TLR-8, and TLR-9 (**Figure 4**). They each distinguish different extracellular material, but TLR-7 and TLR-8 are phylogenetically similar, and perform similar functions within cells. TLR-7 recognizes RNA homologs such as imiquimod, resquimod and loxoribine, as well as synthetic single-stranded RNA that is rich in guanosine or uridine, suggesting that TLR-7 can recognize ssRNA from viruses such as HIV and influenza (Lund et al., 2004). TLR-8 can also recognize HIV-derived ssRNA, but studies show that TLR-8 may have a species specific function (Heil et al., 2004). Lastly, TLR-9 recognizes unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs that are present in bacterial and viral DNA (Hemmi et al., 2000). Upon recognizing these PAMPs, these TLRs start a pathway ultimately leading to the production of IFN-α (**Figure 4).**

One particular TLR of specific interest to this MQP project is TLR-3. TLR-3 is located in a cell's endosomal membrane, and is responsible for dendritic cell activation necessary for virus-specific T-cell responses and initiating an IFN pathway (**Figure 5)**. TLR-3 recognizes dsRNA, as shown by Kawai and Akira (2006) in their experiments using an analog of viral dsRNA, polyinosinic acid-cytidiylic acid (polyIC) (Alexopoulou et al., 2001). TLR-3 (green in the figure) activates IRF-3 (blue, diagram center) and NF-κB (yellow, diagram center) using TRIF (purple), an adaptor molecule. This TLR-3/TRIF fusion allows the activation of inhibitor kappa-B (IκB) which becomes degraded, exposing a nuclear translocation signal allowing NF-κB to translocate to the nucleus, thus transactivating inflammatory and interferon genes (Kawai and Akira, 2006). TLR-3 also activates the IRF-3 pathway (diagram left side) and the MAPK pathway (diagram right side) to further activate inflammatory and interferon genes.

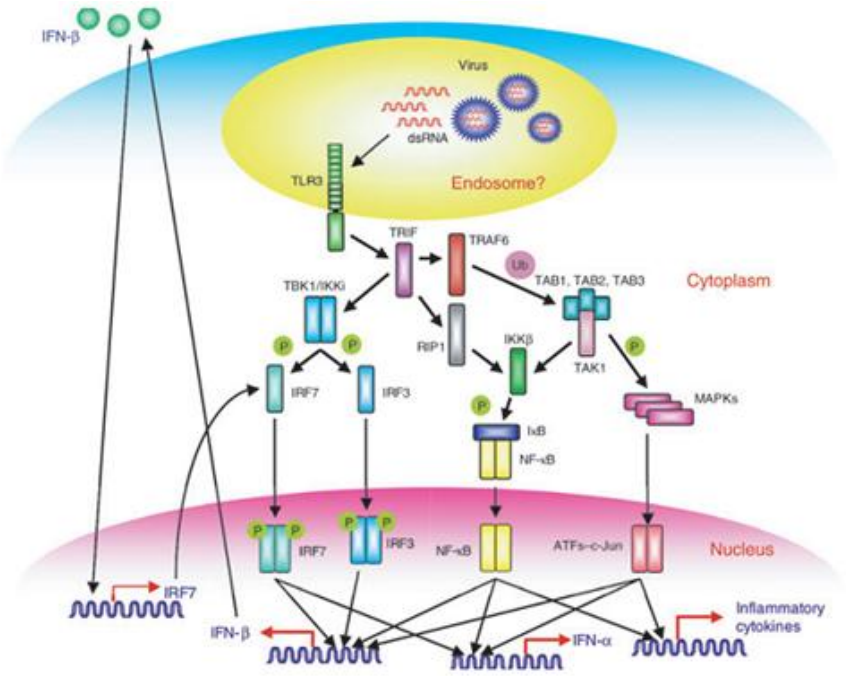

Figure 5: Diagram of the TLR-3-TRIF Signaling Pathway. After recognizing foreign dsRNA, TLR-3 (green) transmits signals through TRIF (purple) which interacts with TBK1, RIPI, and TRAF6. These proteins then continue through the pathway to activate NF-κB and finally produce IFN-β (Kawai and Akira, 2006).

In addition to TLR-3, another PRR is retinoic acid-inducible gene I (RIG-I) (**Figure 6**). RIG-I is a cytoplasmic protein (shown as purple in the figure) that acts as an RNA helicase (Yoneyama et al., 2004). It has two caspase-recruiting domains, or CARD-like domains, which are responsible for interacting with IPS-1 (diagram center), which also contains a CARD-like domain which mediates interaction with RIG-I and Mda5, ultimately activating downstream signaling mediators. IPS-1 is also known by several other names, including mitochondrial antiviral signaling protein (MAVS), virus-induced signaling adaptor (VISA), and CARD adaptor inducing IFN-β (Cardif), and is known to localize to mitochondria via its C-terminal hydrophobic region (Yoneyama et al., 2005), suggesting that mitochondira may be important in

antiviral immune responses. The main outcome of IPS-1 signaling is to activate NF-κB or IRF-3 to start the interferon cascade (Seth et al., 2005).

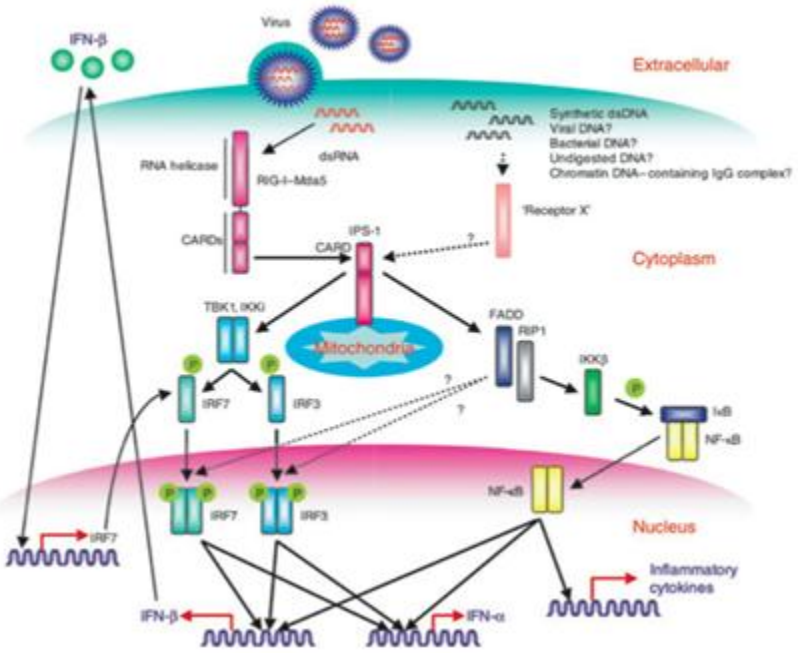

Figure-6: Diagram of the RIG-1-IPS-1 Signaling Pathway. This figure shows the signaling pathway triggered by RIG-1 and Mda5. When viruses enter the cytoplasm, they replicate and produce dsRNA which is then recognized by RIG-1 and Mda5 (pink). RIG-1 then interacts with IPS-1 (pink, center) which then activates IRF-3 (blue) and NF-κB (yellow) which transactivates genes to produce IFN-α and IFN-β (Kawai and Akira 2006).

Classic Viral Detection Methods

Currently, a number of assays are available for use in diagnosing viral diseases, which may be placed in two categories: serological screens and viral isolation assays. Serological assays include RT-PCR, hemagglutination inhibition (HI), complement fixation (CF), viral neutralization tests (NT), and two types of Enzyme-Linked Immunosorbent Assays (ELISAs). For flaviviruses, viral isolation assays include mosquito inoculation and mosquito cell culture.

There is a wide range in application between these tests, but they all eventually lead to diagnosis of a viral infection.

Reverse transcriptase PCR (RT-PCR) is a sensitive method used to detect viral infections. To perform this assay, primers known to occur in a viral gene are used to amplify RNA of a possibly infected sample. Using RNA from the virus of interest as the template, RT-PCR is performed. The presence of a band indicates a viral infection because if no virus were present, the primers could not bind to the sample and amplify the fragment (Freymuth et al, 1995). Although this method is highly sensitive and relatively easy to execute, it requires use of a thermocycler which can be expensive, and the whole process can take several hours.

A hemagglutination inhibition (HI) assay is based on the ability of certain viruses to agglutinate erythrocytes (red blood cells) spontaneously. Upon viral infection, the body initiates an immune response that prevents this agglutination. The sera of a test patient can be tested for this inhibition of agglutination, which would indicate the presence of antibodies. The HI test was originally used to detect antibodies to rubella virus (Rawls and Chernesky, 1976), but is now being used to test for other viruses such as influenza, arboviruses, reoviruses, and certain enteroviruses (Kendal et al, 1985). There is currently no HI assay for flaviviruses.

Complement fixation and neutralization tests are two types of lytic assays. Complement fixation (CF) involves using a patient's complement to lyse indicator erythrocytes. In this test, an antibody reacts with an antigen in the presence of a complement. An example of an appropriate complement is rabbit serum. If the antigen-antibody complex is present in a patient's serum, the complement cascade is activated. Erythrocytes coated with a red blood cell antibody are added to the reaction mixture to act as the indicator; if the complement has been activated, the indicator cells are not lysed. Conversely, if the antigen-antibody pair is not

present, the complement cascade will not begin, and lysis of the indicator cells will not occur (James, 1990). As with HI, CF does not distinguish IgM from IgG antibodies, but CF can be used to detect either viral antigens or antibodies. One example of the use of CF in diagnostic medicine occurs with the identification of coxsackievirus which causes hand foot and mouth disease (Stites and Rodgers, 1987). One advantage of CF is that it keeps the majority of test parameters constant, and therefore many antigens can be tested to determine a particular population's exposure to a rare virus.

A different lytic assay is a neutralization test. This assay involves the lysis of erythrocytes by extracellular toxins called hemolysins, which are produced by certain pathogens. In addition to lysing red blood cells, hemolysins stimulate the production of antibodies, which eventually neutralize the hemolysins. For both complement fixation and the neutralization test, viruses are detected when cells are negative for hemolysis. In a negative test (no viruses are present), the antibodies are not produced and therefore the erythrocytes will lyse (Kohler et al., 1984).

One of the most well known assays for viral detection is an Enzyme-Linked Immunosorbent Assay (ELISA). There are several types of ELISAs, but IgM capture (MAC-ELISA) and indirect IgG ELISAs are the most widely used. For MAC-ELISA, IgM antibodies are detected, while IgG antibodies are identified in an indirect ELISA. In both cases, an antigen of choice is coupled to a plate surface, and a serum sample is added. If antibodies are present in the test serum (meaning that the test will be positive for infection), the antibody will bind to the corresponding antigen on the plate. Then, an anti-human IgG or IgM antibody conjugated to an enzyme is added which will bind to the IgG or IgM antibody present and cause a color change. If there is no infection, the serum sample will not contain antibodies, nothing will bind, and no

color formation will occur. Furthermore, the intensity of the color is directly proportional to the amount of antibody present in the serum sample (Lanza et al., 1993).

Virus isolation is another viral detection test. For Flaviviruses, one such method is mosquito inoculation, used for detection of mosquito-born viruses. This system of inoculation is the most sensitive for virus isolation, but is labor-intensive and requires a large number of mosquitoes for inoculation. Four species of mosquitoes are commonly used: *Aedes aegypti, A. albopictus, Toxorhynchities amboinensis,* and *T. splendens.* Although Flaviviruses replicate in most mosquito tissues, salivary glands and brain tissue are most often used. Then, direct fluorescent-antibody (DFA) tests are used to determine the infection of that insect tissue (Gubler, 1998). Another such method for viral isolation is mosquito cell culture; this is currently the most widely used system as it is quicker and easier than mosquito inoculation. In this system, *A. albopictus* cells are used to attempt to grow flaviviruses from a test serum. This method therefore provides a more sensitive and economical method for isolating viruses, especially Dengue Virus (Gubler, 1998). Although mosquito cell culture is more reliable than direct inoculation, it is not a very rapid diagnostic test.

Mammalian Two-Hybrid Systems

Two-hybrid systems are important because they are a good method for detecting protein to protein interactions *in vivo*. Studying these interactions is necessary because important regulatory events in cells such as transcription, metabolism, and signal transduction are often aided by interactions between proteins.

The two-hybrid system was originally developed in yeast, but has recently been adapted for use in mammalian cells (Promega, 2010). Fields and Song (1989) originally developed the yeast two-hybrid system to screen for protein-protein interactions by using a specific yeast

transcription regulatory factor named GAL4. In this yeast two-hybrid system, the DNA binding domain and the transcriptional activation domain of GAL4 are produced by separate plasmids, and are therefore closely associated when one protein (known as the "bait") fused to a GAL4 DNA binding domain interacts with a second protein (known as the "prey") fused to a GAL4 transcriptional activation domain. The physical interaction between proteins brings the two functional regions of the GAL4 protein in close proximity and enables the activation of reporter gene expression when placed in the proximity of a particular promoter (Chien et al., 2001).

Mammalian Two-Hybrid Systems were adapted from this original yeast system. In the mammalian-based system, transcription factor activity comes from two different protein domains that are expressed from separate vectors (Sadowski et al., 1988). The strength of this system lies in its ability to confirm recognized protein-protein interactions in mammalian cells versus yeast cells which may lack the proteins. Furthermore, a two-hybrid system for mammalian cells allows characterization of mammalian protein-protein interactions within a cellular environment that more closely mimics the native protein environment. Also, types of protein modifications or unique factors or modulators present in mammalian cells may influence the ability of protein domains to interact (Buckholz and Gleeson, 1991). Finally, using a two-hybrid system with mammalian cells can potentially eliminate false positives better than yeast two-hybrid systems, and confirm true positive results in a more native context (Promega, 2010).

GFP, YFP, and Engineered Viral Detection Systems

Green fluorescent protein (GFP) was discovered in 1961, when Osamu Shimomura and Frank Johnson extracted a calcium-dependent bioluminescent protein (which they named aequorin) from an *Aequorea victoria* jellyfish (Shimomura et al., 1962). These two scientists

determined that aequorin and the green fluorescent protein work together in the light organs of the jellyfish to convert calcium-induced luminescent signals into the green fluorescence characteristic of the species (Piston et al., 2007). GFP is extremely stable, and produces significant fluorescence, though the excitation maximum is very near to the ultraviolet range. This range of light has the ability to damage live cells, meaning that this protein as an imaging product has limits. However, by one single point mutation (altering serine at 65 to a threonine) the excitation maximum shifted from approximately 395 nm to about 488 nm (Johnson et al., 1962). This mutation, termed enhanced GFP (EGFP) is commercially available in a wide range of cloning vectors, and is the most popular variant of GFP. EGFP and GFP are not without flaws, as they contain a slight sensitivity to temperature, pH and chloride ions (Ward et al., 1982; Chalfie et al., 1994; Piston, 2007).

Yellow fluorescent proteins (YFP) were investigated after it was discovered that the threonine residue 203 (Thr203) of green fluorescent protein could be mutated to a tyrosine to produce a stable excited state dipole moment of the chromophore (Piston, 2007). This mutation caused a 20-nanometer shift to longer wavelengths, including the excitation and emissions spectra (**Figure-7**).

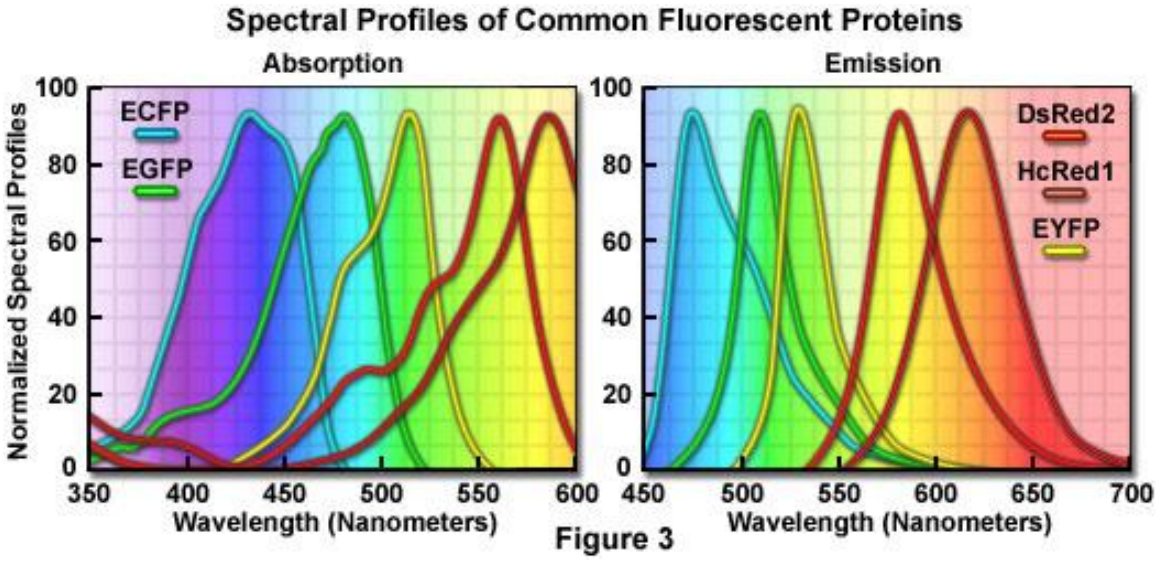

Figure-7: Absorption and Emission Profiles of Various Fluorescent Proteins. Figure is taken from Piston et al., 2007.

Modifications to YFP produced an enhanced yellow fluorescent protein (EYFP), which is one of the most widely used and brightest fluorescent proteins (Piston et al., 2007). EYFP has its maximum excitation at 534 nm, with an emission maximum at 527 nm, and has a relative brightness of 151% that of EGFP. YFP is not perfect however, as it is very sensitive to pH (it loses approximately 50% of its fluorescence at pH 6.5); it is also sensitive to chloride ions, and photobleaches more readily than GFP.

To combat these issues, modifications were continued to decrease the sensitivity to pH and Cl^- ions (Nagai et al., 2002). Venus is a mutated variant of YFP, having its maximum excitation at 515 nm, an emission maximum at 528 nm, and has a relative brightness of 169% that of EGFP (Piston et al., 2007). A novel mutation, F46L, greatly accelerates the maturation of the Venus chromophores at 37°C. As a result of other mutations (F46L/M153T/V163A/S175G) the protein also folds well and is relatively tolerant of exposure to acidosis and Cl- (Nagai et al., 2002). These mutations make Venus the fastest maturing and brightest of the yellow variant

reporters, and it works well at physiological conditions making it an excellent reporter for cellular cloning experiments.

Bimolecular Fluorescence Complementation

Bimolecular fluorescence complementation (BiFC) is a tool which enables the direct visualization of protein interaction in living cells (Kerppola, 2006). This assay was based on the discovery that two fragments of a fluorescent protein, which do not fluoresce themselves, can associate to form a fluorescent complex (see **Figure 8**). One way to achieve fluorescence is to fuse the two fragments to proteins that are known to interact with each other.

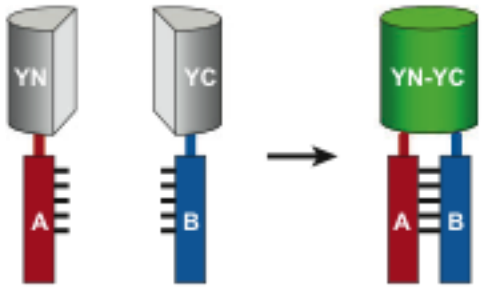

Figure 8: Schematic Representation of the BiFC Assay. Two non-fluorescent fragments (YN and YC) of a yellow fluorescent protein are fused to putative interaction partners (A and B). The association of the interaction partners allow the formation of a bimolecular fluorescent complex (green).

There are both advantages and limitations to the BiFC assay, although the limitations in this case do not present a pressing problem. One limitation is the time necessary for fluorophore maturation, which is reflected in the chemical reactions for formation of the cyclic fluorophore. And under *in vitro* conditions, the formation of the bimolecular fluorescent complex is essentially irreversible (Hu et al., 2002). Lastly, fluorescent protein fragments have a finite

ability to associate with each other, whether or not the proteins to which they are fused are interacting, which accounts for a high background signal when conducting the assay.

There are strengths to using the BiFC assay as well, which is why this particular assay was chosen for use in this project. It enables direct visualization of protein interactions and does not rely on their secondary effects. Interactions can also be viewed in living cells; meaning potential artifacts caused by fixation or cell lysis can be eliminated. The most important of these strengths is that BiFC does not require specialized equipment beyond that of an inverted fluorescent microscope that allows for imaging of fluorescence in cells.

PROJECT PURPOSE

The purpose of this project was to develop a new diagnostic tool for rapid detection of flaviviral infections by using a mammalian plasmid two-hybrid system with Bimolecular Fluorescence Complementation (BiFC). Two different plasmids will be constructed encoding TRL-3 and TRIF fused to half of a YFP reporter. The plasmids will be stably transfected into Vero cells. The cells will then be mixed with samples containing virus. The viral infection should allow the plasmid expressed TLR-3 and TRIF proteins to dimerize, causing the halves of the Venus (YFP) protein to also dimerize and fluoresce. This new detection system can be used to quickly detect whether viral RNA is present in particular cells.

METHODS

Primer Design

Primers were designed to include restriction cut sites necessary for cloning. Two sets of primers were created: one for PCR of the Venus plasmid halves, and the other for the RT-PCR of the mammalian host proteins of interest, TLR-3 and TRIF. Shown below are the primer sequences for the Venus plasmid halves, henceforth to be named N172-stop and C172-stop for their terminal positions on the proteins. Stop codons were added to the N172-stop and C172-stop primers because these genes were fused to the C-terminus of TLR-3 and TRIF. Shown below are the primer sequences designed for the TLR-3 and TRIF RT-PCR. All primers were ordered from Integrated DNA Technologies (IDT) at 310 pM/µl, and then diluted to 10 pM/µl and stored at 4˚C.

pcDNA Neomycin
5` N172: gcggcaGAATTCatggtgagcaagggcgaggagctgttc
3' C172 Stop: agccgcGATATCtcagatgttgtggcggatcttgaagtt

pcDNA Hygromycin
5' N172: gcggcaGGATCCgaggacggcggcgtgcagctcgcc
3` C172 Stop: gtccgcGATATCtcactcgtccatgcggagagtgatccc

5' TLR-3
gcggacAAGCTTatgagacagactttgccttgtatctacttt
3' TLR-3
gtccgcGAATTCatgtacagagtttttggatccaagtgctac

5'TRIF
gcgcatGCTAGCatggcctgcacaggcccatcacttcctagc
3'TRIF
atgcgcAAGCTTttctgcctcctggctcttgtcctcgggcgc

Polymerase Chain Reaction (PCR)

The following reaction was used to amplify the Venus fragments, N172-stop and C172-stop, to be cloned into pcDNA3.1.

PCR Component	Amount (µl)
Buffer	5
$MgCl_2$	1.5
dNTPs	1
Primer (+)	1
Primer (-)	1
Template	1
Taq	0.5
H_20	39
Total	**50 µl**

The above ingredients were placed into a thermocycler, and run as follows (30 cycles):

PCR Program	Temperature	Time (minutes)
Denaturing Temp.	94°	2
Melting Temp.	94°	0.5
Annealing Temp.	54°	0.75
Extension Temp.	72°	0.75
	72°	5
	4°	Pause

After the cycle was complete, the entire PCR reaction was analyzed on a 1.2% agarose gel in 1X TAE buffer, containing ethidium bromide. The gel was run for twenty minutes at 100V. After the PCR was complete and the band size verified using both a 1.0kb and a 100bp marker, the correct band was extracted and purified using a Qiagen Gel Extraction Kit.

Reverse-Transcript PCR (RT-PCR)

RT-PCR was performed on total cellular RNA to amplify the host proteins of interest, TLR-3 and TRIF, using the Superscript One Step RT-PCR for Long Templates kit. The reactions were set up as follows:

RT-PCR Component	Amount (µl)
2X Reaction Buffer	25
RNA Template	10
Primer (+)	1
Primer (-)	1
RT Taq	1
H_20	12
Total	**50 µl**

Total cellular RNA from Vero cells and from human dendritic cells were used as RNA templates. The reaction was run as follows in a thermocycler (30 cycles):

RT-PCR Program	Temperature	Time (minutes)
Reverse-Transcriptase Activation	50°	30
Denaturing Temp.	94°	2
Melting Temp.	94°	0.5
Annealing Temp.	54°	0.75
Extension Temp.	68°	3
	72°	5
	4°	Pause

The reactions were again verified on an agarose gel, and the amplicons were purified according to the above-stated conditions.

Restriction Digestions

As previously stated, each primer was designed to contain a restriction site, either on the 3' or 5' end of the sequence, as shown in the Table below.

Primer Sequence	Restriction Sites Inclused
N172-Stop	EcoRI, EcoRV
C172-Stop	BamHI, EcoRV
TLR-3	HindIII, EcoRI
TRIF	NheI, HindII

Figure-9 shows the pcDNA 3.1 vectors containing both Neomycin and Hygromycin resistance and the restriction sites used for each construct. All restriction enzymes cut sites were verified using NEB Cutter from the New England Biolabs website.

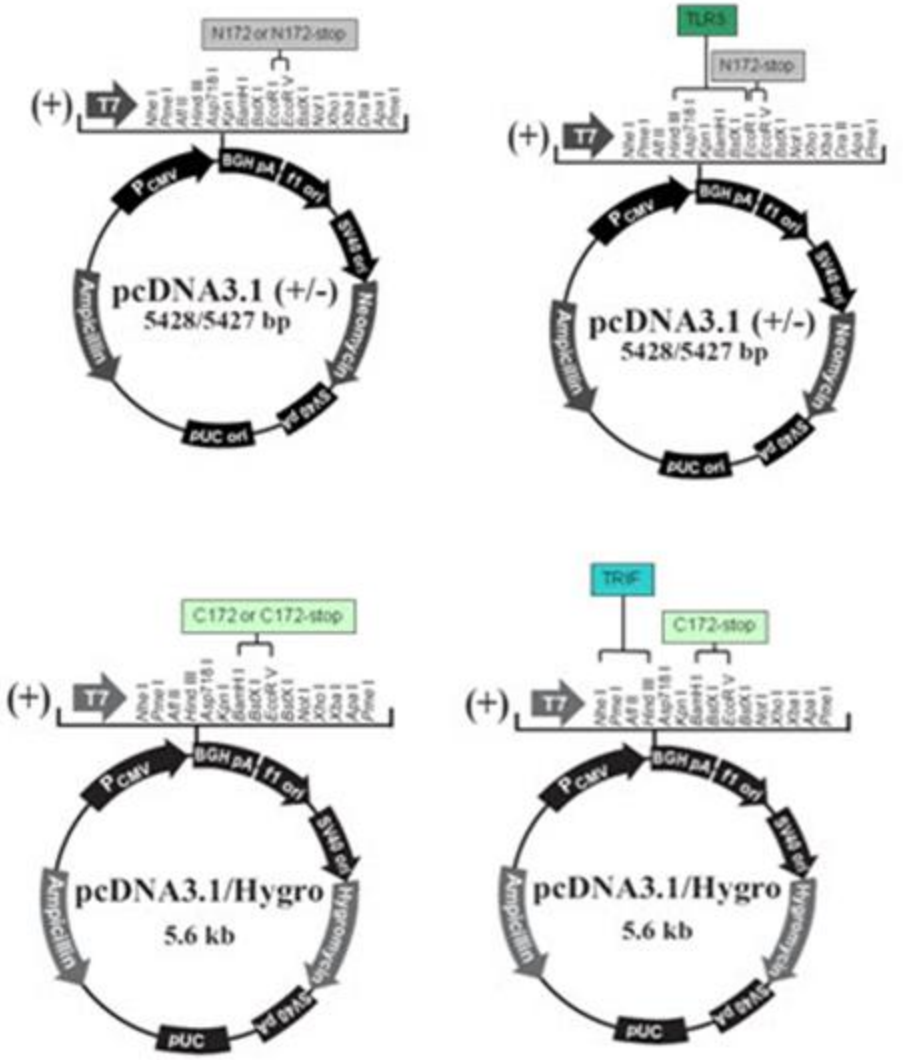

Figure-9: Diagram of the Four pcDNA3.1 Vectors Cloned in This Project. The panels deonte, from upper left to lower right N172 or N172-Stop, TLR-3/N172-Stop, C172 or C172-Stop, and TRIF/C172-Stop.

Both pcDNA3.1 vectors (pcDNA 3.1-Neomycin and pcDNA-Hygromycin) were digested with the appropriate restriction enzyme (see tables below) at 37˚ for two hours. After 75 minutes into the restriction digestion, 1µl of Calf Intestinal Alkaline Phosphatase (CIP) (Invitrogen) was

added to each tube to prevent self-ligation. The restriction digestion reactions were set up as follows:

pcDNA Neomycin (Vector)	Amount (µl)
pcDNA Neo	2
10X BSA	5
10X EcoRI Buffer	5
EcoRI Enzyme	1.5
EcoRV Enzyme	1.5
H_20	35
Total	**50 µl**

pcDNA Hygro (Vector)	Amount (µl)
pcDNA Hygro	2
10X BSA	5
NEB Buffer 3	5
EcoRV Enzyme	1.5
BamHI Enzyme	1.5
H_20	35
Total	**50 µl**

Next, the N172-Stop and C172-Stop purified PCR products discussed previously were digested at 37˚C, as follows. This time however, CIP was not added:

N172/N172 Stop	Amount (µl)
DNA	20
10X BSA	5
10X EcoRI Buffer	5
EcoRI Enzyme	1.5
EcoRV Enzyme	1.5
H_20	17
Total	**50 µl**

C172/C172 Stop	Amount (µl)
DNA	20
10X BSA	5
NEB Buffer 3	5
EcoRV Enzyme	1.5
BamHI Enzyme	1.5
H_20	17
Total	**50 µl**

The RT-PCR products of TLR-3 and TRIF were also digested to match the N172-stop and C172-stop plasmids. These reactions were run at 37˚C for two hours, and CIP was added to the vector tubes after one hour. The enzymes used for TLR-3 could not be incorporated as a double digest, and therefore a sequential digest was performed on both the TLR-3 PCR product and its N172-Stop vector. The double digests were set up as follows:

C172 Stop	**Amount (µl)**
pcDNA Construct	5
10X BSA	5
Buffer	5
Enzyme	1.5
Enzyme	1.5
H_20	32
Total	**50 µl**

TRIF	**Amount (µl)**
PCR	12
10X BSA	5
Buffer	5
Enzyme	1.5
Enzyme	1.5
H_20	25
Total	**50 µl**

The sequential digests were set up as follows:

Step 1 (N 172 Stop)	**Amount (µl)**
pcDNA Construct	7
NE Buffer 2	5
Hind III	1.5
H_20	36.5
Total	**50 µl**
***Add CIP at 75 minutes**	
Step 2	
Step 1 Purification	7
EcoRI Buffer	5
EcoRI Enzyme	1.5
H_20	36.5
Total	**50 µl**

Step 1 (TLR 3)	**Amount (µl)**
PCR	15
NE Buffer 2	5
Hind III	1.5
H_20	28.5
Total	**50 µl**
Step 2	
Step 1 Purification	30
EcoRI Buffer	5
EcoRI Enzyme	1.5
H_20	13.5
Total	**50 µl**

After the restriction digestion reactions were completed, 4 µl of each sample were run for 25 minutes at 100 V on a 1.2% agarose gel in 1X TAE buffer and stained with ethidium bromide. Both a 1.0 kb and a 100 bp marker were used to confirm that band sizes were correct.

Ligation

The digested PCR products (N172-Stop and C172-stop) were then ligated into cut pcDNA3.1 Neo or pcDNA3.1Hygro vectors. The digested RT-PCR products were ligated into their respective vectors as well (TLR-3 → N172-Stop, TRIF → C172-Stop). The N172-Stop vector was diluted 1:40 in ddH_20, while the C172-Stop vector was diluted 1:30 in the same media. The ligation reactions were set up as follows:

Ligation Reaction Component	Amount (µl)
10X Ligation Buffer	1
10 mm ATP	1
Vector DNA	1
Insert	6
Fast-Link DNA Ligase	1
Total	**10 µl**

This reaction was allowed to incubate at room temperature for five minutes, and then at 70˚C for an additional fifteen minutes to stop the ligation reaction.

Transformation

Following the ligation, 2.5 µl of each ligation reaction was added to a separate vial of thawed TOP10 competent *E. coli* cells (Invitrogen). These vials were allowed to incubate on ice for 30 minutes, and were then placed into a 42˚C waterbath for 30 seconds. Next, the cells were again incubated on ice for two minutes before 250 µl of SOC medium was added. Then, the cells were placed in a 37˚C horizontal shaking incubator for one hour. Following this incubation, 100 µl from each vial was spread on an LB-Ampicillin plate and allowed to incubate at 37˚C overnight.

Plasmid Purification

After the transformation, 8-14 white colonies were asceptically taken from each plate and used to construct overnight bacterial suspensions. Each colony was transported to a 15mL sterile tube containing 3 ml of LB and Ampicillin (100 µg/ml final concentration). These cultures were incubated in a 37˚C horizontal shaking incubator overnight. Following roughly 24 hours of

incubation, the cultures were processed using a QIAprep Spin Mini-prep kit (Qiagen), in order to obtain purified plasmid DNA. One change from the normal Qiagen protocol was that the DNA was eluted from the column in 40 µl of Elution Buffer (instead of the listed 50 µl) resulting in a slightly more concentrated sample.

Screening Positive Clones

Finally, each cloned sample was screened to test whether the correct insert was present. Another restriction digest was set up using enzymes *HindIII and XhoI*, which encompass all potential inserts. The digests were set up as follows:

Digestion Screening Reactions	**Amount (µl)**
DNA	5
10X BSA	2
NE Buffer 2	2
Hind III Enzyme	0.5
Xho I Enzyme	0.5
H_20	1
Total	**20 µl**

These screening digestion reactions were incubated at 37˚C for one hour. Following the restriction digests, each sample was analyzed by running 4 µl for 20 min at 100 V on a 1.2% agarose gel in 1X TAE buffer, stained with ethidium bromide. Positive clones were verified using a 1.0 kb and 100 bp markers.

TA Cloning

Due to problems cloning the TLR-3 gene, TA cloning was performed on this sequence using the TOPO TA Cloning® Kit for Sequencing (Invitrogen). First, a 3' adenine residue overhang was added to each end of the PCR TLR-3 sequence to allow annealing with the T-tailed vector. The reaction was set up according to the following:

Adding a 3' A-Tail	**Amount (µl)**
PCR Product	8
Taq Buffer	1
Taq Polymerase (NEB)	0.5
ATP (10mM)	0.5
Total	**10 µl**

Then, the tailed reaction was directly ligated into vector pCR2.1-TOPO by mixing the following:

TA Ligation Reaction	**Amount (µl)**
PCR Product with A overhangs	4
Salt Solution	1
Topo Vector	1
Total	**6 µl**

This ligation mixture was mixed gently, and then incubated for one hour at room temperature. Transformation into competent *E. coli* cells took place immediately following the ligation.

For the transformation, 2 µl of the ligation reaction was added to a vial of thawed TOP10 competent cells (Invitrogen) and placed on ice for five minutes. Then, the cells were heat shocked in a 42˚C waterbath for 30 seconds and retransferred to ice. Next, 250 µl of room temperature SOC medium was added to the tube, and it was incubated at 37˚C in a horizontal

shaker for one hour. The cells were then spread in volumes of 100 µl and 25 µl onto LB/ampicillin/X-gal plates, and allowed to incubate at 37˚C overnight. After roughly 24 hours, the plates were checked for colony formation; white colonies indicated the insert had been incorporated into the vector, and these colonies were screened for the correct clone.

RESULTS

Mammalian 2 Hybrid Design

To design a mammalian two-hybrid system for detecting flaviviruses, many steps must be taken. First, bacterial cells containing the Venus plasmid (obtained from Addgene Inc.) had to be grown in LB medium so the Venus plasmid could be isolated by miniprep. Primers must also be designed to insert fragments into expression vectors, N-172 Stop and C-172 Stop, as well as for host proteins TLR-3 and TRIF. These primers will be used in PCR and RT-PCR reactions to amplify the appropriate amplicons. Next, the PCR product will be ligated and transformed into Top 10 competent *E. coli* cells. The transformed cells will then be plated onto LB plates containing ampicillin to select for bacterial cells that took up the vectors encoding ampicillin resistance. The resulting colonies will be selected and expanded in an overnight suspension of LB plus ampicillin, followed by a screening digestion to ensure the appropirate insert is present.

PCR Amplification of Vector Inserts N172-Stop and C172-Stop

PCR was used to amplify the N172-Stop and C172-Stop fragments using the Venus plasmid as a template. To verify the presence of these inserts, the PCR products were run on 1.2% agarose gels containing EtBr. The amplification of N172-Stop is shown in **Figure 10**. Lane-1 contains a 1.0 Kb marker, while lane 2 shows the PCR reaction with a very strong 519 bp fragment, the expected length of N-172 Stop fragment being amplified.

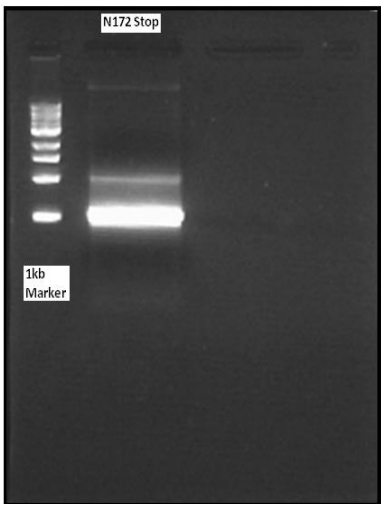

Figure 10: PCR Amplification of Fragment N-172-Stop. The strong band at approximately 500 bp in lane 2 corresponds with the expected size. Sample was run on 1.2% agarose gel containing EtBr then visualized by UV.

The PCR was repeated for the C-172 Stop fragment (**Figure 11)**. Lanes 1 and 4 each contain a 100 bp marker, while lanes 2 (C-172) and 3 (C-172 Stop) show strong PCR bands approximately 200 bp long which correspond to the expected length (198 bp) of the fragment.

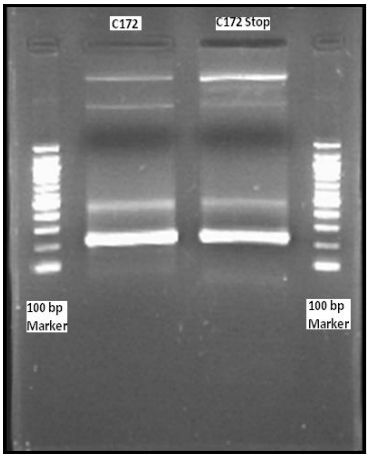

Figure 11: PCR of Fragments C-172 and C-172 Stop. Strong bands at approximately 200 bp in lanes 2 and 3 correspond with expected size. Samples were run on 1.2% agarose gel containing EtBr, then visualized by UV.

Cloning of N172-Stop, and C172-Stop into Venus Expression Plasmid pcDNA3.1

Next, the PCR products were cut with the appropriate restriction enzymes and gel purified. Fragments N172-Stop and C172-Stop were cloned into Venus expression plasmid pcDNA3.1 cut with corresponding enzymes. The digestion screening is shown in **Figure 12**, with lanes 2 and 6 denoting positives.

Figure 12: Digestion Screening of Plasmid pcDNA3.1-N-172-Stop (Lane 2) and Plasmid pcDNA3.1-C172-Stop (Lane 6). Samples were run on 1.2% agarose gel containing EtBr, then visualized by UV. The bands in lanes 2 and 6 show linearized pcDNA that has taken up the insert fragment so is sensitive to digestion.

RT-PCR Amplification of Host TLR-3 and TRIF

Creating host-Venus fusion proteins was a multistep process. To begin, RT-PCR was used to amplify either TLR-3 or TRIF from total cellular RNA, and the amplicons were analyzed by 1.2% agarose electrophoresis. The TLR-3 amplification is shown in **Figure 13.** Lanes 1 and 5 each contain a 1.0 Kb marker, while lanes 2, 3, and 4 show three separate RT-PCR reactions with a strong band approximately 2,700 base pairs (bp) long, which corresponds to the expected length of the TLR-3 gene being amplified.

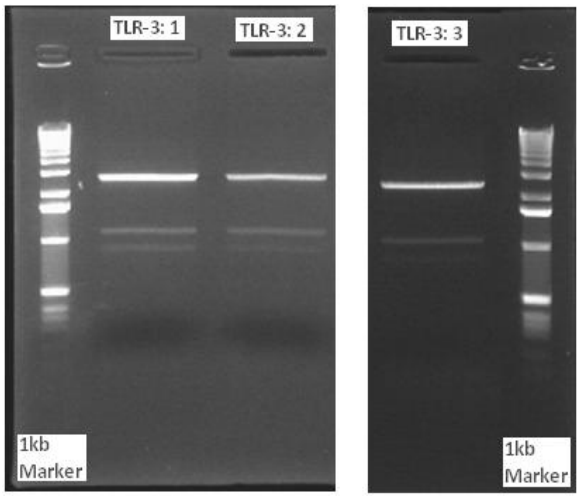

Figure 13: RT-PCR Reactions for Gene TLR-3. A strong 2,700 bp band is seen in lanes 2, 3 and 4, at the expected size. Samples were run on 1.2% agarose gel containing EtBR, then visualized by UV.

The RT-PCR was repeated for the TRIF gene (**Figure 14)**. Lanes 1 and 5 again contain a 1.0 Kb marker, while lanes 2, 3, and 4 show three separate RT-PCR reactions with a strong band approximately 2,100 bp long, corresponding to the expected length of the TRIF gene.

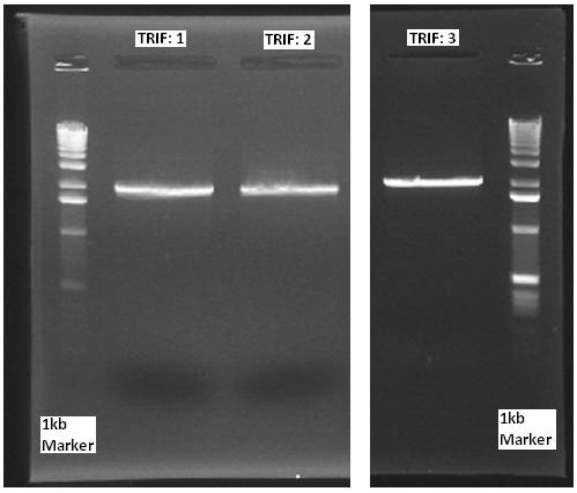

Figure 14: RT-PCR Analysis of Gene TRIF. A strong 2,100 bp band seen in lanes 2, 3 and 4 corresponds with the expected size. Samples were run on a 1.2% agarose gel containing EtBr, then visualized by UV.

Cloning of TLR-3 and TRIF into pcDNA Plasmids

The TLR-3 amplicon was cut with the appropriate restriction enzymes and ligated into plasmid pcDNA3.1-N-172-Stop cut with the same enzymes. The TRIF amplicon was also cut with enzymes, and ligated into pcDNA3.1-C-172 Stop. The digestion screening for TRIF-pcDNA is shown in **Figure 15**. Lanes 2, 5, 7 and 9 (shown by asterisks) were positives containing linearized pcDNA upper band (2,300 bp) plus the TRIF insert band at approximately 2,100 bp. For unknown reasons, insert TLR-3 was not successfully cloned using this procedure.

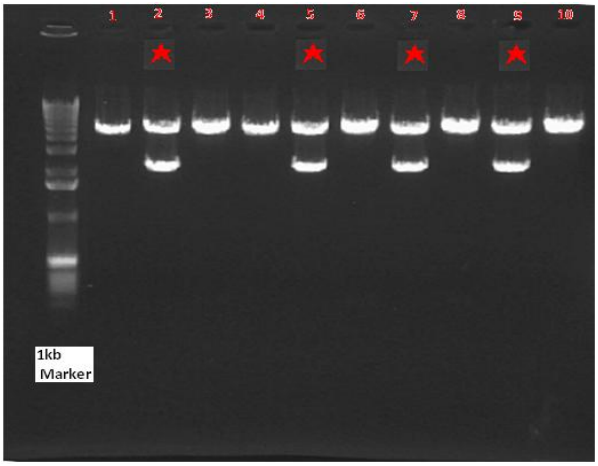

Figure 15: Digestion Screening of TRIF Cloned into Plasmid pcDNA3.1-C172-Stop. Positive clones are shown in lanes 2, 5, 7 and 9 (asterisks). Positives contained linearized plasmid (upper band) and TRIF insert (lower band). Samples were run on 1.2% agarose gel containing EtBr, then visualized by UV.

Since TLR-3 could not be cloned using this approach, TA cloning was performed on this amplicon. 3' Adenine overhangs were added to each end of the RT-PCR amplicon to allow for annealing to the 3' T-tailed vector. The ligation was immediately transformed, and the cells plated onto LB/ampicillin/X-gal. Both blue-green (negative) and white (positive) colonies were observed. Plasmid DNA was isolated from white colonies, and screened by *EcoRI* digestion to

remove the TLR-3 insert (**Figure 16).** Three positive clones were identified (asterisks in the figure).

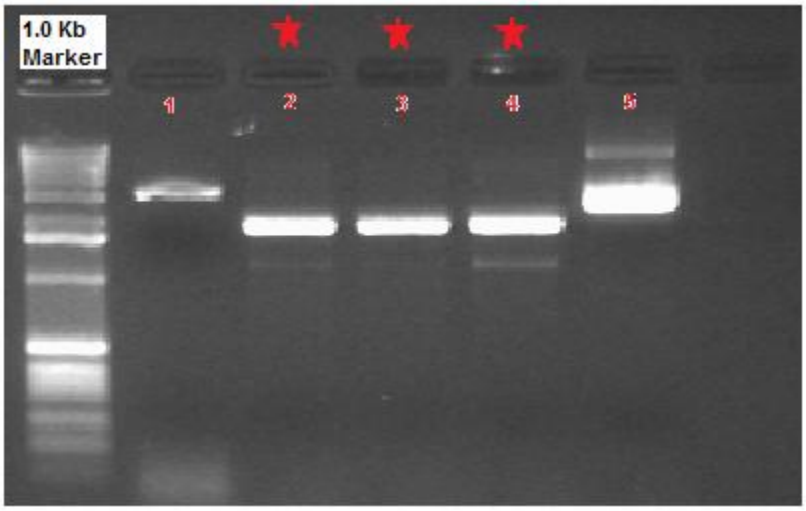

Figure 16: Digestion Screening of TLR-3 Cloned into the pCR2.1-TOPO Vector. Positive clones that took up the insert are shown in lanes 2-4 (asterisks) which contain linearized plasmid (main band at roughly 4.0 kb) plus a lower fainter insert band representing the 2.7 kb TLR-3 insert. Samples were run on a 1.2% agarose gel containing EtBr, then visualized by UV.

DISCUSSION

The purpose of this project was to develop a new diagnostic tool for rapidly detecting flaviviral infections using a mammalian plasmid two-hybrid system with Bimolecular Fluorescence Complementation (BiFC). The design of this assay was based on the construction of two different plasmids encoding TRL-3 and TRIF, each fused to half of a YFP reporter. The plasmids followed a specific design to enable stable transfection into Vero cells. Upon viral infection, the expressed TLR-3 and TRIF proteins should dimerize as the signal transduction pathway containing these proteins is activated by flaviviral infection. Dimerization of TLR-3/TRIF (each fused with half a Venus YFP reporter protein) causes the Venus protein to also dimerize and fluoresce. Thus, the fluorescence of cells expressing Venus (YFP) should indicate the presence of foreign dsRNA inside the cell.

The first step in constructing the fusion proteins was to create modified pcDNA3.1 Venus expression plasmids containing N172-Stop and C172-Stop sequences using PCR. The insert PCR amplicons were obtained, digested, and successfully ligated into pcDNA3.1. Digestion screening showed the presence of insert bands at the expected 519 bp and 198 bp sizes, respectively, for N172-Stop and C172-Stop, thus showing successful cloning.

The second step in the construction of the fusion proteins was to amplify the TLR-3 and TRIF host genes. Amplification was performed by RT-PCR to avoid non-coding introns in the genes. RT-PCR amplicons of the expected sizes, 2,700 bp for TLR-3, and 2,100 bp for TRIF were obtained. The RT-PCR products were digested and ligated into the previously designed pcDNA3.1 Venus plasmids. Digestion screening indicated a successful cloning of TRIF into

pcDNA3.1-C172-Stop (with its 2,100 bp TRIF insert), but no positives were obtained for TLR-3-pcDNA3.1.

To prove that TLR-3 was in fact clonable in *E. coli*, a TA cloning approach was used. This approach allows the direct ligation of an A-tailed PCR amplicon into a 3' T-tailed vector, and is an efficient way to clone PCR amplicons. Overnight incubation of the transformed plates yielded a large number of potentially positive white colonies, and three positive clones were subsequently identified by *EcoRI* screening, showing that TLR-3 does have the ability to be cloned.

This project contained some success, as positive clones for pcDNA3.1-N172-Stop, pcDNA3.1-C172-Stop, and pcDNA3.1-TRIF were obtained. Also, the clonability of TLR-3 in *E. coli* was confirmed by TA cloning. In the future, to complete the rapid detection test, a clone of TLR-3 in pcDNA3.1 must be obtained. This could be possible by using a high fidelity Taq polymerase when conducting RT-PCR, or by taking specific steps to minimize the loss of product during DNA purification steps. The use of different restriction sites, or a different intermediate vector, could also potentially produce positive results. Once the TLR-3 clone is obtained, both TRIF and TLR-3 pcDNA plasmids will be stably transfected into Vero cells, that are permissible for a flaviviral infection. Lastly, it would be necessary to measure the specificity of this assay; this would be performed by testing an array of different flaviviridae (i.e. Dengue, West Nile, Yellow Fever, etc) that are known to activate the TRIF/TLR-3 pathway, as well as non-related viruses that do not activate the pathway.

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