UNIVERSITI TEKNOLOGI MALAYSIA

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CELL & MOLECULAR BIOLOGY FOR BIOINFORMATICS

LET'S CLONE IT!

ENVELOPE PROTEIN (E) - DENGUE VIRUS 2

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1.0 INTRODUCTION

What is Virus? Virus is a small life form that infects living organisms. It is an infectious substance that generally consists of a nucleic acid molecule surrounded by a protein coat. It is too small in size to be seen by light microscope and is only able to multiply within the living cells of a host. Like other viruses, the dengue virus has a microscopic structure that can only replicate inside a host organism. It also means that, in order to survive, the dengue virus needs a host to live in. The gene we have chosen Envelope protein in Dengue Virus 2. Dengue is a mosquito-borne viral disease that has rapidly spread in all regions according to World Health Organization (WHO) in recent years, significantly in the tropics, such as Africa, the Caribbean, Central America, Central Pacific, China, India, the Middle East, South America, Southeast Asia, and the South Pacific as the levels vary depending on rainfall, temperature, relative humidity, and unplanned rapid urbanisation.

The sequence of DNA that made up the Envelope protein is shown as below.

```
 901 ttcatcttac tgacagctgt cactccttca atgacatgc gttgcatagg aatgtcaaat
 961 agagactttg tggaaggggt ttcaggagga agctgggttg acatagtctt agaacatgga
1021 agctgtgtga cgacgatggc aaaaaacaaa ccaacattgg attttgaact gataaaaaca
1081 gaagccaaac agcctgccac cctaaggaag tactgtatag aggcaaagct aaccaacaca
1141 acaacagaat ctcgctgccc aacacaaggg gaacccagcc taaatgaaga gcaggacaaa
1201 aggttcgtct gcaaacactc catggtagac agaggatggg gaaatggatg tggactattt
1261 ggaaagggag gcattgtgac ctgtgctatg ttcagatgca aaaagaacat ggaaggaaaa
1321 gttgtgcaac cagaaaactt ggaatacacc attgtgataa cacctcactc aggggaagag
1381 catgcagtcg gaaatgacac aggaaaacat ggcaaggaaa tcaaaataac accacagagt
1441 tccatcacag aagcagaatt gacaggttat ggcactgtca caatggagtg ctctccaaga
1501 acgggcctcg acttcaatga gatggtgttg ctgcagatgg aaaataaagc ttggctggtg
1561 cacaggcaat ggttcctaga cctgccgtta ccatggttgc ccggagcgga cacacaaggg
1621 tcaaattgga tacagaaaga gacattggtc actttcaaaa atccccatgc gaagaaacag
1681 gatgttgttg ttttaggatc ccaagaaggg gccatgcaca cagcacttac aggggccaca
1741 gaaatccaaa tgtcatcagg aaacttactc ttcacaggac atctcaagtg caggctgaga
1801 atggacaagc tacagctcaa aggaatgtca tactctatgt gcacaggaaa gtttaaagtt
1861 gtgaaggaaa tagcagaaac acaacatgga acaatagtta tcagagtgca atatgaaggg
1921 gacggctctc catgcaagat ccctttgag ataatggatt tggaaaaaag acatgtctta
1981 ggtcgcctga ttacagtcaa cccaattgtg acagaaaaag atagcccagt caacatagaa
2041 gcagaacctc cattcggaga cagctacatc atcataggag tagagccggg acaactgaag
2101 ctcaactggt ttaagaaagg aagttctatc ggccaaatgt ttgagacaac aatgaggggg
2161 gcgaagagaa tggccatttt aggtgacaca gcctgggatt ttggatcctt gggaggagtg
2221 tttacatcta taggaaaggc tctccaccaa gtctttggag caatctatgg agctgccttc
2281 agtggggttt catggactat gaaaatcctc ataggagtca ttatcacatg gataggaatg
2341 aattcacgca gcacctcact gtctgtgaca ctagtattgg tgggaattgt gacactgtat
2401 ttgggagtca tggtgcaggc cgatagtggt tgcgttgtga gctggaaaaa caaagaactg
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Figure 1: The DNA sequence of DENV-2 for the gene "Envelope protein E"

2.0 SIZE OF THE DNA AND PROTEIN OF INTEREST

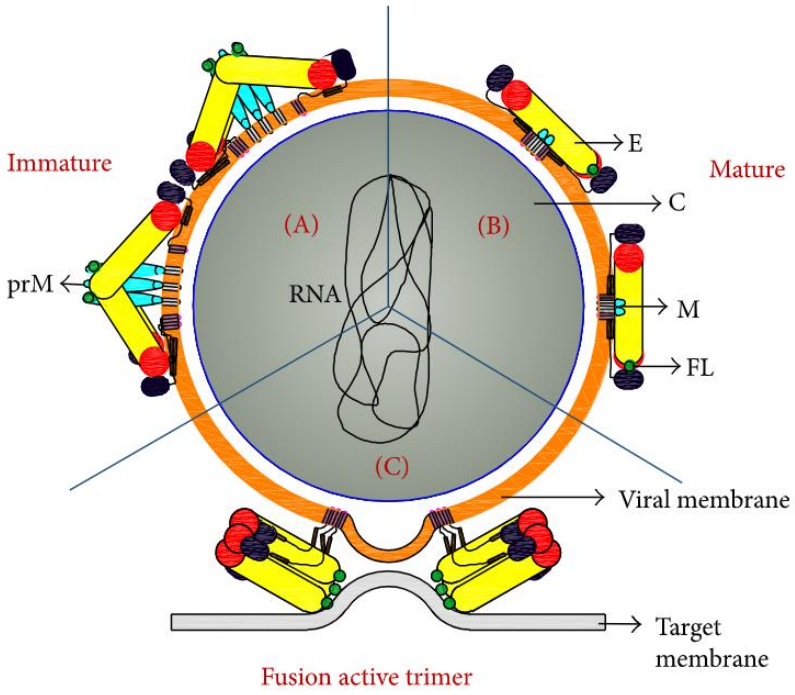

Figure 2: Structure of E protein on Dengue Virus

Based on our research, there are a few unique sequences in Dengue Virus 2. The Dengue Virus genome is a single strand of RNA which has the structure of a spherical virus. The diameter of this virus is in an approximation of 50 nm with an isometric nucleocapsid of 25 to 30 nm, in an estimation of 10.7 kb. The presumed molecular weights for the protein of interest ranges around 20 to 40 kDa in size, or bigger in the bounds of 57 to 130 kDa. DENV has a positive polarity RNA virus with the length of 1484 bases in a genome. The RNA encodes 3 structural proteins which are C, prM and E. They form the components of the virion, and 7 non-structural proteins (NS1, NS2A/B, NS3, NS4A/B, NS5) which are involved in viral RNA replication. The virus will turn into a ‘spiky’ or ‘smooth’ form depending on the pH of the surrounding area.

3.0 DISEASES RELATED TO THE GENE OR PROTEIN

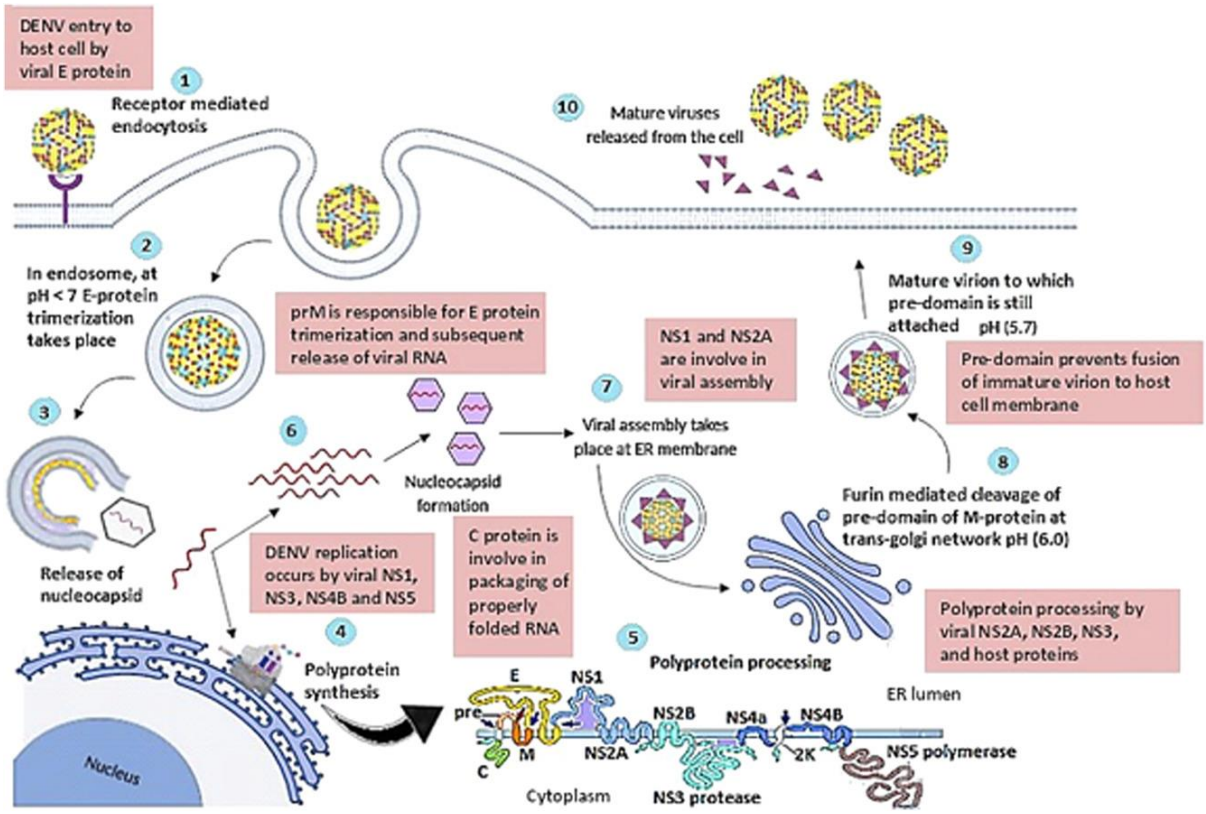

Figure 3: Life cycle of dengue virus inside the cell and role of viral proteins during this process

Female mosquitoes, mostly *Aedes aegypti* and *Ae. albopictus*, carries dengue virus. Dengue fever is characterized as a high fever with flu-like symptoms, caused by a virus from the Flaviviridae family which consists of four different serotypes of the virus, DENV-1, DENV-2, DENV-3, and DENV-4. Dengue haemorrhagic fever is a severe form of dengue fever that can result in major bleeding, a drop in blood pressure and death. There are plenty of symptoms to indicate if someone has dengue fever, but many people do not show any sign of the infection. The common symptoms are severe headache, pain behind the eyes, muscle and joint pains, nausea, vomiting, swollen glands, and rashes, whereas severe abdominal pain, persistent vomiting, rapid breathing, bleeding gums, fatigue, restlessness, and blood in vomit take place more severely.

Dengue fever cannot be contracted by being in the presence of an infected individual. Someone can have long-term immunity to the type of virus that has infected them but not to the other three types of dengue fever virus. This means that you could still potentially be infected by other three virus types in the future. If you encounter dengue fever for the second, third or fourth time, your chances of acquiring severe dengue fever is high.

There are three types of dengue fever transmissions. The first transmission is mosquito-to-human. Infected female mosquitoes, typically the *Aedes aegypti* mosquito, transmits the virus to people through bites. The virus replicates in the mosquito midgut after it feeds on a DENV-infected person before spreading to secondary tissues, such as the salivary glands. Once infectious, the mosquito is capable of transmitting the virus for the rest of its life. The second transmission is human-to mosquito. Mosquitoes can get infected with DENV by biting people who are viraemic. The last transmission made is another mode of transmission. Mosquito vectors are the principal method of transmission of DENV between people while vertical transmission rates appear to be modest, the risk of vertical transmission appears to be connected to when the dengue infection occurs during pregnancy. When a mother has a DENV infection while pregnant, her baby may be born prematurely, have a low birthweight or even experience deadly complications.

4.0 THE ROLE OF GENE IN PATHOGENESIS

Envelope protein is a glycoprotein that has been studied for its antigenicity and immunogenicity and especially explored as an epitope type. It is also a multifunctional protein that plays a role in cell receptor binding and virus entrance by fusion with the host cell membranes. It possesses multiple epitopes that respond with neutralising antibodies and is necessary for adhesion to cell membranes during infection. E proteins are found on the surface of dengue viruses and play an important role in cell entry where the shape of the protein influences how the virus interacts with the host cell. The genome and nucleocapsid, which contain the genetic information and surrounding proteins, are surrounded within a lipid-based viral envelope. By allowing viruses to enter host cells, E proteins play a critical function in the dengue life cycle. As a result, modifications on E protein may have an impact on the virus' path. During endocytosis, the E proteins undergo structural changes, and targeting the proteins could be an efficient antiviral therapy against dengue. Neutralising antibodies are mostly directed against E protein. Despite the fact that recent research has revealed many new details about the role of E proteins, the precise processes by which the function is still unknown.

The pathogenesis of severe manifestation is unknown, although it is thought that the diverse presentation is owing to intricate virus replication and the host immune response. There has been considerable advancement in DENV vaccine development, however, there is still no effective antiviral on the market. ER stress was found to be higher in viral strains that replicate quickly. This shows that UPR may have a role in DENV pathogenesis and severity. Antibodies to DENV have the potential to be a double-edged sword. As the numbers of cross-reactive antibodies decline over time, these antibodies may become more harmful. The antibody-dependent enhancement (ADE) theory, which states that sub-neutralizing quantities of antibodies might create immune complexes that interact with FcγRs (Fc receptor) in the brain, is the most commonly accepted explanation for this phenomenon. Usually occur in dendritic cells, monocytes, and macrophages leading to increased uptake and infection.

5.0 CLONING METHODS

5.1 PCR AMPLIFICATION OF THE GENE OF INTEREST AND PLASMID VECTOR

Polymerase Chain Reaction (PCR) is used to amplify a segment of DNA of interest or produce copies continuously from a small sample. PCR allows a little sample of the DNA to be amplified and eases the process of studying. A typical amplification reaction includes a double stranded DNA template, two oligonucleotide primers, a thermostable DNA polymerase that catalyses new formation of DNA strands, nucleotides (dNTPS), and a reaction buffer that contains $MgCl_2$. The reaction is then placed in a thermal cycler, an apparatus that exposes the reaction to a series of different temperatures for predetermined periods of time. One cycle of amplification is defined as a series of temperature and timing modifications. Each PCR cycle doubles the amount of targeted sequence (amplicon) in the reaction, theoretically.

Template denaturation, primer annealing, and primer extension are all steps in the PCR cycle. The target DNA is denatured in the first stage by heating it to 94°C or higher for 15 to 20 minutes. The two entangled strands of DNA split during the denaturation process, resulting in a single-stranded DNA template that is required for replication by the thermostable DNA Polymerase. The temperature drops to around 40°C to 60°C which enables oligonucleotide primers to form stable connections with the denatured target DNA and serves as DNA polymerase primers. This phase takes approximately 15 to 60 seconds to complete. As the reaction temperature increases optimally, the synthesis of new DNA begins. The temperature is in the range of 70°C to 74°C for most thermostable DNA polymerases. The extension process takes about 1 to 2 minutes, and the next cycle begins with a denaturation step at 94°C.

For each template and primer pair combination, each step of the cycle should be optimised. If the temperatures in the annealing and extension processes are similar, the two can be integrated into a single step that includes both primer annealing and extension. The amplified result can be evaluated for size, amount, sequence, and other properties after 20–40 cycles, or employed in subsequent experiments (Promega, n.d.). After successfully amplifying the desired DNA, it can be cloned into a TA vector. TA vectors are T vectors which are linearized plasmids that have been treated to add T overhangs to match the A overhangs of the PCR product.

5.2 PURIFICATION OF THE DNA

Purification of the DNA can be done by using the QIAamp UltraSens Virus Kit. This kit is commercially used for the purification of the DNA, RNA, and protein. According to QIAGEN, this kit allows viral DNA from 1ml samples to be highly concentrated, prior to being purified using QIAamp silica-membrane technology.

To make a pellet, buffer AC and carrier DNA are added to the plasma sample and these complexes are centrifuged at low speed. Then, the pellet will be resuspended in Buffer AR and proteinase K, and incubated for 10 minutes at 40°C. In order to bind the DNA to the QIAamp membrane, the binding conditions are adjusted by adding Buffer AB, and the lysate to a QIAamp spin column. After that, the sample is centrifuged. During centrifugation, RNA and DNA selectively bind to the QIAamp membrane as contaminants pass through. The remaining contaminants and enzyme inhibitors are efficiently removed by centrifugation in two wash steps. Lastly, the pure viral nucleic acids are eluted in low-salt buffer AFE, and we got the product of purified DNA.

5.3 RESTRICTION ENZYME TREATMENT OF THE DNA

Restriction enzymes are enzymes that cleaves DNA at specific sites, known as recognition sites. Due to this, the use of restriction enzymes is vital in this research to genetically engineer genes of interest. Restriction enzymes can be categorized as type I and type II restriction enzymes, with type II being the more favoured option. For this research, NotI restriction enzyme was chosen to be used to cleave the DNA in DENV to acquire the envelope protein E. NotI restriction enzyme is a type II restriction enzyme specifically used for 6 to 8 base pairs which produce sticky ends when cleaned. By forming a vast array of direct and water-mediated interactions with individual nucleotide bases, the NotI enzyme approaches DNA from the main groove side and recognises its 8-bp target sequence (Tamulaitiene & Siksnys, 2008). The NotI restriction enzyme will recognize the recognition site 5′-GCGGCCGC-3′ and should be incubated at 37°C in the O buffer to achieve optimum performance.

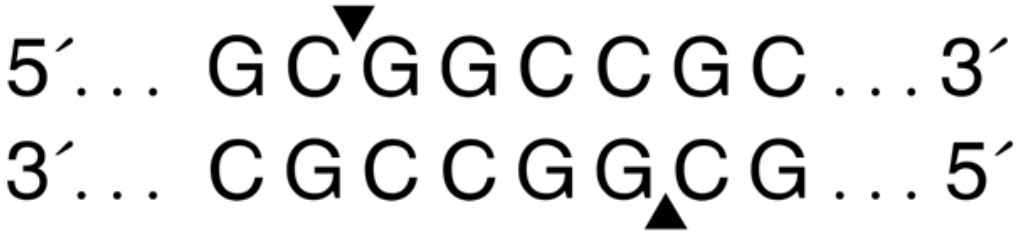

Figure 4: The base sequence of NotI Recognition Site

5.4 SEPARATION OF THE DNA VIA ELECTROPHORESIS

To conduct the agarose gel electrophoresis, we need to use the horizontal gel electrophoresis apparatus. The gel is made by dissolving the agarose powder in a boiling buffer solution. Then, the solution is cooled and poured into a casting tray which serves as a mould. A well-formed template is placed across the end of the casting tray to form wells when the gel solution solidifies. The gel is then submerged in a buffer-filled electrophoresis chamber which contains a positive (anode) and a negative (cathode) electrode at each side of the apparatus. The samples are prepared for electrophoresis by mixing them with a loading dye. Loading dyes in gel electrophoresis are used to provide colour and simplify the loading process. The dyes move at standard rates through the gel, allowing for the estimation of the distance that DNA fragments have migrated. Then, these samples are delivered to the sample wells with a clean micropipette.

A power source is connected to the electrophoresis apparatus and electrical current is applied. The charged molecules in the sample enter the gel through the walls. Molecules that have a net negative charge migrate towards the anode while net positive charged migrate towards cathode. The buffer serves as a electricity conductor. Since DNA has a strong negative charge, it migrates through the gel towards the positive electrode during electrophoresis. The bluish-purple dye allows for visual tracking of sample migration during the electrophoresis. The gel is run until the dye has migrated to an appropriate distance. The agarose gel will have to be post stained after electrophoresis. Ethidium bromide (EtBr) is the most commonly used stain for visualizing DNA. EtBr stains the DNA orange if viewed under UV light. After the orange bands are located, they are cut out of the gel and the DNA is extracted to yield a pure fragment.

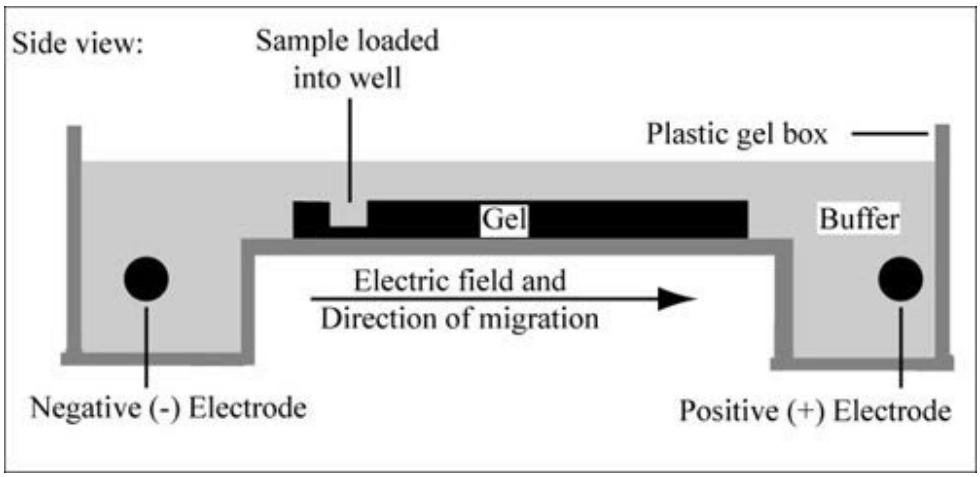

Figure 5: Separation of DNA in the Horizontal Gel Electrophoresis Apparatus

5.5 DNA LIGATION

PCR cloning is a method where the amplified fragments are ligated directly into a vector. Two simplest PCR cloning methods are represented by TA cloning and blunt-end cloning, and it depends on the nature of the vector and the type of PCR enzymes used in cloning. Ligation is the process such as gluing two ends together which can be seen as a vector and PCR-amplified. When ligation is a success, PCR cloning has succeeded.

A thermostable Taq DNA polymerase capable of amplifying short DNA sequences is used in TA cloning. This enzyme has a terminal transferase activity that adds an additional deoxyadenosine to the 3′ end of the amplicons (3′ dA), but no 3′ 5′ proofreading activity. The 3′ dA overhangs in the PCR products can easily be cloned into a linearized TA cloning vector with complementary 3′ deoxythymidine (3′ dT) overhangs.

Meanwhile, the ligation of an insert into a linearized vector with no overhangs is referred to as blunt-end cloning. High-fidelity DNA polymerases with 3′5′ exonuclease or proofreading activity can be used to make blunt-end inserts. Their proofreading activity increases the sequence accuracy of the amplified products; nonetheless, limitations include reduced ligation efficiencies and the inability to clone directionally when inserting into blunt-end cloning vectors. By incubating the amplicons with a Taq DNA polymerase and dATP in a technique known as "3′ dA tailing" (incubate 20–30 minutes at 72°C), then filtering the 3′ dA-tailed products, ligation efficiency can be increased. (Scientific, T., n.d.)

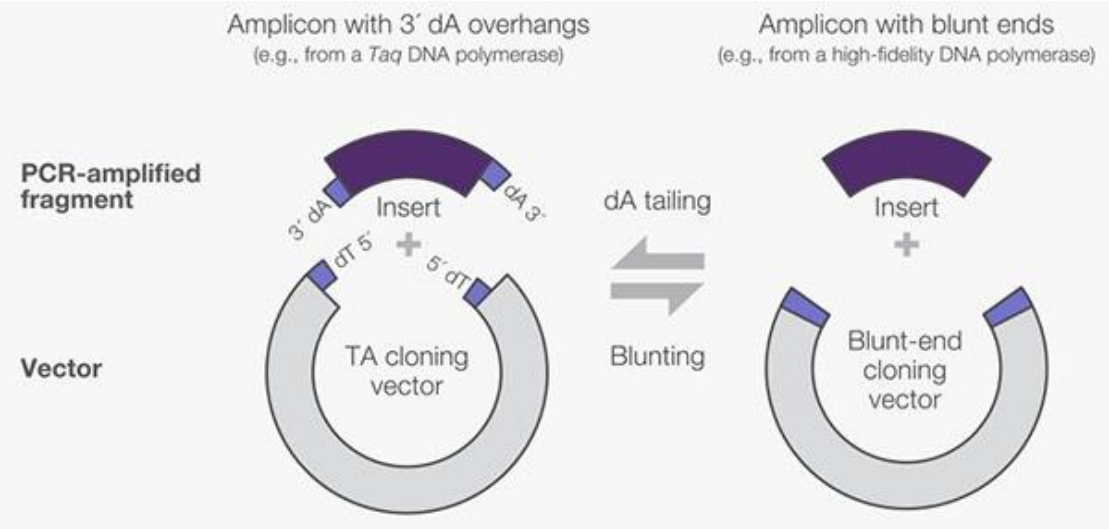

Figure 6: The process of cloning/transformation process

5.6 SELECTION FOR THE CORRECT CLONES AND VERIFICATION

After successfully transforming the plasmid vector containing our cloned gene into the bacterial cell, we have to select the correct clone and verify if the cloned gene is present in the bacterial cell. There are several methods of selection and verification that we can use to determine the correct clones. These include using antibiotics, brute force and ignorance and blue/white screening. For this research, the blue/white screening method was chosen as it is very efficient in determining recombinant bacteria. An article by Julin (2018) states that blue-white screening is a quick and easy approach to tell the difference between bacterial colonies or phage plaques that have a cloning vector with a DNA insert and those that have empty vectors with no insert DNA. The approach is based on the blue pigment that results from the hydrolysis of the synthetic substrate X-gal by beta-galactosidase (Julin, 2018).

According to Grooms (2019), the plasmids have a multiple cloning region within the coding sequence of the α-fragment for blue/white colony screening. The reading frame is interrupted when a sequence is placed into this cloning region, resulting in a non-functional α-fragment. α-complementation is not possible with this fragment. You can distinguish between bacterial colonies created from cells having plasmid with insert and those created from cells having plasmid without insert by growing transformed bacteria on a plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). As a result of the α-galactosidase activity, any colony bearing the plasmid (and hence the functioning α-galactosidase gene) will turn blue. This is referred to as α-complementation. The colour of the colonies carrying plasmids with an insert may be differentiated from those without an insert, with blue being the colony that contains the α-galactosidase gene and white being the colony without the α-galactosidase gene. Since the α-galactosidase gene was disrupted by the insert, the colonies will be white (Grooms, 2019).

6.0 CONCLUSION

In conclusion, the dengue virus is very deadly to human beings. Based on statistics, Malaysia has reported 130,101 cases (over a 60% increase from 2018) & 182 deaths in 2019. (Prudential, 2019). The sequence of E protein can be used in cloning methods according to the steps such as PCR amplification, purification of the DNA, restriction enzyme treatment of the DNA, separation of the DNA via electrophoresis, DNA ligation, and selection for the correct clones and verification. By learning more about E protein, implementing cloning methods, and making research regarding it, it is possible that we can prevent this deadly disease from being spread. Aside to that, there are several other ways that we can do to prevent dengue from infecting our community such as reducing the mosquito habitat by getting rid of stagnant water, which can enable them to breed such as tires, plastic covers, and flowerpots. Other than that, we can wear protective clothing such as long sleeves to prevent mosquitoes from biting. We also can use mosquito repellents by applying creams on the skin, which can prevent mosquitoes from biting.

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