

CLONING OF DENGUE VIRUS TYPE 3 (INDONESIAN STRAIN D3-1703) NON STRUCTURAL-1 GENE INTO pYES2/CT VECTOR

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Abstract

Dengue is an infectious disease caused by dengue virus. Dengue endemic region includes America, Western Pacific, Africa, East Mediterranean, and South East Asia including Indonesia. An early diagnostic system specific for Indonesia is needed to control dengue in Indonesia. In this research, cloning of Non Structural 1 (NS1) gene from dengue virus type 3 (Indonesian strain D3-1703) into pYES2/CT vector was performed. In the long run, NS1 recombinant protein will be expressed in *Saccharomyces cerevisiae* for diagnostic materials. Polymerase Chain Reaction (PCR) amplification of NS1 gene fragments were done with optimal annealing temperature at 55 °C. NS1 gene fragment and pYES2/CT were cut by *Bam*H I and *Not* I enzymes. The digested pYES2/CT was dephosphorylated using Calf Intestine Alkaline Phosphatase enzyme. Ligation with the vector:insert ratio of 1:12 and 1:20 resulted in 6 and 5 recombinant colony candidates respectively. Restriction enzyme and PCR verifications showed that 5 recombinant plasmids contained NS1 gene. Sequencing of the first 600 bp of one recombinant plasmid was performed. The *blastn* analysis showed that it had a 99% identity with dengue virus type 3 strain FW06. Finally, it was shown that NS1 clone within pYES2/CT was in the correct Open Reading Frame and ready to be expressed in *S. cerevisiae*.

Keywords: cloning, dengue, NS1, Saccharomyces cerevisiae

1. Introduction

Dengue fever is a disease caused by dengue virus and is transmitted through the bite of *Aedes aegypti* (the main vector) and *Aedes albopictus* [1-2]. This disease afflicts any ages and might cause death. Dengue viruses cause various degrees of diseases, namely Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) [3]. In 1998, DF became the major tropical infectious disease after malaria and has endemic areas in America, Southeast Asia, Western Pacific, Africa and Eastern Mediterranean where there is more than 2/5 of total world population occupies these areas that are susceptible to dengue disease [4-6]. In DF epidemic in Jakarta in 1998, there were as many as 72,113 cases with 1,414 deaths [2-4].

DF disease is often misdiagnosed with other illnesses such as influenza or typhoid. This occurs because the infection caused by dengue virus is asymptomatic or having vague symptoms [2]. Various diagnostic techniques of dengue disease have been progressed in the world [4-8]. Detection of NS1 antigen has become

the basis of ELISA antigen capture, an early diagnosis method that is recently counted on [9-13].

Genome of Dengue virus is composed of structural proteins (Envelope), membrane, and Capsid and non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). NS1 protein has size between 42-50 kDa and contains 353-354 amino acids. The function of NS1 in the virus replication process is not yet known, but it is suspected that this protein plays an important role in virion maturation process [14]. Anti-NS1 antibodies have been detected in patients infected with dengue virus for the second time (secondary infection) [15]. In the production of vaccines for dengue virus, NS1 protein is an important component since this protein is expressed on the surface of infected cells, making these cells become targets for immune cytolysis [16].

DNA cloning is the process of multiplying the number of recombinant DNA through the bacterial cell proliferation. This is done by inserting a recombinant DNA into host cells and growing them at optimal

temperature, so the cell can multiply exponentially [17]. In most cases, introduction of foreign DNA into host cells requires a vector that can be a form of plasmid. The shuttle vector is a type of plasmid that can be replicated into two or more host cells which is different from each other [18]. The early stages of DNA manipulation are done in the bacterial cell, and then its protein expression system is performed in yeast cells [19].

Saccharomyces cerevisiae yeast is the first eukaryotic organism that was developed in the expression system because as a unicellular organism, *S. cerevisiae* can be genetically manipulated using a technique commonly used in the bacterium *E. coli* and as eukaryotic organisms, *S. cerevisiae* is more suitable used as a host in the production of eukaryotic proteins. Besides, *S. cerevisiae* is also safe to be used in the production of recombinant pharmaceutical ingredients because it does not produce toxic compounds [20].

Previously, NS1 of dengue virus type 1 has been successfully cloned and expressed in *Escherichia coli* [21]. However, inclusion bodies became a difficult purification phase in the *E. coli* system. In addition, expressions of NS1 from previous studies were not from Indonesian isolates, so there are concerns that they cannot detect cases of dengue in Indonesia specifically.

In this study, the NS1 gene of dengue virus strains from Indonesian D3-1703 isolates have been successfully cloned in the *S. cerevisiae* expression vector. It is expected that the production of NS1 recombinant protein in this system will not experience inclusion bodies problems and it can be used as Indonesian specific early dengue diagnosis material.

2. Experiment

Strain and plasmid. Dengue virus strain used in this study was Indonesian isolate, D3-1703. pYES2/CT plasmid (5963 bp, Invitrogen Corporation, CA, USA) was used as a cloning vector. Bacterial strain used as host cells was *E. coli* DH5 α (wild type; Invitrogen Corporation, CA, USA).

Cloning construction. cDNA synthesis of dengue virus RNA has been performed. NS1 gene fragment was amplified using PCR with this cDNA as a source of DNA. Primers used were primer 1-F and 1-16R. The resulting fragments were purified and cut using *Bam*H I and *Not* I. pYES2/CT cutting using the same enzyme was also performed. pYES2/CT which was cut and dephosphorylated using CIAP enzyme. The NS1 fragment was then ligated to the sites of *Bam*H I and *Not* I of vector pYES2/CT. Ligation reaction was transformed into competent cells of *E. coli* DH5 α .

Transformants were selected on LB agar medium containing ampicillin (100 μ g/mL).

Cloning verification. Recombinant plasmid contained in the candidate recombinant colonies was verified by digestion of enzymes *Bam*H I and *Not* I, then it was digested using internal enzyme *Nco* I. Verification was followed by PCR amplification using primers 1-F and 1-16R. Finally sequencing was carried out on one of the recombinant plasmid positively verified using the dideoxy chain-termination reactions at the 5'-end using T7 universal primer.

3. Results and Discussion

PCR amplification of the NS1 gene showed the expected band of 1050 bp. Figure 1 shows the band of 1050 bp when the NS1 gene was amplified from cDNA of dengue virus strains D3-1703. There are non-specific bands when amplification performed using annealing temperature below 52 $^{\circ}$ C, but when the temperature was raised to 55 $^{\circ}$ C, a specific band around 1050 bp was showed. The tape is was then isolated and purified to be used in digestion and ligation reaction steps.

The NS1 gene fragment and pYES2/CT were then digested using *Bam*H I and *Not* I. Digestion of *Bam*H I and *Not* I againsts pYES2/CT vector generated a band of 5.9 kb (Figure 2). Digestion using *Bam*H I or *Not* I enzymes were done to ensure that each enzyme works properly. After purification, a vector was obtained with concentration of 119 ng/mL and NS1 gene fragment with concentration of 24 ng/mL. The NS1 gene fragment that was digested by *Bam*H I and *Not* I will look similar to the undigested NS1 gene fragment (data not shown). This is because cutting on some nucleotides of the NS1 gene fragments will not be seen in 0.8% electrophoresis gel.

pYES2/CT vector ligation process and the NS1 gene insertion using *in vitro* ligation technique as well as the recombinant plasmid transformation into competent cells of *E. coli* strain DH5 α produced 6 colonies of

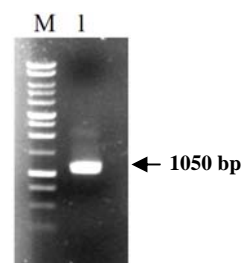


Figure 1. PCR Amplification of NS1 Gene Fragments from cDNA of Dengue Virus Strains D3-1703. M: λ /Hind III Markers; Line 1. NS1 Gene Fragment Amplified with the Annealing Temperature of 55 $^{\circ}$ C. 0.8% Agarose Gel (w/v), 75 Volt, EtBr 1 μ g/mL

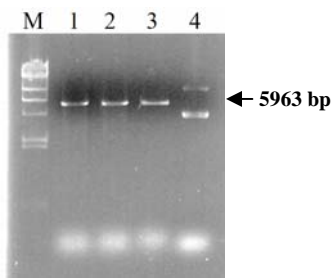


Figure 2. BamH I and Not I Digestion Against pYES2/CT. M. λ /Hind III Markers; Line 1. BamH I Digestion; Line 2. Not I Digestion; Line 3. BamH I and Not I Digestion. Line 4. Intact pYES2/CT. 0.8% Agarose gel (w/v), 75 Volt, EtBr 1 μ g/mL

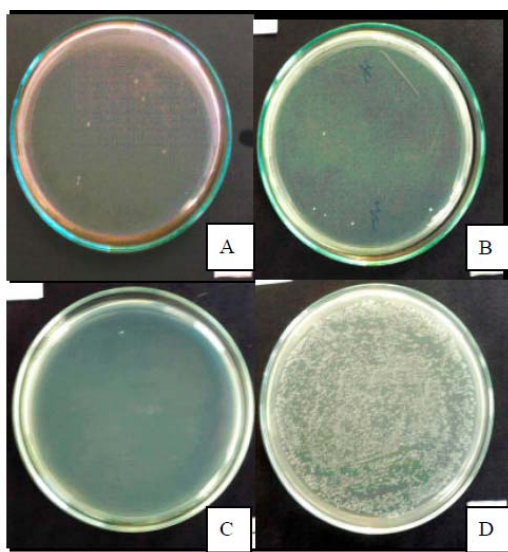


Figure 3. The Results of Ligation of Plasmid Vector pYES2/CT and NS1 Gene Insert Transformed into *E. coli* DH5 α in SOB + Ampicillin Screening Medium with a Ratio of Vector: Insert 1:12 (A) and 1:20 (B). (C) Ligation Reaction of Vector with CIAP Treatment without the Addition of the Insert (Negative Control), (D) The Transformation of Intact pYES2/CT Vector (Positive Control)

bacteria for ligation reaction composition with a ratio of vector:insert of 1:12 (Figure 3-A). In addition, 5 recombinant colonies were obtained for ligation reaction composition with a ratio of vector:insert at 1:20 (Fig. 3-B). The amount of T4 DNA ligase enzyme was 3 units in a 10 μ l reaction. Incubation was performed at room temperature for 30 minutes and continued at 16 $^{\circ}$ C for 16 hours. This ligation condition was optimum at preliminary test on pYES2/CT plasmid religation.

The verification process was performed by isolating plasmid from all recombinant colony candidates. Verification by using the BamH I and Not I restriction enzymes showed positive results with the appearance of

two DNA bands, sized 5.9 kbp indicating pYES2/CT plasmid vector and 1.05 kbp indicating NS1 gene insert (Fig. 4).

Recombinant plasmid candidate positively verified by BamH I and Not I cutting and re-verified using the Nco I restriction enzyme that has introduction site on the NS1 gene insert and vector pYES2/CT. The cropped was visualized by agarose gel electrophoresis. The positive result was shown by the appearance of two DNA bands, sized 3.2 and 3.6 kbp (Figure 5).

Verification using PCR was then performed to ensure the insert was present in the obtained recombinant plasmid. In addition, this verification was used for preliminary test prior to sequencing of recombinant plasmid DNA. The results of amplification of the recombinant plasmid candidates were visualized by agarose gel electrophoresis and showed positive results, with the presence of DNA bands from NS1 gene at 1050 bp (Fig. 6).

Verification of PCR was then performed on the recombinant plasmid which showed positive results on the verification digestion using BamH I and Not I restriction enzymes. The results of PCR amplification produced DNA bands sized approximately 1 kbp (Fig. 6). Each recombinant plasmid was positively verified

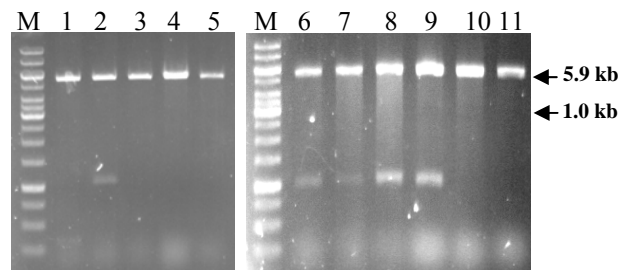


Figure 4. Verification on pYES2/CT Recombinant Resulted from Ligation Reaction with Vector : Insert 1:12 (Line 1-4) and 1:20 (Line 6-10) Using BamH I and Not I Digestion. M: 1 kb DNA Ladder Marker; Line 5 and 11: pYES2/CT. 0.8% Agarose gel (w/v), 75 Volt, EtBr 1 μ g/mL

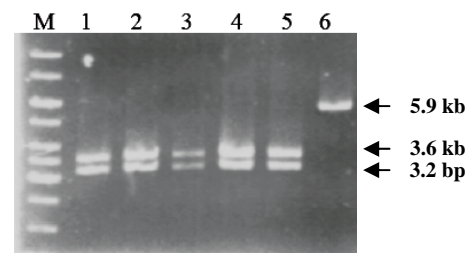


Figure 5. Verification of pYES2/CT Recombinant Using Nco I Digestion. M: 1 kb DNA Ladder Marker; Line 1-5: pYES2/CT Recombinant; Line 6: pYES2/CT. 0.8% Agarose gel (w/v), 75 Volt, EtBr 1 μ g/mL

using this technique indicating that the NS1 gene was successfully cloned into the pYES2/CT vector. Recombinant plasmid was named pYES2/CT-NS1-1, -2, -3, -4, and -5.

Sequencing on pYES2/CT-NS1-2 using T7 primer produced nucleotide readings as much as 613 bases. Homology study was performed using *blastn* at the BLAST program (Basic Local Alignment Search Tool) which is a facility of the NCBI (National Center for Biotechnology Information). *Blastn* analysis showed that the recombinant plasmid sequences have a level of similarity with 100 sequences in GenBank, all of which are DNA sequences of dengue virus type 3 with different serotypes. The highest value is shown by the sequence gi|57544893|gb|AY858041.1| Dengue virus type 3 strain FW06 with bit score: 1061 (535), expect value: 0.0, the percentage identities: 550/555 (99%), and the percentage gaps: 0/555 (0%) (Figure 7).

The results of *blastn* analysis on the recombinant plasmid sequences indicate a low E values, which is 0.0. Expect value (E value) describes the expected chance level of similarity when the data search process takes place. The smaller the value of E value, the higher the

level of confidence of sequence similarity and not a mere coincidence [22-23]. This illustrates that the recombinant plasmid sequences have undoubted level of similarity to the sequences contained in GenBank.

The subsequent parameter was the percentage of identities, which was the calculation results of the ratio of query sequence with unfitted sequences in GenBank divided by the matched query sequence, then multiplied by 100%. While the percentage of gaps is the result of

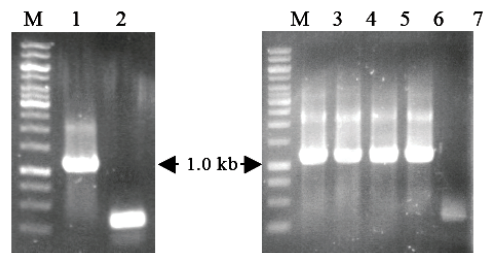


Figure 6. PCR Verification of pYES2/CT Recombinant. M: 1 kb DNA Ladder Marker; Line 1, 3, 4, 5, 6: pYES2/CT Recombinant; Line 2 and 7: Positive Control PCR. 0.8% Agarose Gel (w/v), 75 Volt, EtBr 1 µg/mL

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gi|57544893|gb|AY858041.1| Dengue virus type 3 strain
FW06, complete genome
Length=10687
Score = 1061 bits (535), Expect = 0.0
Identities = 550/555 (99%), Gaps = 0/555 (0%)
Strand=Plus/Plus

Query 1   ATGGGGTGTGTCATAAACTGGAAGGTAAAGAACTCAAATGTGGAAGTGGAAATTTTCGTC 60
          |||
Sbjct 2406 ATGGGGTGTGTCATAAACTGGAAGGCCAAAGAACTCAAATGTGGAAGTGGAAATTTTCGTC 2465

Query 61  ACTAATGAGGTCCACCTGGACAGAGCAATACAAATTTCAAGCAGACTCCCCAAAAGA 120
          |||
Sbjct 2466 ACTAATGAGGTCCACCTGGACAGAGCAATACAAATTTCAAGCAGACTCCCCAAAAGA 2525

Query 121 CTGGCGACAGCCATTGCAAGCGCTTGGGAGAATGGAGTGTGCGGAATCAGGTCGACAACC 180
          |||
Sbjct 2526 CTGGCGACAGCCATTGCAAGCGCTTGGGAGAATGGAGTGTGCGGAATCAGGTCGACAACC 2585

Query 181  AGAATGGAGAACCCTCTGTGGAAGCAAATAGCCAAATGAACATAAACTACATATTATGGGAA 240
          |||
Sbjct 2586 AGAATGGAGAACCCTCTGTGGAAGCAAATAGCCAAATGAACATAAACTACATATTATGGGAA 2645

Query 241  AACACATCAAATTAACGGTAGTTGTGGGTGATATAATTGGGGTCTTAGAGCAAGGGAAA 300
          |||
Sbjct 2646 AACACATCAAATTAACGGTAGTTGTGGGTGATATAATTGGGGTCTTAGAGCAAGGGAAA 2705

Query 301  AGAACACTAACCCACAACCCATGGAGCTAAAATATTTCATGGAAAACATGGGGAAAGCGG 360
          |||
Sbjct 2706 AGAACACTAACCCACAACCCATGGAGCTAAAATATTTCATGGAAAACATGGGGAAAGCGG 2765

Query 361  AGGATAGTGACAGCTGAAATACAAAATTCCTCCTTTATAATAGATGGGCCAACACACCA 420
          |||
Sbjct 2766 AAAATAGTGACAGCTGAAATACAAAATTCCTCCTTTATAATAGATGGGCCAACACACCA 2825

Query 421  GAGTGTCCAAATGCCTCAAGAGCATGGAATGTGTGGGAGGTGGAAGATTACGGGTTCCGGA 480
          |||
Sbjct 2826 GAGTGTCCAAATGCCTCAAGAGCATGGAATGTGTGGGAGGTGGAAGATTACGGGTTCCGGA 2885

Query 481  GTCTTCACAACCAATATATGGCTGAAACTCCGAGAGATGTACACCCAACACTATGTGACCAC 540
          |||
Sbjct 2886 GTCTTCACAACCAATATATGGCTGAAACTCCGAGAGATGTACACCCAACACTATGTGACCAC 2945

Query 541  AGGCTAATGTCGGCA 555
          |||
Sbjct 2946 AGGCTAATGTCGGCA 2960
    
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Figure 7. The Results of *Blastn* Analysis of the First 613 bp Sequence from pYES2/CT-NS1-2

counting the number of "gaps" that occur as a result of the 84 nucleotide query sequences are not homologous with sequences in GenBank [24]. The results of *blastn* analysis on the recombinant plasmid sequences showed that the highest percentage identities were 99%, this suggests that the level of similarity of query sequence to the sequence data in GenBank is at 99%. While the percentage of gaps is 0%, this suggests that the absence of "gaps" that occur as a result of the query sequences not homologous with sequences in GenBank. Finally, analysis of these sequences showed that the NS1 was contained in this recombinant plasmid with appropriate Open Reading Frame and could be expressed in *S. cerevisiae*.

Some literatures have reported that NS1 has been expressed in several expression systems. Expression in bacteria produced NS1 protein as much as 10-30 mg/L [25] and 25 mg/L for dengue multi-epitope protein [26]. NS1 protein has also been expressed in baculovirus systems and mammalian but this expression system requires high cost and difficult maintenance level [27-31]. Bacterial expression is the expression system that most often used for the production of un-glycosylated recombinant proteins. This organism is relatively easy to be manipulated and small scaled analysis can be optimized in a short time. This allows the rapid identification and optimization for growth and induction conditions for medium-scale production.

The downside of bacterial expression systems such as *E. coli* is eukaryotic genes are not expressed efficiently due to differences in codon tendency and toxicity of recombinant protein as well as mRNA instability. Recombinant protein also has no post-translational modifications. Often, protein expression is very high and that protein tends to agglomerate and forms inclusion bodies [32-33]. It is rare to find protein expression in *E. coli* that is secreted into media solution. Yeast system such as *S. cerevisiae* becomes an alternative for the production of NS1 recombinant protein. NS1 recombinant protein expression was performed in yeast with a yield of 70 mg/L [32]. With the acquisition of the NS1 gene cloning from Indonesian dengue virus isolates in this study, the protein is expected to be expressed immediately in the *S. cerevisiae*. Protein obtained can be developed for the production of monoclonal antibodies and furthermore can be used for the development of specific Indonesian dengue early diagnosis.

4. Conclusion

NS1 gene from dengue virus type 3 (D3-1703 strain Indonesian isolates) has been successfully cloned in pYES2/CT expression vector with suitable open reading frame. This NS1 DNA recombinant will be sequenced completely and ready to be expressed in *S. cerevisiae*.

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