Construction of Recombinant Plasmids encoding the sACE2-Fc Gene for the Development of SARS-CoV-2 Neutralization Test

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Abstract

Background: COVID-19 infection is caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The presence of neutralizing antibodies in the body of an infected person is necessary to prevent viral infection. The presence of neutralizing antibodies in seroconvalesen or post vaccinated sera can be measured by several techniques. Competitive Elisa using recombinant RBD spike antigens and ACE2 receptors is one of techniques that viable to be developed since this technique can be applied in facility that does not have a BSL 2 facility. In this research was aimed at obtaining a recombinant plasmid that could be used for the production of the soluble ACE2 recombinant (sACE2). To enhanced its activity, the sACE2 was fused to the C-terminal portion of Imunoglobin F (Fc region).

Methods: The sACE2 coding gene was inserted within the NheI and BamHI sites replacing sRBD gene in the pcDNA3-SARS-CoV-2-S-RBD-Fc vector. The presence of sACE2 gene was confirmed using restriction enzyme analysis and sequencing.

Results: The result showed that the recombinant pcDNA3-sACE2(WT)-Fc plasmid was successfully verified using restriction enzymes and sequencing so that it can be used for the production of recombinant soluble ACE2 using mammalian cells.

Conclusions: The construction process of sACE2 into the pcDNA3 SARS-CoV-2-S-RBD-Fc was successfully carried out and verified.

Keywords : SARS-CoV-2, sACE2-Fc, Recombinant DNA

Abstrak

Latar Belakang: *Infeksi COVID-19 disebabkan oleh Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Keberadaan antibodi netralisasi dalam tubuh seseorang yang terinfeksi sangat diperlukan untuk mencegah infeksi virus. Antibodi netralisasi dalam serum konvalesen atau serum paska vaksinasi dapat dideteksi dengan beberapa teknik. Elisa kompetitif menggunakan antigen rekombinan RBD spike dan reseptor ACE2 merupakan salah satu teknik yang layak untuk dikembangkan karena teknik ini dapat diterapkan pada fasilitas yang tidak memiliki fasilitas BSL 2. Pada penelitian ini bertujuan untuk mendapatkan plasmid rekombinan yang dapat digunakan untuk produksi rekombinan soluble ACE2 (sACE2). Untuk meningkatkan aktivitasnya, sACE2 digabungkan ke bagian C- terminal dari Imunoglobulin F (Fc region).*

Metode: *Gen pengkode sACE2 dimasukkan ke dalam situs NheI dan BamHI menggantikan gen S-RBD dalam vektor pcDNA3-SARS-CoV-2-S-RBD-Fc. Keberadaan gen sACE2 dikonfirmasi menggunakan analisis restriksi enzim dan sekuensing.*

Hasil: *Hasil penelitian menunjukkan bahwa plasmid rekombinan pcDNA3-sACE2(WT)-Fc berhasil diverifikasi menggunakan enzim restriksi dan sekuensing sehingga dapat digunakan untuk produksi rekombinan soluble ACE2 menggunakan sel mamalia.*

Kesimpulan: *Proses konstruksi sACE2 kedalam plasmid pcDNA3-SARS-CoV-2-S-RBD-Fc telah berhasil dilakukan dan diverifikasi.*

Kata kunci : SARS-CoV-2, sACE2-Fc, DNA rekombinan

INTRODUCTION

COVID-19 infection is caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). COVID-19 can cause complications such as long-term lung damage, pneumonia, acute respiratory distress syndrome (ARDS), peripheral nerve and olfactory damage, multi-organ failure, septic shock, and death. WHO declared the COVID-19 outbreak a pandemic on March 11, 2020. COVID-19 cases were reported to have reached 380 million cases in 188 countries with a death rate of more than 5.6 million as of February 2, 2022.[1] Various attempts have been made to control the COVID-19 pandemic, such as the development of vaccines, drugs, and therapies as well as the detection of SARS-CoV-2.[2,3]

SARS-CoV-2 belongs to the genus Coronaviridae. The viral genome encodes four structural proteins those are Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N), about 16 nonstructural proteins, and eight accessory proteins. Protein S plays an important role in the attachment of the virus to its receptor and the entry of the virus into the cell. The virus infects cells through the host Angiotensin Converting Enzyme 2 (ACE2) receptor. Virus entry into cells depends on the binding between the receptor binding domain (RBD) of S protein and ACE2 on the cell surface.[4] The presence of neutralizing antibodies can interfere with the interaction of the S protein and ACE2.[5]

The presence of neutralizing antibodies in the body of an infected person is necessary to prevent viral infection and to determine the presence of neutralizing antibodies needed to be developed.[6] Neutralizing antibody detection can use virus neutralization tests and pseudovirus-based virus neutralization tests, but these two tests require biosafety level 3 and level 2 facilities respectively.[7] To overcome this limitation, several studies based on serological assays have been developed one of which is the competitive Elisa. The test is designed to mimic the interaction between the virus and its receptor on the ELISA well plate.[6,8,9] In this assay, the neutralizing antibody will compete with soluble recombinant ACE2 receptor to bind recombinant RBD spike.[6,8]

ACE2 is a type 1 transmembrane domain, has a 740 amino acid ectodomain, and is expressed in the nasal respiratory epithelium, pneumocytes type II, and elsewhere.[4,6] ACE2 normally plays a role in cardiovascular homeostasis by cleaving angiotensin II, the key agonist of the renin-angiotensin-aldosterone system (RAAS) that regulates blood pressure and electrolytes. It also plays a protective role in the physiology of hypertension, cardiac function, heart function, and diabetes. A recombinant soluble human ACE2 (rhACE2) was recently reported to block SARS-CoV-2 infection in cell culture and human organoids.[6,8] One study showed the multimeric state of ACE2 protein affecting its interaction with RBD.[5,8] In this study, we constructed a plasmid that bearing a gene coding a dimeric sACE2. To get the dimeric structure the sACE2 was fused with the C-terminal of the IgG heavy chain (Fc). The study aims to construct a plasmid coding a sACE2-Fc recombinant that can be used in the production of sACE2 in mammalian systems.

METHODS

Soluble ACE2 coding gene. Plasmid bearing soluble ACE2 gene (pcDNA3-sACE2(WT) was obtained from Addgene.[10]

Subcloned gen coding sACE2 protein into pcDNA3 SARS-CoV-2-S-RBD-Fc. The sACE2 coding gene was inserted within the NheI and BamHI sites replacing the S-RBD gene in the pcDNA3-SARS-CoV-2-SRBD-Fc vector. The bacteria used for plasmid propagation was Top10 strain E.coli. Propagation was carried out for the preparation

of vectors and inserts. pcDNA3-sACE2(WT) and pcDNA-SARS-CoV-2-S-RBD-F were digested with BamHI with composition reaction 10 µg of plasmid DNA, 1x buffer E, 1x BSA (Promega), and 40.000 units of BamHI (Promega) and nuclease-free water (Ambion) to a volume 100 µl. The mixture was incubated overnight at 37^0C. Further, DNA was desalted using the Qiaex II gel extraction kit (Qiagen) following the procedure described by the manufacturer. Further, the DNA was restricted by NheI. DNA that had been cut with BamHI was put into a tube containing 1x Cutsmart (NEB), 40.000 units of NheI (NEB), and nuclease-free water up to a volume of 100 µl followed by the overnight incubation at 37^0C. The restricted DNA was desalted using the Qiaex II gel extraction kit (Qiagen). DNA was run on 1% LMA containing crystal violet (Invitrogen) and purified from LMA using the Qiaex II gel extraction kit (Qiagen). Ligation was performed using a ratio of a vector and insert = 1:3. The ligation reaction was vector 100 ng, insert 92,33 ng, 1x ligation buffer (NEB), 2,5 units of T4 ligase (NEB) and nuclease-free water to a volume of 20 µl. The ligation reaction was incubated at 16^0C overnight. Ligation was transformed by heat shock method to E.coli strain Top10 which was made chemically competent.

Selection of recombinant cloned. Clones were grown with selective LB agar containing 50 µg/ml ampicillin. Colonies were picked up from LB agar and grown in a 4 ml LB medium containing 100 µg/ml ampicillin, incubated at 37^0C, and shaken at 150 rpm overnight. Furthermore, the recombinant plasmids were isolated using Qiaprep miniprep (Qiagen) according to the procedure described by the manufacturer. The recombinant pcDNA3-sACE2(WT)-Fc plasmid was then analyzed with restriction enzymes by making a restriction reaction consisting of 1x buffer 3.1 (NEB), 1x BSA (NEB), 40 units PstI (NEB), then incubated at 37^0C for 1 hour. Visualization of the resulting DNA restriction by electrophoresis of 0,8% agarose, 100 V, 40 minutes. Clones that show the presence of recombinant DNA are continued to the sequencing analysis stage.

RESULTS

Subcloned gen coding sACE2 protein into pcDNA3-SARS-CoV-2-S-RBD-Fc

The preparation stage of the SARS-CoV-2-S-RBD-Fc vector plasmid was restricted with NheI and BamHI to produce 6034 bp and 651 bp DNA fragments. The 6034 bp DNA fragment contained important elements or gene expression, Fc coding gene, origin of replication, and selectable marker gene that will be used as backbone vector. The 651 bp fragment was an RBD coding gene. Meanwhile, restriction of plasmid pcDNA3-sACE2(WT) with NheI and BamHI to produce DNA fragments with sizes of 5353 bp and 1857 bp. The sACE2 insert DNA has a size of 1857 bp. The 5353 bp DNA fragment was pcDNA3.1 backbone and the 1857 bp DNA fragment was the sACE2 coding gene (Figure 1).

The vector and insert fragments were obtained from 1% LMA which had been purified, then the vector and insert were ligated to obtain recombinant DNA. Following are the visualization result of DNA inserts and vectors on 0,8% agarose gel (Figure 2).

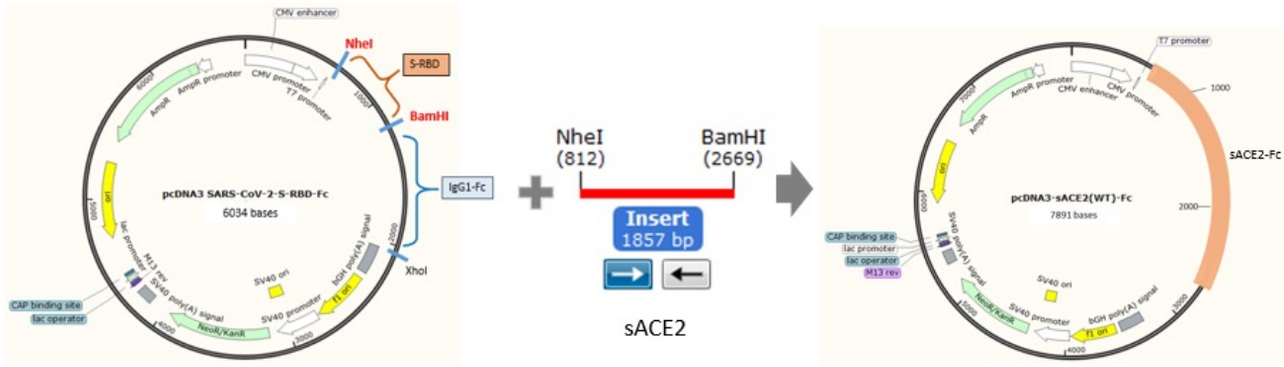
Selection of recombinant cloned

Recombinant pcDNA3-sACE2(WT)-Fc plasmid ligation transformation colonies grew as many as 16 colonies, furthermore isolation and restriction enzyme analysis were carried out. The results of restriction plasmid analysis pcDNA3-sACE2(WT)-Fc with PstI produced bands with size of 4313 bp, 2635 bp, and 943 bp. Meanwhile, the vector restricted by PstI will be linear with a size of 6685 bp (Figure 3, 4,5).

Colony numbers 5, 6, 7, 8, 10, 15, and 16 showed the expected bands, then colony number 5 was selected for isolation and sequencing analysis to determine the base arrangement and the accuracy of the insertion DNA orientation in the plasmid vector.

The sequencing results showed no base change compared to the reference sequence (pcDNA3-sACE2(WT) and pcDNA3 SARS-CoV-2-S-SRBD-Fc) and the orientation of the inserted DNA matched and there was no change in the reading frame of the sACE2-Fc gene. The sequencing results also show that the plasmid expression system is complete, starting from the signal peptide, promoter enhancer, and Fc receptor, and at the junction insert vector, there are no insertions or deletions that cause a shift in the reading frame (the full sequence of sACE2-Fc was not shown) (Figure 6).

A



B

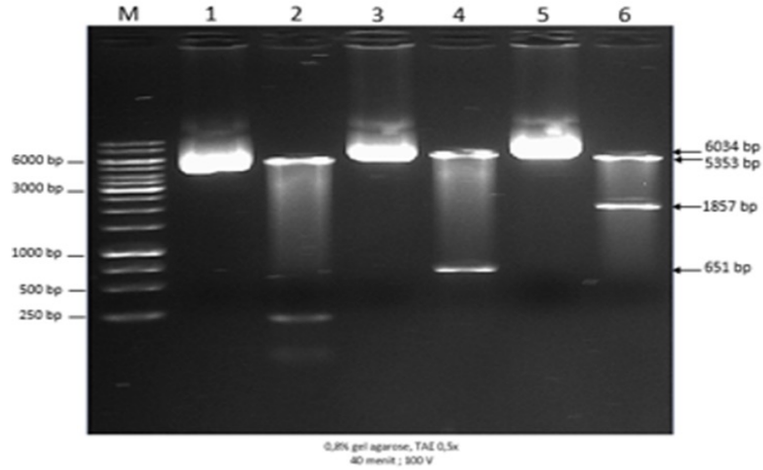


Figure 1. A. Schema of subcloning plan, B. Result of NheI and BamHI restriction enzymes, M: Marker DNA Ladder 1 Kb (Thermo Scientific), Lane 1: pcDNA3.1 Ires Foldon-7xHis uncut, Lane 2: pcDNA3.1 Ires Foldon-7xHis cut, Lane 3: pcDNA3-SARS-CoV-2-S-RBD-Fc uncut, Lane 4: pcDNA3-SARS-CoV-2-S-RBD-Fc cut, Lane 5: pcDNA3-sACE2(WT)-Fc uncut, Lane 6: pcDNA3-sACE2(WT) cut.

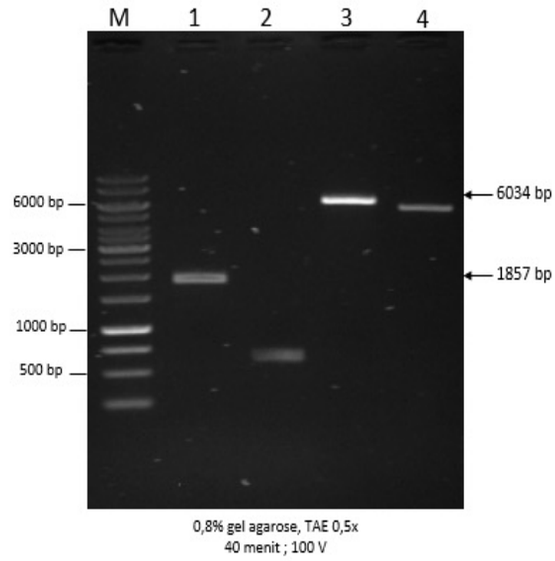


Figure 2. Result of DNA Purification from 1% LMA, M: Marker DNA Ladder 1 Kb (Thermo Scientific), Lane 1: Insert, Lane 3: Vector

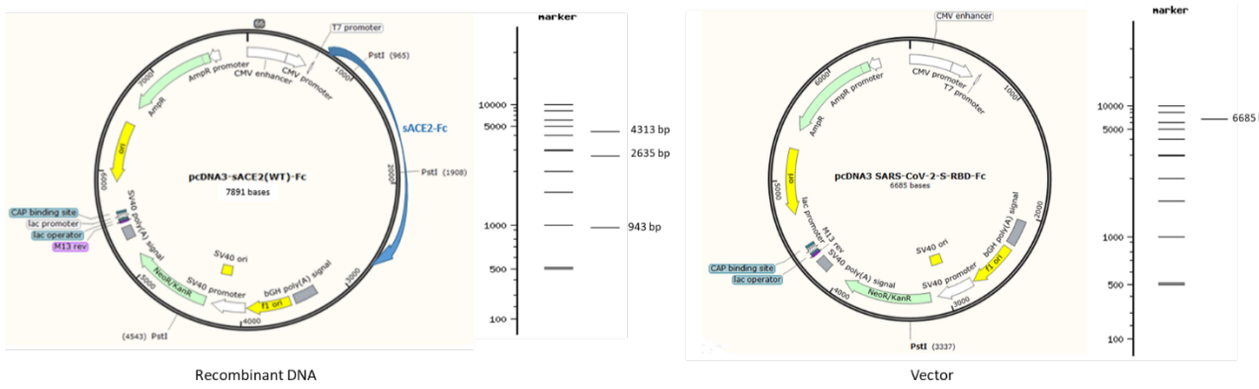


Figure 3. Recombinant DNA and Vector Restriction Maps

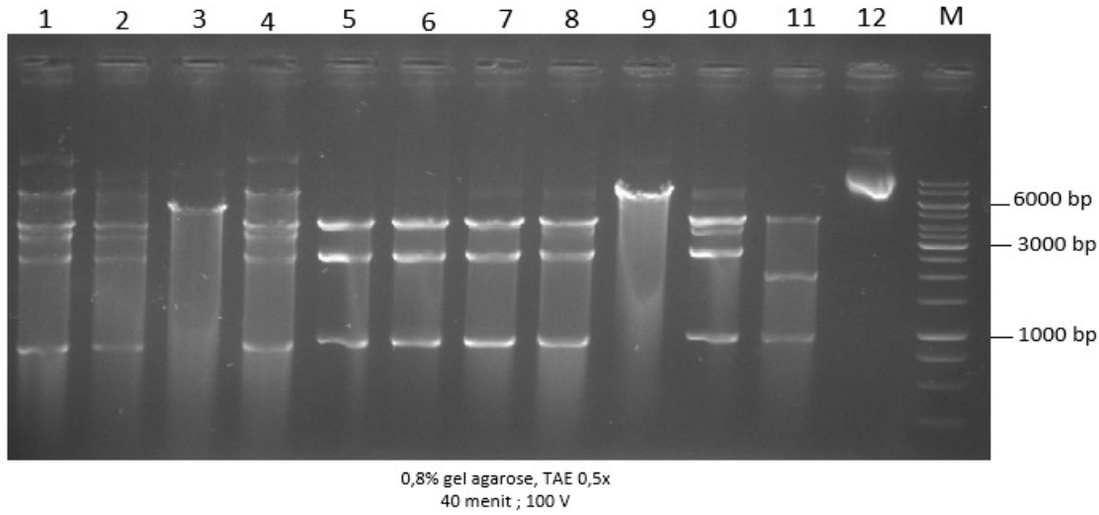


Figure 4. Result of Restriction PstI, M: Marker DNA Ladder 1 Kb (Thermo Scientific), Lane 1: colony no.1, Lane 2: colony no.2, Lane 3: colony no.3, Lane 4: colony no.4, Lane 5: colony no.5, Lane 6: colony no.6, Lane 7: colony no.7, Lane 8: colony no.8, Lane 9: colony no.9, Lane 10: colony no. 10, Lane 11: pcDNA-sACE2(WT) cut , Lane 12: pcDNA-sACE2(WT) uncut.

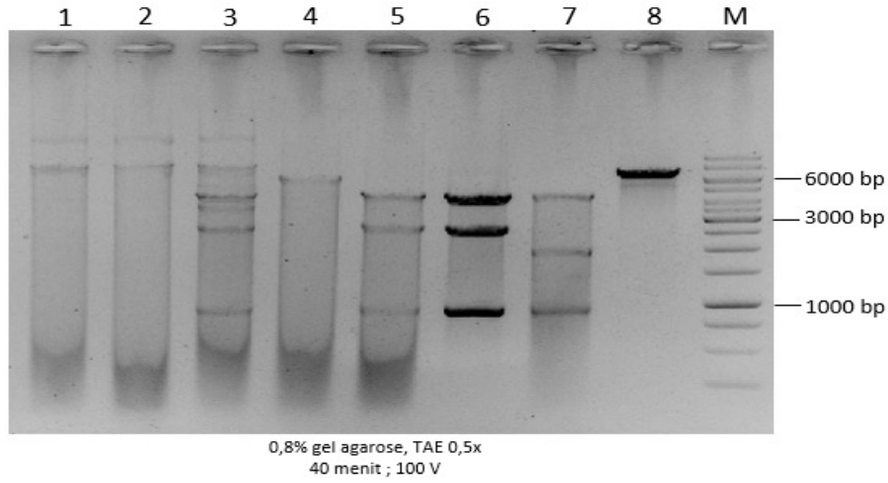


Figure 5. Result of Restriction PstI, M: Marker DNA Ladder 1 Kb (Thermo Scientific), Lane 1: colony no.11, Lane 2: colony no.12, Lane 3: colony no.13, Lane 4: colony no.14, Lane 5: colony no.15, Lane 6: colony no.16, Lane 7: pcDNA-sACE2(WT), Lane 8: pcDNA3-SARS-CoV-2-S-RBD-Fc.

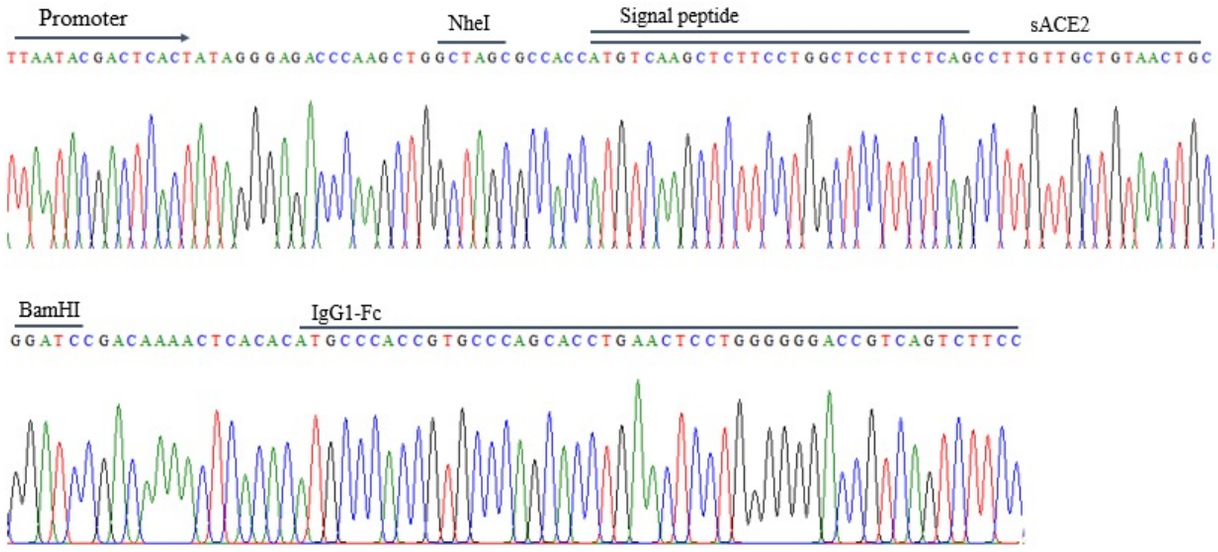


Figure 6. The sequencing result showed the position of the sACE2-Fc gene

DISCUSSIONS

The plasmid pcDNA3-SARS-CoV-2-S-RBD-Fc was used in this study because it already contains the IgG1-Fc gene which will form a dimerization of the expressed protein. The insertion of the sACE2 coding gene in place of RBD to make the protein can be expressed in a homodimer resembling its native form. The vector plasmid has an important element needed in the expression of recombinant protein in the mammalian system those are enhancer, CMV promoter, and poly-A tail. Moreover, the vector also contained a DNA fragment coding a signal peptide peptidase (SPP) that destinated the recombinant protein to be exported extracellularly. In this construct, the signal peptide was fused with sACE2. The promoter has the advantage of being able to regulate gene expression broadly in several types of mammalian cells. Vector plasmids also contain genes coding ampicillin resistance as selectable markers that are used for the selection of transformant clones with ampicillin antibiotics.[6,10,11,12]

Vector plasmids and inserts were cut with the endonuclease enzymes NheI and BamHI. Both of these enzymes have the activity of only cutting one site and producing a piece of a sticky end. The ligation process will be more efficient in cloning techniques with enzymes that produce sticky ends because free sharp ends can be paired with other complementary sharp ends through hydrogen bonds, which can form relatively stable bonds.[13]

Ligation was performed to join the phosphodiester bond between the sACE2 insert DNA and the vector plasmid. Ligated DNA was transformed into competent E.coli using cold $MgCl_2$ and $CaCl_2$ solutions accompanied by heat shock to see the success of the ligation process. High salt concentration accompanied by heating will disrupt the permeability of the E.coli cell membrane so that the DNA around the bacteria can enter the cytoplasm.[13,14]

Colonies grown as a result of the transformation of pcDNA3-sACE2(WT)-Fc ligation were cultured on Luria Bertani (LB) agar medium containing ampicillin and LB broth medium containing ampicillin. Bacterial cultures in Luria bertani broth media were harvested for plasmid isolation. Colony PCR was not carried out for the recombinant plasmid pcDNA3-sACE2(WT)-Fc, this was because there was no specific primer to recognize the target gene, and also the DNA polymerase enzyme used for PCR was unable to amplify the target gene by more than 2000 bp when using CMV Forward and BGH Reverse primers (unpublished data). Thus to confirm used restriction enzymes.[14,15]

The colony selection method using restriction enzyme analysis is intended to determine the success of ligation between the vector and the insert. In this study, the PstI enzyme was used to confirm the recombinant plasmid pcDNA3-sACE2(WT)-Fc because this enzyme can distinguish recombinant plasmids from vector plasmids. There are 3

restriction sites for the PstI. The recombinant plasmid pcDNA3-sACE2(WT)-Fc has 3 restriction sites for the PstI, one of which is contained in the inserted DNA. Furthermore, the colony selection method uses sequencing analysis.

The sequencing results showed that the inserted gene matched the reference sequence and there were no changes or base shifts or changes in the orientation of the inserted DNA in the vector.[15] There is a CMV promoter and enhancer located before the insert. The plasmid expression system is complete, starting from enhancers which function to enhance transcriptional activity, promoters which are useful for initiation of transcription, signal peptides which function as target proteins for secretion are translocated from the endoplasmic reticulum (ER) to the Golgi to the extracellular medium, the Fc receptor has fused with sACE2 to function for protein expression with homodimers and at the junction, the insert vector does not experience insertions or deletions which causes a shift in the reading frame.[12,13,14]

In this study, the construction of pcDNA3-sACE2(WT)-Fc was verified based on the suitability of the size of the DNA bands resulting from the restriction enzymes and sequencing. The recombinant plasmid that has been constructed will later be used to express the recombinant sACE2-Fc protein so that it can be used as a recombinant antigen for the development of neutralization test detection.

CONCLUSION

The construction process of sACE2 into the pcDNA3 SARS-CoV-2-S-RBD-Fc was successfully carried out and verified, it was proven that there were no mutations and the cloned inserted DNA was in the correct orientation in the vector.

Acknowledgment

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Conflict of Interest

The authors declare that they have no competing interest

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