Cloning and Expression of Human Monoclonal Antibody with Potent Binding to 2019 Novel Coronavirus

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ABSTRACT Coronavirus disease 2019 or COVID-19 is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Currently, there is no vaccine or specific medication to prevent and treatment of the infection. Human monoclonal antibody (mAb) CR3022 has been shown to specifically bind to SARS-CoV and cross react to SARS-CoV-2 with high affinity. We therefore construct and produce mAb CR3022 by cloning and expression in mammalian cell line. Immunoglobulin genes coding for mAb CR3022 were synthesized and cloned into human IgG1 Fc expression vector, then transfected into HEK293 cells. The expressed mAb CR3022 was harvested and purified. The obtained yield of purified mAb CR3022 was 16.5 mg/L of culture. The purified mAb CR3022 could bind to recombinant SARS-CoV-2 spike RBD with an affinity constant of 24.6 nM as determined by biolayer interferometry (BLI) binding assays. These indicated that the mAb CR3022 has the potential to be developed as diagnostic and therapeutic agents.

Keywords: Coronavirus, Monoclonal antibody, COVID-19, CR3022, SARS-CoV-2

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Introduction

The 2019 novel corona virus (2019-nCoV)[1] or Severe acute respiratory syndrome virus 2 (SARS-CoV-2)[2] is causing an outbreak of coronavirus disease 2019 or COVID-19 that was first reported in December 2019 in Wuhan city, Hubei province, China. Subsequently, the virus has been spread all over the world.[3, 4, 5] As of 22 June, 2020, WHO reported more than 9.2 million confirmed cases and 479,133 death in 216 countries around the world.[6] This pandemic situation has forced scientists to revise strategies to fight an infectious disease through drugs, treatment, and control measures. Currently, various diagnostic test kits are available, and repurposing therapeutics has shown to be clinically effective. However, the global demand for diagnostic and therapeutic agents continues to rise, it is important to rapidly develop various algorithms to successfully identify and control the virus.[7]

SARS-CoV-2 is classified into the family Coronaviridae, genus *Betacoronavirus* in which SARS-CoV and MERS-CoV are. These coronaviruses are enveloped, positive-sense single-stranded RNA virus. The viral genome sequences of SARS-CoV-2 consist of 29,891 bases and shares 79.6% sequence identity to SARS-CoV.[8] It encodes four structural proteins, Nucleocapsid (N) protein, Membrane (M) protein, Spike (S) protein and Envelop (E) protein and several non-structural proteins (nsp). Spike protein is the major surface antigen consists of S1 and S2 subunits. The S1 subunit contains a receptor binding domain (RBD), which is responsible for recognizing and binding to the cell surface receptor while the S2 subunit contains other basic elements needed for the membrane fusion. This facilitates viral entry into the host cell by attaching to angiotensin converting enzyme 2 (ACE2) receptors that is found on the surface of many human cells.[9, 10] Therefore, spike protein is the main target for neutralizing antibodies and vaccines.[11] As the sequence similarity between SARS-CoV-2 and SARS-CoV, it raises the possibility that cross-reactive epitopes may exist.[12]

The CR3022 antibody has previously been generated by the screening of an antibody-phage library derived from lymphocytes of a convalescent SARS patient. This antibody is shown to neutralize SARS-CoV by targeting the receptor binding domain (RBD) of SARS-CoV[12, 13] and potently binds to SARS-CoV-2 with high affinity but is not tight enough to neutralize and stop infection.[14] However, CR3022 antibody can synergize with other SARS-CoV-2 RBD-targeted monoclonal antibodies for neutralization and remains under investigation.[15] We, therefore, constructed CR3022 antibody and expresseed as scFv-Fc human IgG1 molecule in mammalian cell to facilitate development of effective antiviral therapeutic and diagnostic agents.

Materials and Methods

Construction of CR3022 antibody expression plasmid

Mammalian expression vector was constructed by inserting a human IgG1 Fc portion and signal sequence into plasmid pcDNA3.4 vector (Thermo Fisher Scientific, USA). Genes encoding

human immunoglobulin gamma-1 consists of C_H2 and C_H3 constant domain according to the UniProt, accession number P01857, were designed to link with secretory signal through a hinge region. The sequences were codon optimized and synthesized by GenScript Inc., USA and then cloned into pcDNA3.4 vector. The engineered vector can be used to insert immunoglobulin variable domain at cloning sites, which is between secretory signal and constant domain by blunt-end cloning and it was designated as pcDNA3.4-hIgG1Fc (Figure 1). The immunoglobulin variable heavy (VH) and light (VL) chain genes of CR3022 antibody were retrieved from the GenBank, accession numbers ABA54613.1 and ABA54614.1, respectively. They were designed as single-chain variable fragmant (scFv) consists of VH and VL domains which were linked by a flexible $(Gly_4Ser)_3$ linker. The scFv sequences were codon optimized and synthesized by Gene Universal Inc., USA. These synthetic sequences were PCR amplified by using Q5 DNA polymerase (New England Biolabs, USA) with phosphorylated primers and then inserted into the constructed human IgG1 Fc expression vector by blunt-end ligation and transformed into *E. coli* TOP10. The presence of correct insert orientation was verified by colony PCR using CMV forward primer which was specific to the vector at upstream of the insert and CR3022 scFv reverse primer which was specific to the downstream of the insert. The positive recombinant clones were confirmed by DNA sequencing using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) on ABI PRISM® 3500xL Genetic Analyzer (Thermo Fisher Scientific, USA).

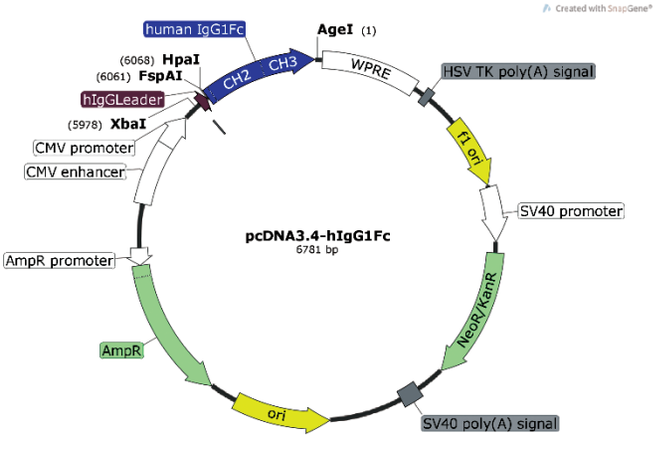

Figure 1 Genetic map of human IgG1 Fc encoding vector. The vector was linearized by double digestion with *FspA*I/*Hpa*I restriction enzymes (Thermo Fisher Scientific, USA) and followed by dephosphorylation with calf intestinal alkaline phosphatase (New England Biolabs, USA) to prevent self-ligation of the vector. The linearized vector was allowed for rapid cloning with PCR product containing immunoglobulin variable gene for expression in mammalian cells.

Expression and purification of CR3022 antibody

Recombinant CR3022 scFv-Fc antibody was transiently expressed by transfecting FreeStyle™ 293-F cell (Thermo Fisher Scientific, USA) which was derived from HEK293

cell line and adapted in suspension culture. Briefly, the cells were cultured in a disposable Erlenmeyer flask with serum-free medium FreeStyle™ 293 Expression Medium (Thermo Fisher Scientific, USA) at 37°C with an orbital shaking at 125 rpm in 8% CO_2 incubator. The culture at an appropriate cell density were transfected with recombinant plasmid by using FectoPRO® transfection reagent (Polyplus-transfection®, France) at the ratio of 1 μg plasmid DNA to 1.5 μL of FectoPRO® transfection reagent according to the manufacturer's protocol. Culture supernatants were harvested 5 days post transfection and antibody was purified from culture supernatant by using Protein G Sepharose® Fast Flow (GE Healthcare, USA). The column was packed with Protein G resin and equilibrated with binding buffer containing 20 mM $NaHPO_4$ pH 7.0. The culture supernatant was applied through the column and washed with binding buffer. The bounded antibody was eluted by using 0.1 M citric acid pH 3.0, and neutralized immediately by using 1.0 M Tris pH 9.0 and then dialyzed against Phosphate Buffered Saline (PBS). The purified antibody was concentrated using an Amicon® Ultra Centrifugal Filters device with a 30 kDa molecular weight cutoff (Merck Millipore, USA) and the concentration was determined by BCA protein assay using BSA as standard protein. The purity was checked by SDS-PAGE and subsequently stained with PageBlue™ protein staining solution (Thermo Fisher Scientific, USA). Western blot analysis was performed after transferring the protein on to a nitrocellulose membrane with polyclonal rabbit anti-human IgG/HRP (Agilent Dako, USA) and followed by TMB substrates (Seracare Life Sciences, USA) color development.

Recombinant SARS-CoV-2 Spike RBD

pcDNA3.4-2019nCoV-RBD was previously constructed. Briefly, the gene encoding RBD (residue 319-541) of 2019-nCoV was amplified from plasmid pCMV3-2019-nCoV-S1 (Sinibiological, China) by PCR using specific primers to introduce the Kozak consensus sequence included Secrecon signal sequences and restriction sites of *HindI*I and *BamHI*. The PCR product was ligated into modified pcDNA3.4 vector containing C-terminal FLAG Tag. The obtained recombinant vector pcDNA3.4-2019nCoV-RBD was then transfected into FreeStyle™ 293-F cells (Thermo Fisher Scientific, USA) as previously described and cultured at 37°C with an orbital shaking at 125 rpm in 8% CO_2 incubator for 3 days. The expressed protein was purified from culture supernatant by Anti-DYKDDDDK G1 Affinity Resin (Genscript®, USA), washed with wash buffer [50 mM Tris-HCl, 150 mM NaCl, pH 7.4] and eluted with alkaline elution buffer [0.1 M Tris, 0.5 M NaCl, pH 12.0], then neutralized with 1M HCl. The purified fractions were dialyzed against PBS and concentrated using an Amicon® Ultra Centrifugal Filter device with a 10 kDa molecular weight cutoff (Merck Millipore, USA) and the concentration was determined by BCA protein assay using BSA as standard protein. Protein purity was examined with SDS-PAGE and specific protein was identified by Western blot using mouse monoclonal anti-FLAG M2-HRP (Sigma-Aldrich, USA).

Analysis of CR3022 antibody binding kinetics

Biolayer interferometry (BLI) assay was performed using an Octet K2 instrument (FortéBio, USA) in order to determine the binding kinetics between the receptor binding domain (RBD) of SARS-CoV-2 and CR3022 scFv-Fc antibody. CR3022 scFv-Fc antibody was immobilized on Protein G biosensors (FortéBio, USA) at 10 μg/ml and six different concentrations (200, 100, 50, 20, 10 and 5 nM) of recombinant SARS-CoV2 spike RBD were used to analyse. The assay was performed in kinetics buffer (PBS, 0.01% BSA, 0.02% Tween 20, and 0.005% NaN_3) at 30 °C with a shake speed of 1,000 rpm. The binding profile of sample was recorded as a wavelength shift in nanometers which represented the difference between the start and the end of the kinetic cycle. Kinetic responses were analyzed using the FortéBio Data Analysis Software HT v11.1 (FortéBio, USA), with a global 1:1 fitting model to obtain values for association rate constants (k_{on}), dissociation rate constants (k_{off}), and the equilibrium dissociation constant (K_D). Curves that could not be reliably fitted with the software ($R^2 < 0.90$) were excluded from further analysis.

Results

Construction of CR3022 antibody expression plasmid

The plasmid pcDNA3.4-hIgG1Fc was successfully constructed as a human IgG1 Fc antibody expression vector. The synthetic scFv sequence encoding CR3022 antibody was amplified and a single PCR product of 742 bp in length was corresponding to the expected size of synthetic scFv sequences as shown in agarose gel electrophoresis was obtained (Figure 2). Then, the purified PCR product was ligated into pcDNA3.4-hIgG1Fc vector and subsequently transformed into competent *E. coli* TOP10 cells.

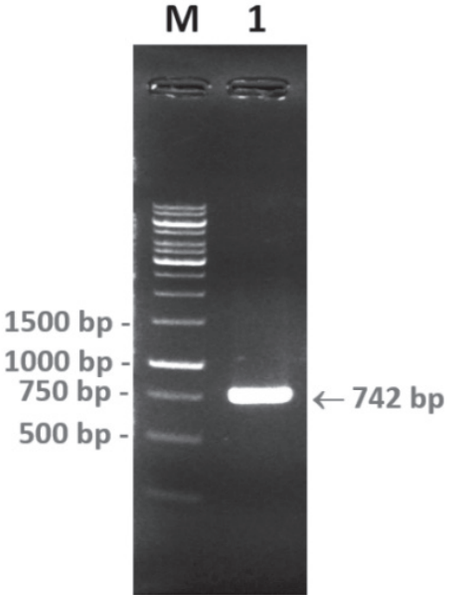

Figure 2 Agarose gel electrophoresis of amplified PCR product from synthetic scFv sequences encoding CR3022 antibody. Lane M: 1 kb DNA ladder (Thermo Fisher Scientific, USA), Lane 1: CR3022 scFV and DNA fragment sizes are indicated.

After transformation, 17 individual colonies were selected and analyzed for the presence of scFv by colony PCR. Two positive clones with 965 bp insert corresponding to the expected size of CR3022 scFv were shown on agarose gel electrophoresis (Figure 3). Sequencing results confirmed that only one of the two positive clones had correct orientation insert of CR3022 scFv in the pcDNA3.4-hIgG1Fc vector (data not shown).

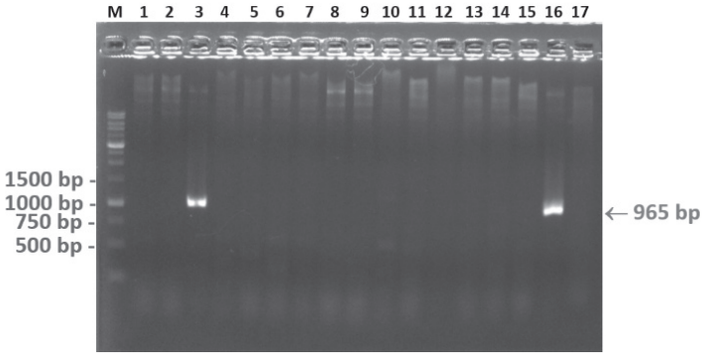

Figure 3 Agarose gel electrophoresis of colony PCR amplified products from transformant colonies. Lane M: 1 kb DNA ladder (Thermo Fisher Scientific, USA), lane 1-17: amplification product of each transformant colonies respectively, and DNA fragment sizes are indicated. Corresponding bands of 965 bp were found on lane 3 and 16, indicating positive clones.

Expression and purification of CR3022 antibody

CR3022 antibody was successfully expressed in the format of scFv-Fc from transiently transfected HEK293 cells with recombinant pcDNA3.4-hIgG1Fc-CR3022scFv plasmid which was secreted into the culture supernatant. The expressed antibody was achieved purification via Protein G affinity chromatography. The purification and dialysis process resulted in the yield of about 16.5 mg of purified antibody from 1 L of transfected culture. The purified antibody

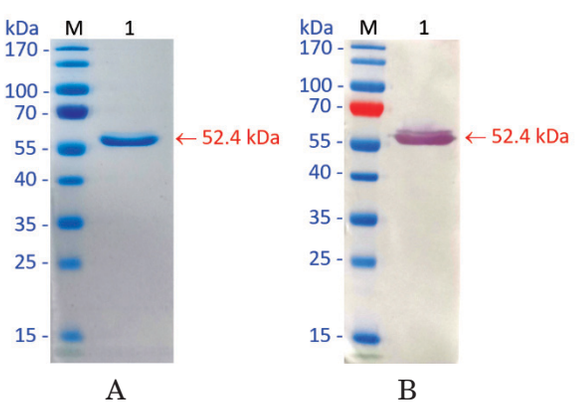

Figure 4 SDS-PAGE and Western blot analysis of purified CR3022 scFV-FcIgG antibody. (A) The purified antibody was separated on 12% acrylamide gel under reduced condition. (B) Western-blot analysis with polyclonal antibody specific to human IgG. Lane M: Protein ladder (Thermo Fisher Scientific, USA), Lane 1: purified CR3022 scFv-FcIgG antibody and molecular weight were indicated.

appeared as a single protein band on SDS-PAGE which was slightly larger than the predicted molecular weight of 52.4 kDa based on calculation from amino acid sequences. This was due to the glycosylation process. The expressed protein could react with anti-human IgG antibody as shown by Western blot analysis (Figure 4).

Recombinant SARS-CoV-2 Spike RBD

The recombinant SARS-CoV-2 Spike RBD protein was expressed as soluble protein and mainly secreted into the culture supernatant. The purification yield obtained from anti-DYKDDDDK affinity chromatography was 11.7 mg/L of culture. The SDS-PAGE of protein obtained from the chromatography showed a single protein band which was slightly larger than the predicted molecular weight of 26.41 kDa based on calculation from amino acid sequences. This was due to the glycosylation process. The expressed protein could react with anti-FLAG antibody which was specific to the fusion tag of the protein as shown by Western blot analysis (Figure 5).

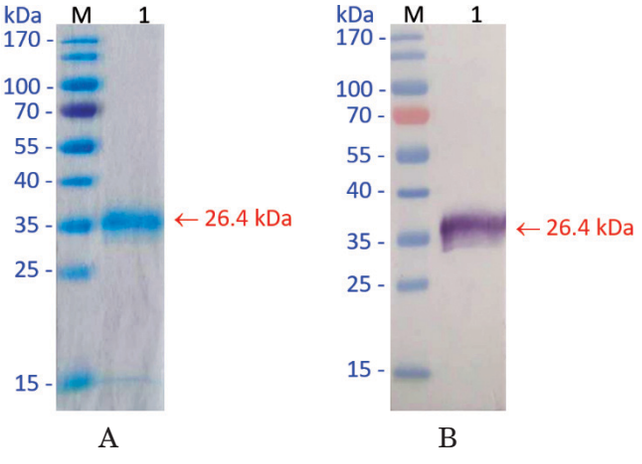

Figure 5 SDS-PAGE and Western blot analysis of purified Recombinant SARS-CoV-2 Spike RBD protein. (A) The purified protein was separated on 12% acrylamide gel under reduced condition. (B) Western-blot analysis with monoclonal antibody specific to FLAG tag of the protein. Lane M: Protein ladder (Thermo Fisher Scientific, USA), Lane 1: purified Recombinant SARS-CoV-2 Spike RBD protein and molecular weights were indicated.

Analysis of CR3022 antibody binding kinetics

The CR3022 scFv-FcIgG1 was shown to bind potently with recombinant SARS-CoV-2 spike RBD as determined by BLI (Figure 6). The processed data were obtained as an association rate constant (K_{on}) of 2.60×10^5 Ms^{-1} and dissociation rate constant (k_{off}) of 6.39×10^{-3} s^{-1}, resulting in an equilibrium binding constants (K_D) of 24.6 nM with an R^2 values greater than 0.97.

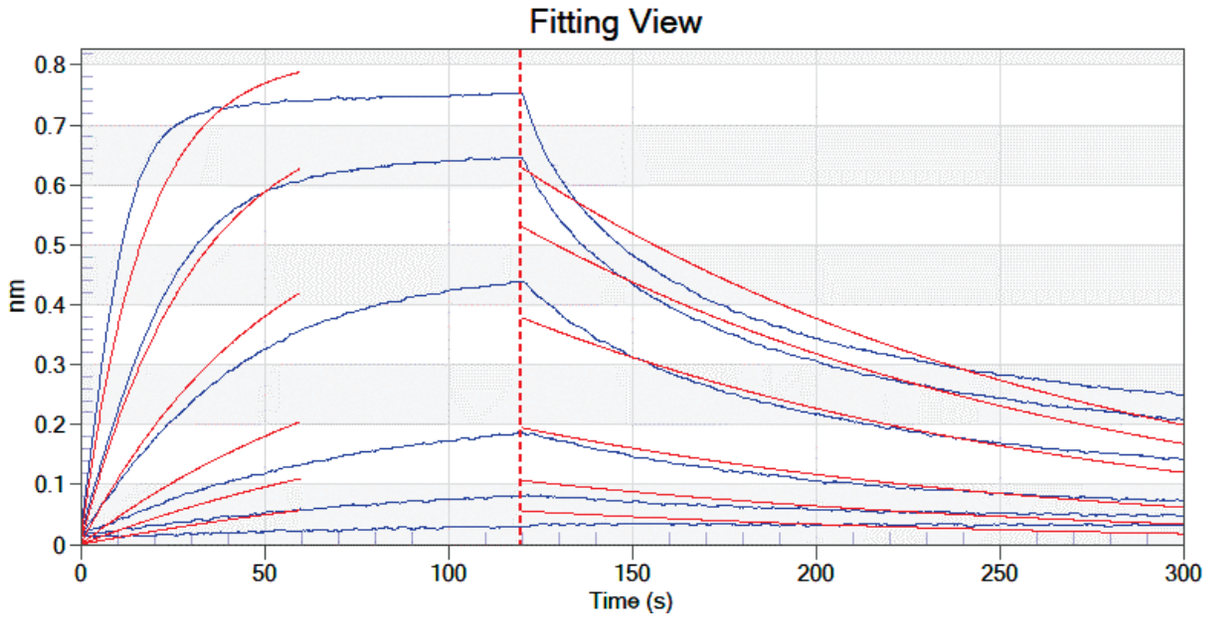

Figure 6 Binding sensorgrams of CR3022 scFV-FcIgG1 antibody interacting with recombinant SARS-CoV-2 spike RBD measured by BLI in Octet K2. Applied analyte concentrations were: 200, 100, 50, 20, 10 and 5 nM. The fit curves were indicated by the red lines, whereas the sensorgrams were shown in blue lines. Binding kinetics was evaluated using a global 1:1 fitting model with FortéBio Data Analysis Software HT v11.1.

Discussion

The results showed that our human IgG1 Fc antibody expression vector could be expressed as secreted scFv-Fc form of CR3022 antibody in HEK293 cell lines. Although, various recombinant antibody expression vectors have been developed and commercialized, a custom vector with signal sequence and optimized human codon usage for higher secreted antibody yield is still required.[16, 17, 18] The most important benefit is to facilitate cloning of several different antibody encoding sequences at a cost-effective timeframe. The original CR3022 antibody was obtained in scFv format by screening from phage display library,[12, 13] which the VH and VL coding sequences were genetically linked in a single transcript and no need to balance the expression of the heavy chain (HC) and light chain (LC). This allows fast production of scFv antibody, in higher yields, and lower costs than full-length IgG antibody in mammalian expression systems.[19] However, an antibody in scFv format retains only the antigen binding activities without Fc mediated effector function, resulting in limitation to be used as therapeutic agents.[20, 21, 22] In order to overcome this drawback, we engineered the CR3022 antibody by fusing scFv with the human IgG1 Fc domain to increase their therapeutic potential. The scFv-Fc format allows for rapid characterization of candidate scFv antibody before conversion into a full-length IgG and offers several advantages over scFv antibody, including bivalent binding, longer half-life, and Fc-mediated effector functions.[23]

Expression of CR3022 scFv-Fc antibody was performed in transiently transfected HEK 293 cells in order to obtain higher yields in combination with simple and robust protocols for production and downstream processing. Human embryonic kidney (HEK) 293 cell lines have been used for transient protein expression because they can be very efficiently transfected with plasmid DNA.[24] Transient mammalian antibody expression is much more suitable for small scale production because it allows rapid production without generating stable cell lines[25]. Moreover, it can be scaled up by using bioreactor processes to more than 150 liter production volumes.[26]

The engineered CR3022 antibody in scFv-Fc format in this study could bind potently to the receptor-binding domain of the SARS-CoV-2 as well as original scFv construct, as previously reported[13] while the absolute kinetic values could also vary due to different materials and experiment conditions.[27] Although CR3022 scFV-Fc antibody itself could not neutralize SARS-CoV-2 in an in vitro microneutralization assay (data not shown) as previously reported, it could be combined with other SARS-CoV-2 RBD-targeted monoclonal antibodies for synergistic effect on neutralizing activity.[12, 13] Moreover, engineered Fc variants of CR3022 antibody could be optimized for effector function which will be able to reduce the viral replication in an in vivo assay.[14]

The results indicated that CR3022 antibody has the potential for further development as therapeutic and diagnostic agents for treatment and control of 2019 coronavirus infections.

Conclusion

Recombinant human monoclonal antibody CR3022 was successfully generated in scFV-Fc format and could be produced by transiently expressed in HEK 293 cell lines. The expressed antibody was predominantly secreted into the culture medium as a result of the leader signal introduced in the constructed human IgG1 Fc vector. The purification could be achieved by protein G affinity chromatography. This recombinant antibody has been proven to bind potently with recombinant SARS-CoV-2 spike RBD.

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การโคลนและการแสดงออกโมโนโคลนอลแอนติบอดีของมนุษย์ที่มีศักยภาพในการจับกับไวรัสโคโรนาสายพันธุ์ใหม่ 2019

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บทคัดย่อ โรคติดเชื้อไวรัสโคโรนา 2019 หรือ COVID-19 เป็นโรคติดต่อที่เกิดจากเชื้อไวรัส SARS-CoV-2 ปัจจุบันยังไม่มีวัคซีนป้องกัน หรือการรักษาที่เฉพาะเจาะจง โมโนโคลนอลแอนติบอดีของมนุษย์ CR3022 สามารถจับกับเชื้อไวรัส SARS-CoV ได้อย่างจำเพาะและเกิดปฏิกิริยาข้ามกลุ่มกับเชื้อไวรัส SARS-CoV-2 ด้วยปฏิสัมพันธ์สูง การศึกษานี้ได้สร้างและผลิตโมโนโคลนอลแอนติบอดีของมนุษย์ CR3022 ในเซลล์สัตว์เลี้ยงลูกด้วยนม โดยการสังเคราะห์ยีนที่สร้างโมโนโคลนอลแอนติบอดี CR3022 และโคลนเข้าสู่พาหะสำหรับแสดงออกแอนติบอดี IgG1 ของแอนติบอดีมนุษย์ในเซลล์สัตว์เลี้ยงลูกด้วยนม จากนั้น transfection เข้าสู่เซลล์ HEK 293 จากการแสดงออกสามารถแยกโมโนโคลนอลแอนติบอดี CR3022 ให้บริสุทธิ์ได้จำนวน 16.5 มิลลิกรัมต่อลิตร โดยโมโนโคลนอลแอนติบอดีที่ได้หลังจากทำให้มีความบริสุทธิ์แล้วสามารถจับกับโปรตีนลูกผสมส่วน spike RBD ของเชื้อไวรัส SARS-CoV-2 ได้โดยมีค่าคงที่ของปฏิสัมพันธ์ (K_D) เท่ากับ 24.6 nM โดยการวิเคราะห์ด้วยเทคนิค Biolayer interferometry (BLI) แสดงให้เห็นว่าโมโนโคลนอลแอนติบอดี CR3022 ที่ผลิตขึ้นนี้มีศักยภาพในการนำไปพัฒนาการตรวจวินิจฉัย และแอนติบอดีเพื่อการรักษาได้

คำสำคัญ: ไวรัสโคโรนา, โมโนโคลนอลแอนติบอดี, COVID-19, CR3022, SARS-CoV-2