A study protocol to prepare an RBD protein for vaccine against COVID-19 [version 3; peer review: 1 approved with reservations, 1 not approved]

ZMG Sarwar Jahangir, Arleta Helena Marnik

Department of Biological Sciences, Kingsborough Community College of The City University of New York, Brooklyn, NY, 11235, USA

**Abstract**

**Background:** SARS-CoV-2 pandemic is a global threat to humans and the world's economy. Effective and safe vaccines against this virus are essential to control and eradicate the pandemic. The currently applied vaccines carry SARS-CoV-2 spike-protein mRNA/cDNA. These vaccines go through several cellular processes in the recipients for producing antigens. On the contrary, the SARS-CoV-2 RBD (receptor binding domain)-protein is an antigen. It will directly stimulate antibody production against SARS-CoV-2. Hence, we propose to produce SARS-CoV-2 RBD-protein as a fast acting, effective and safe vaccine.

**Methods:** We propose to reconstruct a plasmid carrying three types of DNA sequences: RBD cDNA, FP (fusion peptide) DNA and sfGFP (superfolder-green-fluorescent-protein) cDNA creating the RBD-FP-sfGFP DNA within an **orf** (open-reading-frame). *Escherichia coli*, C2566H, transformed with the reconstructed plasmid will express RBD-FP-sfGFP fusion protein producing green fluorescent **cfu** (colony forming unit). The RBD-protein will be separated from the sfGFP using an FP specific enterokinase, and eluted by HIC (**hydrophobic-interaction-chromatography**), detected with a BioVision-Elisa-Kit, and quantified by spectrophotometry at UV280 nm and immune simulation will be carried out using C57BL mice.

**Results:** The plasmid reconstruct will carry amp^R^ (ampicillin-resistant) gene as a selective marker and a T7 promoter controlling the expression of RBD-FP-sfGFP fusion protein. The transformed *Escherichia coli* will efficiently express the RBD-FP-sfGFP fusion protein. The highly efficient sfGFP fused within the RBD-FP-sfGFP will produce green fluorescent **cfu**. The RBD-FP-sfGFP protein extract from the green **cfu**, digested by enterokinase and separated by the HIC will produce pure immunoreactive RBD protein.

**Conclusion:** A positive BioVision-ELISA test detects <10 pg RBD protein/ml of the sample. A larger sample of the purified RBD protein
can be used as a vaccine following a standard formulation and safety protocols. Once administered, the RBD protein will stimulate antibody production against the SARS-CoV-2 virus. The RBD protein has no potential to recombine with human genome.

**Keywords**
receptor angiotensin-converting enzyme 2, amino acids, colony forming unit, hydrophobic interaction chromatography, Luria Bertani microbial culture medium containing ampicillin, fusion peptide, open reading frame, receptor binding protein, Spike protein, severe respiratory syndrome coronavirus 2, superfolder green fluorescent protein.

This article is included in the Emerging Diseases and Outbreaks gateway.
Introduction
A brief history of coronavirus infection

Using the “RNA-Dependent RNA Polymerase Molecular Clock”, it was estimated that the common ancestor of coronavirus (CoV) appeared about 10,000 years ago. The first human upper respiratory tract infection (URTI) caused by human CoV (H-CoV) was reported in 1965. The first severe acute respiratory syndrome caused by CoV (SARS-CoV) was reported from Guangdong, China, in 2002, that ultimately spread over many countries causing a pandemic in the Americas, Europe, and Asia, infecting over 8,098 people and killing about 774 of the infected. SARS-CoV has 99.6% genome sequence homology to CoV found in masked palm civets (Paguma larvata) and 88%–95% homology to CoV found in several horseshoe bats, Rhinolophus pusillus, R. macrotis, R. pearsoni and R. sinicus.

This was followed by another outbreak of a CoV pandemic in 2012 that started in Saudi Arabia, which is known as Middle East respiratory syndrome (MERS) and is caused by MERS-CoV. It spread over 27 countries, reaching Western Africa to the west and South Korea to the east, infecting over 2,400 and killing over 850 people. The survivors suffered from many diseases including heart, kidney, and multiorgan failures. In November 2019, another CoV pandemic emerged in Wuhan, China, found to be caused by SARS-CoV-2 infections, leading to a global pandemic. That pandemic infected 606,879,638 people and killed 6,490,816 as of Aug 30, 2022. As observed by many, the COVID-19 pandemic is not over yet as reported on Sept 8, 2022. However, vaccines being developed may change the illness. Coronavirus infections are not a new challenge to human survival. Some of those challenges, we know, others are unknown to us. However, it is clear that the recent pandemic of 2019 will not be the last, and we have to be alert and keep us ready to be safe for the future.

The benefits and importance of SARS-CoV-2 vaccines

All types of viruses mutate and evolve as they replicate. Their prolonged presence in an uncontrolled environment favors development of new variants. That ability to generate de novo diversity in a short period of time, as well as the rate of spontaneous mutation, vary among viruses. Furthermore, mutation rates in RNA viruses are higher than DNA viruses and are higher in single-stranded viruses than double-stranded viruses.

Hence, vaccinating a smaller segment of a population against SARS-CoV-2 may favor generation of new variants with new infectivity. In that scenario, even the vaccinated individuals would face risks from arrivals of new variants. This may only be brought under control by administering a safe and effective vaccine as soon as possible to a significantly large part of the population. Successes of such efforts will reduce the development of new variants and, thus, help the global human population attain herd immunity.

Furthermore, structure-based analyses found that the VH3-53/3-66 class-derived public neutralization antibodies (NAbs) interact with naturally occurring SARS-CoV-2 RBD multiple variants. Hence, the RBD protein (a domain of the spike protein) vaccine we are proposing will be effective against multiple SARS-CoV-2 variants.

As cases of coronavirus disease 2019 (COVID-19) were growing globally, it brought together the efforts of worldwide biotechnologists, scientists, experts, pharmaceuticals, and investors to develop effective vaccines against SARS-CoV-2 as soon as possible. More than 50 such vaccine candidates were put into human trials in 2020, and a total of 250 vaccine candidates were in the process of being developed. The safety and effectiveness of a vaccine are measured by the vaccine’s long-term antigenicity and immunogenicity for its therapeutic application as a vaccine. However, we still do not have direct evidence of the long-term efficacy of the short life mRNA and long-life cDNA antigenic vaccines.

SARS-CoV-2 mutates at a rate four times slower than the influenza virus, and the new variants have a minimal effect on antigenicity of the virus. Although the coronaviruses mutate at a slower rate, some of the new variants of
SARS-CoV-2, such as D 614 G, that mutated outside the receptor-binding domain (RBD) residue have a higher rate of infectivity by boosting viral replication in lung and respiratory tract tissues. This suggests that the RBD residue although not fully conserved, the RBD protein vaccines are effective against multiple SARS-CoV-2 and SARS-CoV-1 variants.31

The RBD protein structure

The RBD is a small segment of the spike protein (S-protein) located on the outer membrane of the SARS-CoV-2 virion. It plays a critical role in binding the virus to the angiotensin-converting enzyme 2 (ACE-2) receptors on human mucosal cells, causing infection leading to COVID-19. Hence, an antibody generated against the RBD protein will be able to strongly prevent any SARS-CoV-2 infection.

According to Huang, et al., the S-protein located on the SARS-CoV-2 virus envelope is composed of 1273 amino acids (aa). The S-protein consists of a 13 aa long signal peptide (1–13 residues), followed by a 672 aa long S1 domain (14-685 residues), and a 588 aa long S2 domain (686–1273 residues). The S1 domain contains a 292 aa long N-terminal domain (14–305 residues) and a 223 aa long RBD domain (319–541 residues). The SARS-CoV-2 encapsulating membrane holds many trimeric S-proteins, each containing three RBD protein monomers, that binds to the ACE-2 receptors present in human cells. Furthermore, Yang, et al., also demonstrated that the S1 domain of the SARS-CoV-2 spike protein directly binds to ACE-2 receptors expressed by the undifferentiated human alveolar cells (A549) facilitating the entry of SARS-CoV-2 into these cells. It was also reported that the SARS-CoV-2 receptor-blocking human antibody, HA001, attaches to amino acid residues A475 and F486 in the RBD of the SARS-CoV-2 spike protein. In this process, a 71 aa long segment of the RBD, known as receptor binding motif (RBM), tightly binds to the ACE-2 receptor. Furthermore, the RBD contains nine cysteine (Cys) residues making four pairs and keeping one free Cys. The three pairs are Cys336–Cys432, and Cys391–Cys525 residues that form the core RBD β sheet related to its 3D structure. The remaining pair, Cys480–Cys488 residue, binds to the N-terminal peptidase domain of ACE-2. All the above information supports that the RBD protein will be a highly effective antigen for vaccine production against SARS-CoV-2.

The RBD protein vaccine and its advantages

Vaccinating across the globe against SARS-CoV-2 is a great scientific, logistical, and moral challenge. Manufacturing protein-based vaccines are potentially cost effective than mRNA vaccines and do not require ultra-cold storage. This would help with a safe, potent, high-volume, and affordable vaccines for a large part of the world, especially in low- and middle-income countries, including Africa where vaccination rates are currently very low. Given a predominance of key biomarker neutralizing antibodies (NAbs) that target RBD following natural infection or vaccination, there is a great justification for selecting RBD protein as the sole vaccine immunogen. It has high-yielding potential, temperature-stable and cost-effective. In addition, the RBD focuses on the immune response to potent and cross-protective domain which is central to the development of future pan-sarbecovirus vaccines. It was also reported that the RBD protein vaccines are equally effective in comparison to full length S-protein vaccine with regard to immune responses against the prototype pandemic SARS-CoV-2 isolate as well as emerging variants of concern.

Clinical evidences supporting RBD subunit protein or S-Protein protein vaccines against SARS-CoV-2

There are several reports on SARS-CoV-2 RBD and spike protein vaccines on trial. In a clinical trial in CUBA, 792 subjects received SARS-CoV-2 RBD protein vaccine named ABDALA during Dec 7, 2020, and Feb 9, 2021. The ABDALA vaccine was found to be safe, well tolerated, and induced humoral immune responses against SARS-CoV-2. For emergency COVID-19 pandemic the results support a 50 μg vaccine dose, applied in a 0-14-28 days schedule was highly effective.

A trial study was conducted on the efficacy and safety of a dimeric tandem repeat of RBD of the SARS-CoV-2 spike protein (Wuhan-Hu-1 strain) vaccine among a total of 28,873 participants conducted during December 12, 2020, and December 15, 2021, at 31 clinical centres across Uzbekistan, Indonesia, Pakistan, and Ecuador with a safety assessment centre in China. In this large cohort of adults, the RBD dimeric vaccine was found to be safe and effective for at least 6 months after full vaccination against symptomatic as well as severe-to-critical Covid-19, without any vaccine-related death. Again, a SARS-CoV-2 recombinant spike protein nanoparticle vaccine in phase 1-2 trials where 83 participants received the vaccine with adjuvant, 25 received the vaccine without the adjuvant and 23 participants received placebo, at random. At 35 days, the nanoparticle vaccine was found to be safe, elicited immune responses that exceeded levels in Covid-19 convalescent serum.

RBD epitope phenotype

The entire RBD protein amino acid chain is involved with the integrity of the RBD epitope 3D structure. The RBD epitope phenotype varies with variations in the amino acid chain in the RBD protein. Altered RBD amino acid chain may
elucidate phenotypic variations in the RBD affecting the epitope.\textsuperscript{44,45} Hence, in this protocol, we will use the entire RBD coding sequence to preserve the epitope structure for producing predominant neutralizing antibodies (NAbs).\textsuperscript{38,39}

The RBD protein vaccine

The RBD vaccine is a sub-unit vaccine and equally effective as spike protein vaccine.\textsuperscript{38,39,46–48} The significance of this study is to:

1. Produce protein vaccine specific against SARS-CoV-2 without any potential for genomic recombination into the recipient’s genome.
2. Production of safer vaccine.
3. Low down the cost of production.
4. Easily transportable and thus accessible across the globe.\textsuperscript{47}
5. There is a limited scope for the RBD vaccine molecule to bind with ACE-2 receptors without hindering the efficacy of the vaccine and will not act as a competitive inhibitor.\textsuperscript{32,41} The amount of RBD molecules per vaccine will be limited and they will have no self-regenerating ability. Most of them will bind to T cells and then instruct the B cells to produce plasma cells for making antibodies against SARS-CoV-2 virus. While the memory B cells will act against any future infection by SARS-CoV-2. However, there will be opportunities for some RBD vaccine molecules to bind the ACE-2 receptors. This opportunity of the RBD protein vaccine is much less than the currently administered spike protein mRNA antigenic vaccines. The mRNA vaccine continuously produces spike protein for a prolong period of time which also binds to the ACE-2 receptors.\textsuperscript{49,50} Hence, the RBD protein vaccine will have little competition in comparison to spike protein mRNA vaccines.

Furthermore, spike proteins produced by the mRNA vaccine has S2 segment that has the potential for facilitating fusion of the free-floating SARS-CoV-2, while the RBD protein vaccine has no potential for viral fusion into human cells and, hence, the RBD protein vaccine is safer.\textsuperscript{31,52}

Our proposal

We propose to reconstruct an amp\textsuperscript{1} plasmid expression vector carrying RBD and superfolder green fluorescent protein (sfGFP) cDNAs linked by an oligo DNA, coding for a fusion peptide (FP), Asp-Asp-Asp-Asp-Lys.\textsuperscript{1} The construct will be expressed using \textit{Escherichia coli}, C2566H, producing the RBD-FP-sfGFP fusion protein. The RBD protein will be separated from the RBD-FP-sfGFP fusion protein by digestion with an enterokinase (specific for the FP) and isolated by hydrophobic interaction chromatography (HIC). The RBD eluate will be analyzed to determine its size by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), tested for its immuno-reactivity with SARS-CoV-2 S-protein antibody using a BioVision ELISA kit, and quantified by spectrophotometry. The purified RBD protein will be available to study its efficacy following approved vaccine formulation and clinical trials.

Proposed methods

This study protocol is a meticulously derived scientific procedure without involving any test animals or test subjects. Hence, it does not require ethical approval at this time. Any further information on this matter, may be obtained from the “Institutional Review Board” (IRB). Once we are ready to submit a grant application and execution of the protocol, we will seek the IRB approval in due course.

Specialized reagents and materials

The following specialized reagents and materials (from the same or alternate sources) will be required in addition to regular materials and reagents available in a running biotechnology laboratory.

1. RBD cDNA: Addgene plasmid, Cat # 141184, pcDNA3-SARS-CoV-2-S-RBD-sfGFP (Amp\textsuperscript{R}, Neo\textsuperscript{R}/ Kan\textsuperscript{R}).
2. Prokaryotic host cells: \textit{Escherichia coli}, NEB, Cat # C2566H.
3. Plasmid DNA extraction kit: Promega PureYield Plasmid Miniprep System I and a Protocol. Promega, Cat # A1222, and Promega Technical Bulletin # TB374.
4. Custom made linker oligo DNA coding for the FP to be used for plasmid recombination: It is a double-stranded linker oligo DNA with ss (single-stranded) 5' overhangs. This will contain a forward strand, 5' GATCGGATGATGATGATAAAC 3', and a reverse strand, 3' CCTACTACTACTATTTGCTAG 5', each carrying a ss 5' GATC 3' overhang at the 5' end, Figure 1, to be obtained from GenScript, NJ. The working concentration will be adjusted to 10^5 units in 1 μl.

5. A chromatography kit: Green Fluorescent Protein Chromatography Kit, Bio-Rad, Cat # 1660005EDU. It is a HIC (hydrophobic interaction chromatography) kit.

6. Protein dialysis tubing: SnakeSkin dialysis tubing with 10 kDa molecular weight cut-off, Thermo Fisher Scientific, Cat # 88243.

7. Power supply unit: Invitrogen PowerEase Touch 600W Power Supply. Thermo Fisher Scientific, Cat # PS0601.

8. Electrophoretic unit: Mini Gel Tank, Thermo Fisher Scientific, Cat # A25977.

9. Protein electrophoresis gel slabs: NuPAGE 4-12%, Bis-Tris, 1 mm, 12-well mini protein gradient gel. Thermo Fisher Scientific, Cat # NP0322PK2.

10. Protein sample buffer: NuPAGE LDS Sample Buffer (4X), Thermo Fisher Scientific, Cat # NP0007.

11. Protein electrophoresis buffer: NuPAGE MES SDS Running Buffer (20X), Thermo Fisher Scientific, Cat # NP0002.

12. Protein standard: Unstained Protein Standard, Broad Range (10-200 kDa) (NEB #P7717).

13. Immunodetection kit: ELISA Kit, BioVision, Cat # E4877.

14. Culture media and other reagents: LB (Luria-Bertini)-agar and LB broth, and SOC (Super Optimal broth with Catabolite repression medium) bacterial cell culture media; Isopropyl-β-D-1-thiogalactopyranoside (IPTG), from Thermo Fisher Scientific or NEB.

15. Enzymes: BamHI (NEB, Cat # R3136S); Enterokinase (NEB, Cat #P8070); Enterokinase removal kit (Sigma-Aldrich, Cat # PRKE); Lysozyme solution (Millipore-Sigma, Cat #L3790); Proteinase K, 800 u/ml (NEB, Cat # P8107S); T4 DNA Ligase (NEB, M0202).

16. Tris-EDTA (TE) Buffer (20/x, pH 9.2): Composed of 0.2M Tris-HCl and 20mM EDTA, pH 7.5 at 25°C, Promega, Cat # A2651; pH to be adjusted to 9.2 by adding 0.1 M NaOH.

17. Tris-buffer containing 20 mM Tris, 200 mM NaCl, pH 8.0, Sigma-Aldrich, Cat # 93283.

18. Protein stain: InstantBlue stain, 1 L, VWR, Cat # 95045-070. It is a Coomassie blue protein stain, ready to use for SDS-PAGE.

Below, we provide a unique study protocol for the production of SARS-CoV-2 RBD protein antigen using recombinant DNA technology. The RBD protein thus produced can be used as a COVID-19 vaccine after formulation, and evaluation for clinical efficacy and safety.

**Figure 1. Oligo DNA Insert.** A 17 bp (base pair) double-stranded DNA with 4 bases long single-stranded (ss) overhangs at the 5' ends. Forward strand, 5' GATCGGATGATGATGATAAAC 3' and the reverse strand, 3' CCTACTACTACTACTATTTGCTAG 5', each carrying a ss (single stranded) 5' GATC overhang.
Replication and purification of the plasmid carrying RBD-sfGFP cDNAs

A sample of *Escherichia coli* from a stock carrying the Addgene plasmid, Cat #141184, will be grown overnight at 37°C in 10 ml LB-ampicillin broth. The plasmid DNA will be isolated from the cells using PureYield Plasmid Miniprep System I (Promega, Cat #A1222 and a Technical Bulletin #TB374). The purity of the plasmid DNA will be determined by the UV OD$_{260\text{nm}}$/OD$_{280\text{nm}}$ (OD = optical density) absorption ratio. A ratio of ≥1.8 is generally accepted as a value for “pure” DNA. If the ratio is <1.8, we will add 0.1 μl (0.8 units) Proteinase K to the sample, incubate it at room temperature for 15 minutes, add two volumes of ice-cold 95% ethanol, mix well, centrifuge at 10,000 g at 4°C for five minutes, pour off the supernatant, bring the DNA into solution in TE, and determine the purity of the DNA following its OD$_{260\text{nm}}$/OD$_{280\text{nm}}$ absorption ratio. The plasmid DNA concentration will be equal to, OD$_{260\text{nm}}$/the dilution factor $\times$ 50 = μg plasmid DNA/ml.

Plasmid reconstruction with modification

*Linearizing the plasmid.* The plasmid in solution will be digested with *Bam*HI in *Bam*HI buffer at 37°C for 2 hours, heat denatured at 65°C for one minute, and chilled at 4°C for 10 minutes. Then, two volumes of ice-cold 95% ethanol will be added into it, mixed well, chilled for 10 minutes at −20°C, and centrifuged at 10,000 g using a Sorvall SS34 Fixed Angle Rotor, at 4°C for five minutes. The supernatant containing the linearized plasmid will be transferred into a fresh microfuge tube. The linearized plasmid will carry the RBD cDNA at one end and the sfGFP cDNA at the other end, as shown in Figure 2.

*Ligating covalently the linker oligo DNA (FP-DNA) into the linearized plasmid.* We will add 1 μl of the linker oligo to the linearized plasmid in the microfuge tube. Then, we will add 1 μl T4 DNA (NEB, M0202) ligase containing 400 – 500 units in 1 x T4 DNA ligase buffer, incubate at 16°C for 2 hours, deactivate the ligase by heating at 65°C for 10 minutes, chill at 4°C for five minutes, add two volumes of ice-cold 95% ethanol, mix well, and centrifuge at 10,000 g at 4°C for five minutes. The supernatant containing the ligated plasmid will be transferred into a fresh microfuge tube and the purity of the plasmid DNA will be determined following UV absorption ratio at OD$_{260\text{nm}}$/OD$_{280\text{nm}}$ reaching ≥ 1.8. If the ratio is <1.8, add 0.1 μl (0.8 units) Proteinase K at room temperature for 15 minutes, add two volumes of ice-cold 95% ethanol, mix well, centrifuge at 10,000 g at 4°C for five minutes, pour off the supernatant, bring the DNA into solution in TE, and then again determine the purity of the DNA following its OD$_{260\text{nm}}$/OD$_{280\text{nm}}$ absorption ratio. The plasmid DNA concentration will be equal to: OD$_{260\text{nm}}$/the dilution factor $\times$ 50 = μg plasmid DNA/ml.

The incorporation of the oligo DNA (Figure 1), into the plasmid (Addgene, 141184) will be accomplished following a standard protocol for plasmid linearization, insertion of the oligo DNA, and ligation using T4 DNA ligase. The insertion of the oligo DNA will take place at the *Bam*HI site located terminally at the RBD cDNA, and 21 bp upstream to the sfGFP cDNA as in Figure 3.

Figure 2. Addgene plasmid, 141184, linearization using *Bam*HI restriction enzyme.
The oligo DNA insert will code for a heptapeptide, DDDDKRS, fused in-between the RBD-sfGFP fusion protein, coding for a novel RBD-FP-sfGFP fusion protein (Figure 4).

The original plasmid carries RBD cDNA-sfGFP cDNA is shown in Figure 5A, and the ligated plasmid reconstruct will carry the RBD cDNA-FP DNA-sfGFP cDNA linked in order within one ORF, as shown in Figure 5B.

**Figure 3.** An excerpt from Addgene, 141184, showing the BamHI site located terminally at the RBD cDNA followed by the sfGFP cDNA.

| Protein sequence | Original translated | Recombined translated |
|------------------|---------------------|-----------------------|
|                  | GSGSSGSGGSGEELFTGVV | GSDDDDDRSGSGSSGSGEELFTGVV |

**Figure 4.** A comparison of the “original translated” RBD-sfGFP fusion protein with the “recombined translated” RBD-FP (underlined) -sfGFP fusion protein. The fusion peptide, DDDDKRS (underlined), is shown in the “Recombined translated”, RBD-FP (underlined) -sfGFP fusion protein produced by the reconstructed plasmid.

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**Transformation and growing Escherichia coli, C2566H, with the reconstructed plasmid**

The *Escherichia coli*, C2566H, carrying T7 RNA polymerase gene will be used as a host. The host, transformed with the reconstructed plasmid, will express the ORF producing RBD-FP-sfGFP fusion protein.

**Transformation.** We will thaw a sample of competent *Escherichia coli*, C2566H, cells for 10 minutes in ice at 4°C, mix well gently, pipette out 50 μl of the cells in suspension into an ice-cold fresh 1.5 ml microfuge tube in ice, add 100 pg reconstructed plasmid DNA, mix well gently without vortexing, chill the microfuge tube in ice for 30 minutes, place the

**Figure 5.** (A) The original Addgene plasmid, 141184, carrying pcDNA3-SARS-CoV-2-S-RBD-sfGFP, 6736 bp (base pair). (B) The reconstructed plasmid carrying RBD cDNA-FP oligo DNA-sfGFP cDNA, 6753 bp. The fusion peptide (FP) carrying a 17 bp long ds oligo, with 4 bases long ss 5' GATC BamHI linker DNA sequence at either ends.
microfuge tube into a Styrofoam holder, transfer the holder into a 42°C water bath, wait for 15 seconds, take out the microfuge tube, and chill it in ice for five minutes without mixing.

Then, we will add 950 μl SOC, maintained at room temperature, into the microfuge tube containing the transformed cells, incubate at 37°C for 60 minutes, and shake vigorously while under incubation using a rotator. This will complete the transformation process.

**Plating the transformed Escherichia coli.** We will warm up prepared petri dishes/plates (100 mm × 15 mm) containing 20 ml LB-Amp-Agar (1.5%) at room temperature for 15 minutes. Using a sterile pipette, we will add aseptically 250 μl SOC medium containing the transformed E. coli cells into the petri dish and add 80 μl filter sterilized 100 mM IPTG solution into the dish over the SOC medium. Then, we will spread the SOC medium with the transformed cells evenly over the agar throughout the dish/plate with a sterilized spreader and incubate it overnight at 37°C. The E. coli cells transformed with the reconstructed plasmid will produce green fluorescent cfu, when observed using a 300 nm UV lamp.

**Growing cells from green fluorescent cfu to produce the RBD-FP-sfGFP fusion protein.** We will select a 15 ml (16 mm × 125 mm) microbial culture tube containing 5 ml sterile LB-Amp broth, warm it up at 37°C for five minutes and add 16 μl filter sterilized 100 mM IPTG solution into the tube reaching a concentration of 0.4 mM IPTG. Then, we will select a green fluorescent cfu from the culture plate, add the cells into the tube, mix gently and grow the cells overnight at 37°C.

Transcription of the SARS-CoV-2 RBD-FP-sfGFP containing ORF from the recombined plasmid will be stimulated by the T7 promoter and regulated by the T7 RNA polymerase produced by E. coli, C2566H, that carries a genomic copy of the T7 RNA polymerase gene inducible by IPTG. Hence, the transformed E. coli, C2566H, will produce SARS-CoV-2 RBD-FP-sfGFP fusion protein from the reconstructed plasmid.

Similar to the RBD-sfGFP fusion protein, the RBD-FP-sfGFP fusion protein will retain its green fluorescence at UV 300 nm. This is supported by the fact that the RBD-sfGFP fusion protein produced by the Addgene plasmid, 141184, expresses green fluorescence in E. coli carrying T7 RNA polymerase.

A comparison of the recombinant RBD protein with the Addgene, 141184, RBD protein sequences are presented in the results section.

**Extraction of RBD-FP-sfGFP fusion protein**

Pipette out 1.5 ml cell culture from the tube into a 1.5 ml microfuge tube, centrifuge it at 5,000 g for 10 minutes at 4°C, pour off the supernatant, add 250 μl 1X TE buffer into the cell pellet, gently resuspend the cells in the pellet by pipetting up and down the buffer along with the cell pellet, add 50 μl lysozyme solution (Millipore-Sigma, Cat # L3790) reaching a final concentration of 0.2 mg lysozyme/ml, mix well, and incubate in a shaker at room temperature for 15 minutes. This will break open the cells and release the RBD-FP-sfGFP fusion protein into the buffer, centrifuge the solution at 2000 g for 10 minutes at 4°C, collect the supernatant containing the RBD-FP-sfGFP fusion protein, and observe the solution using UV 300 nm. The presence of RBD-FP-sfGFP fusion protein in solution will be indicated by a fluorescent green color in UV 300 nm. Hold the tube containing the extract at 4°C for further use.

**Separation of RBD protein from RBD-FP-sfGFP fusion protein**

The RBD-FP-sfGFP fusion protein will be separated by HIC (hydrophobic interaction chromatography) using a BIO-RAD protein extraction kit (Cat #166-0005EDU), https://www.bio-rad.com/webroot/web/pdf/lse/literature/4006099.pdf, using the following steps:

1. Using a pair of scissors, cut off the bottom of the hydrophobic resin prefilled HIC column.
2. Place the column into a 5 ml test tube in a stable rack.
3. Remove the top cap of the column and allow the buffer to drain out up to the top level of the resin in the column.
4. Add 2 ml 2 M (NH₄)₂SO₄ solution on top of the resin and allow it to drain out up to the top level of the resin.
5. Put back the cap on top of the column to stop draining.
6. Side-by-side, add 250 μl RBD-FP-sfGFP fusion protein extract into another microfuge tube.
7. Add 250 μl 4 M (NH₄)₂SO₄ solution and mix well.

8. Transfer the solution into the column (Figure 6, Step 1).

9. Allow the buffer to drain out up to the top level of the resin in the column into a collection tube; discard the fluid from the collection tube.

10. Add 250 μl 1.3 M (NH₄)₂SO₄ solution into the column and drain out the fluid up to the top level of the resin into the collection tube; discard the fluid (Figure 6, Step 2).

The discards will contain all unwanted bacterial protein contaminants while the RBD-FP-sfGFP fusion protein will remain attached to the resin beads in the column.

11. In a separate microfuge tube, add 2 μl enterokinase (NEB, Cat #P8070) in 8 μl of its reaction buffer, 20 mM Tris-HCl, 50 mM NaCl, 2 mM CaCl₂, pH 8.0, and mix well. Add 2 μl buffered enterokinase (NEB Cat #P8070) into the column (Figure 6, Step 3).

12. Incubate the column at 25°C for two hours. This will degrade the FP and thus separate the RBD from the sfGFP from the fusion protein.

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Figure 6. Hydrophobic Interaction Chromatography (HIC) to elute the RBD from the sfGFP. Step 1. Protein extract from *Escherichia coli*, C2566H, cells transformed with the recombined plasmid and treated with the equilibrium buffer (4 M (NH₄)₂SO₄) is being pipetted into the hydrophobic ion exchange column, already equilibrated with a high salt buffer (2 M (NH₄)₂SO₄). This will allow RBD-FP-sfGFP fusion protein to bind tightly into the resins in the column. Step 2. A low salt buffer (1.3 M (NH₄)₂SO₄) is added to wash away the unwanted cell extract, while keeping the RBD-FP-sfGFP fusion protein bound to the column resins. Step 3. Enterokinase is being added to digest the FP to separate the RBD from the sfGFP proteins. Step 4. A low salt buffer (1.3 M (NH₄)₂SO₄) is added to elute the RBD proteins, keeping the sfGFP protein bound to the resins in the column.
13. Add 750 μl 1.3 M (NH₄)₂SO₄ solution into the column and elute the RBD protein into a UV transparent collection tube and save (Figure 6, Step 4). The sfGFP will remain bound to the resin.

14. Take out the collection tube with the eluate, view the eluate using a UV 300 nm lamp. Pure eluate will not emit green fluorescence. The top of the resin column holding the sfGFP will emit green fluorescence at UV 300 nm.

Separation of the RBD protein from the RBD-FP-sfGFP fusion protein after the enterokinase (NEB Cat #P8070) digestion of the FP will be completed by HIC. This is a routine technique to isolate non-hydrophobic proteins from hydrophobic proteins. In this digestion, the enterokinase will digest the DDDDKRS fusion peptide in between DDDDK and RS, leaving DDDDK fused with the RBD protein at its C-terminal. The remaining RS dipeptide will remain fused with the GGSGSG, forming RSGGSGSG. This residue will remain fused with the sfGFP at its N-terminal. Since the sfGFP present in the RBD-FP-sfGFP fusion protein is strongly hydrophobic, the RBD-FP-sfGFP fusion protein will bind to the resin in the HIC column. Once the enterokinase completes the digestion, the RBD protein will become separated from the sfGFP. The separated RBD protein will be eluted by 1.3 M (NH₄)₂SO₄ buffer from the hydrophobic resins in the column, leaving the sfGFP protein bound to the resins (Figure 6).

Removal of (NH₄)₂SO₄ from RBD protein eluate by dialysis

The RBD protein eluate from the hydrophobic column will contain approximately 1.3 M (NH₄)₂SO₄ and stray molecules of enterokinase. The enterokinase molecules will be removed by using an enterokinase removal kit (Sigma-Aldrich, Cat # PRKE) followed by the removal of (NH₄)₂SO₄ as follows:

1. Add 50 μl anti-enterokinase–agarose conjugate pellet (following Sigma-Aldrich, PRKE protocol) to the RBD eluate, mix gently; centrifuge at 1000 g for 2 minutes at 4°C; collect the supernatant containing RBD and <1.3 M (NH₄)₂SO₄. Add v/v Tris-buffer containing 20 mM Tris, 200 mM NaCl, pH 8.0 to the RBD eluate.

2. Take a SnakeSkin dialysis tube prehydrated with the above buffer and close one of its ends with a clip.

3. Place the RBD eluate into the SnakeSkin dialysis tube and close the other end with another clip.61,62

4. Place the dialysis tube in the Tris-buffer at 4°C in a dish for two hours.

5. Transfer the dialysis tube into fresh Tris-buffer two more times and run the dialysis for two hours each.

6. Transfer the dialyzed eluate into a fresh sterile proteinase-free sterile tube and store at 4°C for further tests.

Removal of (NH₄)₂SO₄ from a protein extract using dialysis is a routine procedure.63–65 Berndt, et al.,66 ligated cDNAs of an RBD protein of SARS-CoV-2 with a cDNA coding for a 5′mClover green fluorescent protein gene, which was expressed by a transformed Chlamydomonas reinhardtii. The RBD-mClover fusion protein, expressed by C. reinhardtii, was separated by HIC.66 The eluate RBD molecules retained its full immunogenic activity, as observed by its ability to bind to ACE-2 receptor proteins.66 This supports that (NH₄)₂SO₄ does not affect the structural integrity of the RBD proteins. Furthermore, (NH₄)₂SO₄ is known to stabilize the 3D structure of proteins.67 Park, et al.,68 isolated recombinant colorectal cancer vaccine protein, GA733-FcK, using 50% (5.05M) (NH₄)₂SO₄ in its active form. Hence, we predict 1.3 M (NH₄)₂SO₄ solution used in this protocol will have no impact on the 3D structure of the RBD protein.

Tan, et al.,65 dialyzed RBD-SpyVLP eluate for 16 hours in Tris-buffered saline (TBS). In our protocol, we propose to use a Tris-buffer (20 mM Tris, 200 mM NaCl, pH 8.0) to the RBD eluate, v/v, and put it into a SnakeSkin dialysis tube (Thermo Fisher Scientific, Cat # 88243) with a 10 kDa cut-off, following Tai, et al.62 Since the molecular weight of RBD protein monomer is 25 kDa, the porosity of the SnakeSkin dialysis tube will save the RBD protein inside the tube while allowing (NH₄)₂SO₄ to leach out. Upon completion of the dialysis, storing RBD protein in Tris-buffer, pH 8.0, at 4°C will save the RBD protein from bacterial and enzymatic degradation.

Determination of RBD proteins eluate by SDS-PAGE, Tee, et al.69

We will be using SDS-PAGE NuPAGE 4-12% gradient gel for the electrophoresis as follows:

1. Place the NuPAGE into a Mini Gel Tank, Thermo Fisher Scientific, Cat # A25977.
2. Fill in the chamber with 1 × NuPAGE MES SDS Running Buffer up to the designated level.

3. Put 5 μl Protein Standard in 1 × solution buffer into a well of SDS-PAGE gel.

4. Put 5 μl dialyzed RBD protein sample in 1 × sample buffer into a parallel well in the SDS-PAGE gel.

5. Run the electrophoresis using an Invitrogen PowerEase Touch 600W Power Supply, Thermo Fisher Scientific, Cat # PS0601, at 200 V and 30 – 40 mAmp for 40 minutes.

6. Remove the NuPAGE gel slab and stain it with InstantBlue, following the supplier’s protocol.

7. Measure in cm, using a ruler, the distances traveled by each of the Protein Standard bands as well as by the RBD protein bands and record them in a notebook to be used next for plotting and measurement.

8. Plot a protein standard graph in a semi-log paper using the distance, in cm, traveled by each standard protein band on the Y (log) axis and their respective molecular sizes on the X (linear) axis.

9. Determine the molecular sizes of the RBD protein bands using the protein standard graph, prepared above. The expected sizes of the RBD proteins will be 221 aa (24.3 kDa) for monomers, 442 aa (48.6 kDa) for dimers and 663 aa (72.9 kDa) for trimers.

The SDS-PAGE procedure separates proteins primarily by mass, since SDS denatures and binds to proteins to make them negatively charged. Hence, in an electric field, the SDS-bound RBD proteins will migrate through the gel toward the positively charged electrode based on its mass. A protein molecule of a lower mass size will have higher mobility in comparison to a protein molecule of higher mass size.

This procedure will help us determine the molecular sizes of the RBD proteins in the eluate and compare them with known values.

Immunoreactivity of the dialyzed RBD sample

We will test 1 μl sample of the dialyzed RBD protein for immunoactivity using a SARS-CoV-2 RBD Elisa kit, BioVision, Cat # E4877, for a qualitative determination of the RBD protein, following the supplier’s protocol.

This procedure follows the ELISA principle. It contains SARS-CoV-2 RBD protein samples in solutions, detection solutions, pre-coated RBD antibodies, and all necessary ingredients. This technique is known to be highly sensitive, detecting <10 pg RBD/ml.

In this protocol, a standard RBD concentration graph will be plotted using OD450nm of RBD samples of known concentrations. This will be accomplished by a tagged RBD-antibody-RBD-antigen binding, followed by a chromogenic reaction. An RBD sample from the dialyzed eluate will be tested using the same RBD-antibody-RBD-antigen binding followed by the chromogenic reaction and OD450nm measurement. Based on the OD450nm of the dialyzed RBD protein sample, its concentration will be determined from the standard graph. This procedure will help vaccine development in two ways: it will detect the presence of RBD protein in the HIC eluate that is purified by dialysis, and it will measure the concentration of RBD protein antigen present in the dialyzed sample.

The modification of the RBD protein will not affect its immunogenic ability as demonstrated by Keng, et al. Keng, et al. in an antibody neutralization experiment demonstrated that fragmented spike protein DNA containing variable lengths of RBD proteins, expressed by transformed E. coli, retained the immunogenic ability against SARS-CoV-2 virus. Furthermore, this modification will not affect the internally located RBM, the epitope of the RBD protein. The RBD antigen thus produced can be applied as a safer vaccine after formulation for trials as described by Batty et al.

Immune simulation of the RBD protein

The purified RBD will be tested for immune simulations in 7 weeks old female C57BL mice and tested for neutralizing antibodies produced against SARS-CoV-2 following a protocol presented by Seephetdee et al. (2021).

Mice will be administered a prime-boost immunization intramuscularly (IM), three weeks apart. For antigen formulation, SARS-CoV-2 RBD protein (1 μg for the first dose and 5 μg for the booster dose) will be mixed with 100 μg of aluminum
hydroxide (Invivogen, Cat # vac-alu-250). Serum will be collected for analysis on study days 14, 35, and 56 after the initial immunization.

**Assay for microneutralization assay**

Following a standard protocol as described by Seephetdee *et al.* (2021), the sera will be heat-inactivated 56 °C for 30 minutes, diluted serially starting with 1:10, mixed with equal volumes of 100 TCID50 of SARS-CoV-2, and incubated at 37 °C for 1 hr. A sample of 100 μl of the mixture at each dilution will added in duplicate to Vero E6 cell monolayers in a 96-well microtiter plate in addition to control plates. The last two columns are set as virus control, cell control, and virus back-titration. The plates will be incubated at 37 °C in 5% CO2 in a humidified incubator for two days, the medium was discarded, and the cell monolayer will be fixed with methanol:acetone (1:1) for 20 minutes on ice. Viral protein in the virus-infected cells will be detected by ELISA assay using 1:5000 of SARS-CoV/SARS-CoV-2 Nucleocapsid monoclonal antibody (Sino Biological, Cat#40143-R001) and 1:2000 HRP-conjugated goat anti-rabbit polyclonal antibody (Dako, Denmark A/S, Cat#P0448) followed by TMB substrate (KPL, Cat#5120-0075) and stopped by adding of 1N HCl. Optical density (OD) at 450 and 620 nm will be measured by a microplate reader.

The virus neutralization endpoint titer of each serum will be calculated using the following equation:

\[ X = \left( \frac{\text{average A450 of virus control wells}}{\text{C0} \times \text{average A450 of cell control wells}} \right) + \text{average A450 of cell control wells} \]

The OD values less than \( X \) will be considered positive for neutralization activity. The serum that tests positive at 1:10 dilution will be reported as the NT titer of 20.

Each sample will be carried out in duplicate. All activities with live viruses will be carried out in a certified biosafety level 3 facility.

**Allergen testing**

This protocol will not complete the allergen testing. Once the RBD protein meets the immunity generation test and published, agencies interested in using the protocol for producing a RBD vaccine with clinical formulations as a vaccine has the complete the allergen testing. After vaccine formulation following a standard protocol, the vaccine must meet the allergen tests before application to patients following US FDA recommended vaccine evaluation and management.

**RBD quantitation using spectrophotometry**

We will take a 10 μl sample of the dialyzed RBD protein and determine its concentration by measuring OD at UV 280 nm using a UV-Vis spectrophotometer following Arbeitman, *et al.* The total amount of RBD protein in the dialyzed sample will be equal to \( M = (OD \div L) \times D \). (Where, \( M \) = amount of RBD protein present in mg per ml in the original sample; OD = Optical density at 280 nm; \( L \) = the length of cuvette light path in cm; \( D \) = dilution factor.)

**Evaluation of the modified RBD protein β sheet**

The RBD protein sequences, before (Figure 7) and after modification (Figure 8) will be tested for their alignments with known 2019-nCoV RBD protein sequence available through a computerized program, UniProt Protein Blast (UniProtKB: P00750): https://www.uniprot.org/blast/.

The details of the alignment are presented in the results section following Figure 9 and Figure 10, below:

Alignment of the RBD protein encoded by Addgene, 141184, with the RBD protein generated by 2019-nCoV, SARS-CoV-2 virus, for comparison, using UniProtKB: P00750.

Alignment of modified RBD protein encoded by the recombinant plasmid with the RBD protein generated by 2019-nCoV, SARS-CoV-2 virus, for comparison, using UniProtKB: P00750.

Furthermore, the 3D structure of the RBD protein will be produced using a commonly used program, Phyre2. The above sequences (Figures 7 and 8) of the RBD protein encoded by Addgene, 141184, with the RBD protein generated by 2019-nCoV, SARS-CoV-2 virus, will be entered in Phyre2 to generate their 3D protein structure for comparison. This step will be completed once the protocol is ready for execution.
Storage of RBD protein for future use

Add a measured volume of glycerol to the dialyzed RBD solution, reaching a final concentration of 10% (v/v) glycerol in the solution and mix well. Make aliquots of the RBD solution in separate pre-labeled micro-vials, freeze them quickly in liquid nitrogen, and store them in a –80°C freezer following Tee, et al.69

The aliquots of RBD can be reused for vaccine formulations as needed. Edwards, et al.,80 have observed under electron microscopy that RBD molecules stored at 22°C or 37°C in a buffer (2 mM Tris, pH8.0, 200 mM NaCl, 0.02% sodium azide) for one week displayed well-ordered trimeric structure.80 Hence, we anticipate that the quick-frozen RBD monomers, isolated in the protocol, will form dimers and trimers after thawing at 22°C or 37°C, as a natural phenomenon.

Figure 7. The amino acid sequence of the RBD protein (221 aa long) produced by the Addgene plasmid, 141184.

Figure 8. The amino acid sequence of the RBD protein (215 aa long) fused with the FP (DDDDK) (5 aa) at the C-terminal encoded by the recombined plasmid.

Storage of RBD protein for future use
Add a measured volume of glycerol to the dialyzed RBD solution, reaching a final concentration of 10% (v/v) glycerol in the solution and mix well. Make aliquots of the RBD solution in separate pre-labeled micro-vials, freeze them quickly in liquid nitrogen, and store them in a –80°C freezer following Tee, et al.69

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Since both the RBD dimers and trimers have higher ACE-2 binding activity than its monomers, the dimerization and trimerization will increase vaccination efficacy of the isolated RBD proteins.81,82

Projected results
Comparison of the RBD proteins before and after the recombination
The original plasmid, Addgene, 141184, carries a 639 bp long RBD cDNA linked with a 714 bp long sfGFP cDNA (Figure 5A). The RBD cDNA will code for a 221 amino acid long RBD protein as shown in Figure 7.

The modified RBD protein encoded by the recombined plasmid is shown in Figure 8. As mentioned earlier, the modified RBD protein will have a five amino acid long FP (DDDDK) replacing six amino acids (GGSGSG) at its C-terminal (Figure 8).

The modified RBD protein β sheet remains unchanged
The RBD protein β sheet, critical for its 3D structure, will also remain fully stable since the modification of the RBD protein at the C-terminal has no effect on the pairing of the sulfur containing amino acid Cys336–Cys361, Cys379–Cys432, and Cys391–Cys525.36 We proved this by aligning the RBD protein sequences, before and after modification, with the RBD sequence produced by the 2019-nCoV genome using a computerized program, UniProt Protein Blast (UniProtKB: P00750), https://www.uniprot.org/blast/as), as shown in Figure 9 and Figure 10.

As shown in Figure 9, the RBD protein encoded by the RBD cDNA, Addgene, 141183, matches perfectly with the respective RBD protein sequence produced by 2019-nCoV strain of the SARS-CoV-2 coronavirus. Three pairs of Cys-Cys residues, Cys336-Cys361, Cys379-Cys432 and Cys391-Cys525, encoded by the RBD cDNA, responsible for the core RBD protein β sheet formation, match perfectly with the respective RBD protein sequence produced by the 2019-nCoV strain. The total amino acid length of the RBD protein encoded by the RBD cDNA, Addgene, 141183, is 221, which has 99.0% identity match and 99.5% positivity match with the RBD protein sequence produced by the 2019-nCoV (Figure 9).

Similarly, as shown in Figure 10, the RBD protein encoded by the modified RBD cDNA matches perfectly with the respective RBD protein sequence generated by 2019-nCoV strain of the SARS-CoV-2 coronavirus. Three pairs of Cys-Cys residues, Cys336-Cys361, Cys379-Cys432 and Cys391-Cys525, encoded by the modified RBD cDNA, that form the core RBD protein β sheet, match perfectly with the respective RBD protein sequence produced by the 2019-nCoV strain. The total amino acid length of RBD protein encoded by the modified RBD cDNA is 220, which has 98.5% identity match and 99.5% positivity match with the RBD protein sequence produced by the 2019-nCoV.

Figure 9. Alignment of the RBD protein encoded by Addgene, 141184, with the RBD protein generated by 2019-nCoV, SARS-CoV-2 virus, for comparison, using UniProtKB: P00750.
Assay for microneutralization assay
We expect the O.D. values will be considered positive for neutralization activity. If negative, the immune simulation and microneutralization assay will be repeated to confirm the success of the protocol.

Discussion
The protocol we designed to produce the SARS-CoV-2 RBD antigen, which is responsible for recognition and attachment to the ACE-2 receptors in human cells, is a novel one. Since the RBD protein is not linked to the S2 domain protein, the RBD protein, after binding to ACE-2, will not allow any free-floating virions to enter any human cell. Additionally, the RBD protein alone was found to have effective immunological integration with eight types of ACE-2 variants in human cells. These variants and mostly found in European non-Finnish and African populations except one of the variants in the Latino and another one in Finish populations and absent in Ashkenazi Jewish, East and South Asian populations.

This observation supports that the RBD protein produced by this protocol will remain effective in multiple human recipients, despite their ACE-2 variations. Other authors reported that a single dose of the RBD antigen vaccine delivered to mice has produced a high titer of antibodies effective against both mutant and non-mutant variants of the SARS-CoV-2 virus.

Before vaccine formulation using the RBD protein generated following this protocol will be compared with other variants using UniProtKB: P00750 or any other appropriate program.

Furthermore, the 3D structure of the RBD protein sequences as in (Figures 7 and 8) 2019-nCoV, SARS-CoV-2 virus, analyzed using Phyre are expected to match at the epitope as we observed in our preliminary work, yet to be published.

Although there exist variations in the spike protein, the variations in the RBD region are limited.

In structure-based analyses, the VH3-53/3-66 class-derived public neutralization antibodies (NAbs) interact with naturally occurring SARS-CoV-2 RBD variants. They include B.1.1.7 (UK), B.1.351 (South Africa-(SA)), and P.1 and P.2 in Brazil in addition to others. The most abundant RBD variants are K417N (or T)E484K/N501Y B.1.351, P.1 and P.2. Furthermore, the VH3-53/3-66 class-derived NAbs were commonly used to assess in COVID-19 patients across the globe (Cao, et al., 2020, and Yuan, et al., 2020). Hence, the RBD protein vaccine will be effective against more SARS-CoV-2 variants.
An RBD protein sample, similar to the RBD protein produced by this protocol, was found to induce a potent and functional antibody production in mice, rabbits, and non-human primates (*Macaca mulatta*) within seven to 14 days following single dose administrations. It was also found that the SARS-CoV-2 RBD protein is a highly effective antigen to work as a vaccine by Dai and Gao. Both RBD-dimer and RBD-trimer proteins have been found to increase the immunogenicity of RBD-protein based vaccines effectively, in comparison to RBD protein monomers. RBD proteins, produced by this protocol, will form trimers in a solution as supported by Edwards, et al. All the above reports support that the RBD protein produced by this protocol has the full potential to be an effective vaccine against the SARS-CoV-2 and some of its mutants.

The purified RBD protein molecules become dimers by forming four disulfide bonds between two RBD monomers. Furthermore, the RBD dimers are also much more effective than its monomers in stimulating antibody production (10-100 times immunogenicity) and in neutralizing SARS-CoV and SARS-CoV-2 antibodies. Hence, the RBD protein is a strong vaccine candidate and also potentially effective against multiple coronaviruses: SARS-CoV, MERS-CoV, and SARS-CoV-2. The yield of RBD dimerization from its monomer is high and its production level may be scaled up to meet clinical demands.

Development of a more effective vaccine is the main target of this study protocol. The RBD vaccine antigen, once recognized by the T-cells, will promote secretion of cytokine interferon gamma and interleukin-2 biomarkers, which will stimulate the helper and cytotoxic T cells, B cells, and protective IgG antibodies.

Furthermore, since the glycan shield of the beta-coronavirus (β-CoV) spike glycoprotein acts as a steric block that prevents host immune responses (and thus reduces antibody production), the RBD monomer does not need to be glycosylated, as supported by Henderson, et al.

As the SARS-CoV-2 variants evolve, the RBD DNA is open for modification to produce RBD proteins appropriate and effective vaccine following the same protocol.

**Conclusion**

The RBD protein is an excellent choice for developing a vaccine to prevent COVID-19. The vaccines composed of antigenic mRNA and cDNA are required to go through cellular processes to produce antigens. The RBD protein itself is an antigen and, hence, it will be direct and quick in stimulating the recipients’ immune systems to produce antibody against SARS-CoV-2 virions quickly.

The SARS-CoV-2 RBD protein vaccine can generate a strong immune response and can be used by almost everyone, including people with weakened immune systems and long-term health problems as supported by the United States Department of Health and Human Services. Furthermore, the RBD protein vaccines cannot cause the COVID-19 disease. However, while using the RBD protein vaccine, booster shots may be necessary for immunome compromised recipients to gain sufficient protection against the ongoing SARS-CoV-2 infections.

As needed, the RBD DNA sequence may be modified matching the new and major variants to produce appropriate vaccine following the same protocol. Hence, the RBD protein production and purification protocol that we are proposing is seamless. Hence, the RBD protein thus produced can be used to prepare a vaccine following a standard formulation procedure and clinical trials.

**Dissemination**

The authors are planning to apply for a National Institutes of Health grant in due course to support this project and execute it. After receiving a grant and access to the academic laboratory for the authors is released from the pandemic restrictions, the authors will conduct their research based on this protocol, approved by the IRB, and make their findings available through open access, online, present in conferences and publish in peer reviewed journals.

**Data availability**

No data are associated with this article.

**Acknowledgements**

We would like to acknowledge the contributions made by the editors, Arnab Z. Jahangir, B.Sc. and Dr. Arthee E. Jahangir, Ph.D., and thank them for their editorial support.

A previous version supporting this article can also be found on preprints.org. One of the authors, Arleta H. Marnik,
presented “A Protocol for Producing SARS-CoV-2 RBD Vaccine” at a three-day long 2021 CUNY Research Scholars Summer Symposium, held during July 27-29, 2021. The protocol was highly accepted by the CUNY research scholars present and Mrs. Marnik was one of the winners in the competition receiving a certificate of an award. A list of the winners is available here.

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46. F. Formica, N. Mallory, R. Albert, et al.: Protective immunity against SARS Subunit Vaccine Candidates Based on Spike Protein: Lessons for Coronavirus Vaccination Development. / Immunol Res. 2020; 2020: 7201752. PubMed Abstract | Publisher Full Text | Free Full Text
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PubMed Abstract | Publisher Full Text | Free Full Text

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PubMed Abstract | Publisher Full Text | Free Full Text

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Publisher Full Text
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Current Peer Review Status: ✗ ?

Version 2

Reviewer Report 15 August 2022

https://doi.org/10.5256/f1000research.136287.r146919

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Wen-Hsiang Chen
Baylor College of Medicine, Houston, Texas, USA

In this manuscript version 2, the authors provided additional information to address the reviewers' comments from the first round of review. However, many of them did not seem addressed. Additional issues were also discovered in this revision. Below please find the comments for this round of review. Authors, please address them carefully.

Under the introduction section:
  ○ In the second paragraph, the author addressed that epidemic infected over 179 million people and .... As of Jun 2021. Please change the word “epidemic” to “pandemic”.
  Additionally, please update the statistics to reflect the current status.

Under the “benefits and importance of SARS-CoV-2 Vaccines” section:
  ○ In the first paragraph, the authors stated “Hence, vaccinating a smaller segment of a population against SARS-CoV-2 may favor generation of new variants with new infectivity”. This statement seems to suggest using a smaller fragment will not be preferable due to its tendency of causing mutation? If it is the case, wouldn't using RBD be a worse choice than a full spike as a vaccine antigen? Please justify properly.

  ○ In the fourth paragraph, the authors stated “Although the coronaviruses mutate at a slower rate, some of the new variants of SARS-CoV-2, such as D 614 G.... the RBD protein vaccines are effective against multiple SARS-CoV-2 and SARS-CoV-1 variants”. This paragraph still did not address the previous comment regarding the RBD is a domain within the spike protein that can easily find mutations among different variants, especially when comparing omicron with the original Wuhan variants.

Under the “Advantages for using the RBD protein vaccine” section:
  ○ In the 2nd paragraph, the authors did not address the previous comment: why having 4 cysteine bridges within RBD makes it an effective vaccine. Authors also did not seem to address the previous comment: i.e., why your RBD design is better than the others.

Under the “Purified RBD subunit or S-protein protein vaccine... against SARS-CoV-2” section:
In the 1st paragraph, the authors stated the advantages of using RBD over S protein, this should probably be moved to the previous section (Advantages for using the RBD protein vaccine).

In the 2nd and 3rd paragraphs, the authors provided some information from clinical trials. However, this information did not seem to help strengthen the manuscript. Please consider removing them or making them more concise.

Overall, authors should really streamline the manuscript as it reads very disjointed with unnecessary information.

Under the “RBD epitope phenotype” section:
○ The authors stated that they will use the entire RBD coding sequence to preserve the epitope structure for producing predominant neutralizing antibodies (nAbs). However, as the virus continues to mutate, the antibodies that neutralize one variant may not neutralize the others. Authors may need to revise the statement.

Under the “The RBD protein vaccine” section:
○ The authors stated that their procedures can lower the cost of production, yet only less than 10pg/mL of protein was produced. This result seems to be contradictory to the claim. The authors also stated that RBD is easily transportable, could a reference be provided?

○ Authors stated that the mRNA vaccines facilitate the fusion of the free-floating SARS-CoV-2, yet the references cited only talked about the S2 domain “on” the SARS-CoV-2 virion may facilitate the fusion of virus to cell. The cited manuscripts do not seem to imply that the spike protein expressed by mRNA will facilitate cell entry of the virion. Authors need to be very careful about the statement that were made. Please rewrite this paragraph.

○ As indicated in the previous round of review, it is still unclear why GFP is required for expressing this antigen. Was it because GFP was included to help the protein folding? Was it used for easy purification? Please note that adding a tag and removing it later on by enzymes adds extra steps in the production process, which is not favorable for vaccine production purpose. Please provide a strong justification for making the GFP fusion RBD.

○ The authors add the procedure of “SDS-PAGE” yet did not provide the results. Please run the SDS-PAGE gel and provide it in the results section.

In the section “the modified RBD protein β sheet remains unchanged”:
○ Authors claimed that the 3D structure of the expressed protein remained the same and stable, yet, no experiments, e.g., circular dichroism for secondary structure assessment, CryoEM for overall structure, were provided. Simply aligning the protein sequences unfortunately does not prove the structure is intact or properly folded. Please review those paragraphs and rewrite them accordingly.

Other comments:
1. No functional study was provided. A good way to demonstrate that the produced RBD is properly folded (either fully or partially) is to examine its function, i.e., to evaluate if the RBD still binds to ACE-2. Authors should conduct such evaluation, either by ELISA, BLI, SPR or
any other binding assay.

2. The yield of the purified protein also seemed extremely low (<10 pg/mL out of 1.5mL cell culture). Please address this issue.

3. After addressing the above comments, please revise the discussion and conclusion sessions accordingly.

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Recombinant protein production. Vaccines. Protein biophysical characterization. Protein science

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 10 Sep 2022

**ZMG Sarwar Jahangir,** Kingsborough Community College of The City University of New York, Brooklyn, USA

Wen-Hsiang Chen, Baylor College of Medicine, Houston, Texas, USA

Dear Professor Dr. Chen:

Thank you for reviewing our article meticulously and for your helpful questions, suggestions, and comments. Please find below the specific answers in response to your peer review. In the third revision, we included our answers to your questions to match the format of the article. Please find below the specific answers to all your questions.

**Peer question/comment:**

"In this manuscript version 2, the authors provided additional information to address the reviewers’ comments from the first round of review. However, many of them did not seem addressed. Additional issues were also discovered in this revision. Below please find the comments for this round of review. Authors, please address them carefully.

**Under the introduction section:**

- In the second paragraph, the author addressed that epidemic infected over 179 million people and .... As of Jun 2021. Please change the word “epidemic” to “pandemic”. Additionally, please update the statistics to reflect the current status."

**Answer:** Thank you for your suggestion. We have changed the word Epidemic to Pandemic to all SARS-CoV, MERS and SARS-CoV2 infections with references supporting them as Pandemics. We also updated the number of infections and death to August 30, 2022, following your advice.
Peer question/comment:

"Under the “benefits and importance of SARS-CoV-2 Vaccines” section:

○ In the first (2nd) paragraph, the authors stated “Hence, vaccinating a smaller segment of a population against SARS-CoV-2 may favor the generation of new variants with new infectivity”. This statement seems to suggest using a smaller fragment will not be preferable due to its tendency of causing mutation. If it is the case, wouldn't using RBD be a worse choice than a full spike as a vaccine antigen? Please justify properly."

Answer: We answered all of them in the text with references: There is a vaccination threshold essential for controlling variables (Martcheva, et al., 2007). It was also reported publicly that vaccinated people got infected with new SARS-COV-2 variants. These incidences across the globe support the negative effect of limited vaccination too.

21. Maia Martcheva, Mimmo Iannelli, Xue-Zhi Li. Subthreshold coexistence of strains: the impact of vaccination and mutation. Mathematical Biosciences & Engineering, 2007, 4 (2) : 287-317. doi: 10.3934/mbe.2007.4.287

In low-income countries, mRNA vaccines are not affordable or available as they require specialized transport and storage to keep the vaccine at -80°C (Mabrouk, et al., 2021). In low-income countries, mRNA vaccines are not affordable or available as they require specialized transport and storage to keep the vaccine at -80°C (Mabrouk, et al., 2021).

47. Mabrouk, M. T., Chiem, K., Rujas, E., Huang, W., Jahagirdar, D., Quinn, B., Surendran Nair, M., Nissly, R. H., Cavener, V. S., Boyle, N. R., Sornberger, T. A., Kuchipudi, S. V., Ortega, J., Julien, J., Martinez-Sobrido, L., & Lovell, J. (2021). Lyophilized, thermostable spike or RBD immunogenic liposomes induce protective immunity against SARS-Cov-2 in mice. Science Advances, 7(49). https://doi.org/10.1126/sciadv.abj1476

48. Fliesle, N. 2021. Emerging protein-based COVID-19 vaccines could be game-changing. December 17, 2021 | Basic/Translational, Research Tags: bioengineering, coronavirus, drug development, vaccines. https://answers.childrenshospital.org/protein-based-covid-19-vaccines/

Furthermore, it has been demonstrated, that VH3-53/3-66 class-derived public neutralizing antibodies (NAbs) are effective against multiple SARS-CoV-2 variants (Xu et al, 2021). Hence, the RBD protein vaccine has the potential to be effective against multiple SARS-CoV-2 variants; without claiming it to be effective for all variants.

24. Xu, H., Wang, B., Zhao, T., Liang, Z., Peng, T., Song, X., Wu, J., Wang, Y., & Su, X. (2021). Structure-based analyses of neutralization antibodies interacting with naturally occurring SARS-Cov-2 RBD variants. Cell Research, 31(10), 1126-1129. https://doi.org/10.1038/s41422-021-00554-1

Peer question/comment:

"In the fourth paragraph, the authors stated “Although the coronaviruses mutate at a slower rate, some of the new variants of SARS-CoV-2, such as D 614 G.... the RBD protein vaccines are effective against multiple SARS-CoV-2 and SARS-CoV-1 variants”. This paragraph still did not address the previous comment regarding the RBD as a domain within the spike protein that can easily find mutations among different variants, especially when comparing omicron with the original Wuhan variants."

Answer: Yes, there may be mutants for which the amino acid sequence of the RBD protein vaccine we are proposing may not be equally effective. However, the protocol will be effective. Only the RBD DNA sequence will require modifications to match the variant. We did not claim that the sequence of the RBD protein vaccine we included in our protocol will
eliminate all virus variants. We only said about the advantages of the RBD protein vaccine over mRNA or DNA vaccines. The RBD DNA sequence may be modified as needed.

**Peer question/comment:**

"Under the “Advantages for using the RBD protein vaccine” section:

- In the 2nd paragraph, the authors did not address the previous comment: why having 4 cysteine bridges within RBD makes it an effective vaccine. Authors also did not seem to address the previous comment: i.e., why your RBD design is better than the others."

**Answer:** The answer has been moved to a new section that we have included in the 3rd version. It is named “The RBD protein structure”, and we moved all information addressing relevant information there. We referred to the 4 cysteine bridges to confirm the structure matched the RBD protein region of the spike protein. The RBD protein vaccine is shorter, it is only 223 amino acid (aa) in length while the spike protein is 1273 aa in length. Hence, it will be simpler to produce RBD protein and isolate it while maintaining its structure. We have included this information in the text.

**Peer question/comment:**

"Under the “Purified RBD subunit or S-protein protein vaccine... against SARS-CoV-2” section:

- In the 1st paragraph, the authors stated the advantages of using RBD over S protein, this should probably be moved to the previous section (Advantages for using the RBD protein vaccine).
- Answer: In the 3rd version, we have renamed the above section, “Advantages for using the RBD protein vaccine and its advantages” and we moved the relevant paragraph there. Thank you for the suggestion.

- In the 2nd and 3rd paragraphs, the authors provided some information from clinical trials. However, this information did not seem to help strengthen the manuscript. Please consider removing them or making them more concise."

**Answer:** Another reviewer for this article requested to include evidence supporting the effectiveness of the RBD protein vaccine from clinical trials if any. To satisfy the review, we have included evidence from clinical trials.

**Peer question/comment:**

"Overall, the authors should streamline the manuscript as it reads very disjointed with unnecessary information."

**Answer:** Thank you for the suggestion. We modified the manuscript to rejoin the disjointed information, as you indicated.

**Peer question/comment:**

"Under the “RBD epitope phenotype” section:

- The authors stated that they will use the entire RBD coding sequence to preserve the
epitope structure for producing predominant neutralizing antibodies (nAbs). However, as the virus continues to mutate, the antibodies that neutralize one variant may not neutralize the others. Authors may need to revise the statement.”

**Answer:** We added new relevant information in the text to explain this concern. For example, the VH3-53/3-66 class-derived public neutralizing antibodies (NAbs) are effective against multiple SARS-CoV-2 variants (Xu et al, 2021). Hence, the RBD protein vaccine will be effective against multiple SARS-CoV-2 variants, without claiming it to be effective for all and forever.

24. Xu, H., Wang, B., Zhao, T., Liang, Z., Peng, T., Song, X., Wu, J., Wang, Y., & Su, X. (2021). Structure-based analyses of neutralization antibodies interacting with naturally occurring SARS-CoV-2 RBD variants. *Cell Research*, 31(10), 1126-1129. https://doi.org/10.1038/s41422-021-00554-1

**Peer question/comment:**

"Under the “The RBD protein vaccine” section:
- The authors stated that their procedures can lower the cost of production, yet only less than 10pg/mL of protein was produced. This result seems to be contradictory to the claim. The authors also stated that RBD is easily transportable, could a reference be provided?"

**Answer:** The information refers to the sensitivity of the BioVision-ELISA test. The detects <10 pg RBD protein/ml of the sample. However, the total production of the RBD protein will depend on the input for a production target.

The references for the advantages of transport and others are based on the following references.

46. Hernández-Bernal F, Ricardo-Cobas MC, Martín-Bauta Y, et al.: Safety, tolerability, and immunogenicity of a SARS-Cov-2 recombinant spike RBD protein vaccine: A randomized, double-blind, placebo-controlled, phase 1-2 clinical trial (ABDALA study). *eClinicalMedicine*. 2022;46:101383. 35434578 10.1016/j.eclinm.2022.101383
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48. Fliesle, N. 2021. Emerging protein-based COVID-19 vaccines could be game-changing.

**Peer question/comment:**

"Authors stated that the mRNA vaccines facilitate the fusion of the free-floating SARS-CoV-2, yet the references cited only talked about the S2 domain “on” the SARS-CoV-2 virion may facilitate the fusion of the virus to the cell. The cited manuscripts do not seem to imply that the spike protein expressed by mRNA will facilitate cell entry of the virion. Authors need to be very careful about the statement that were made. Please rewrite this paragraph."

**Answer:** Since the currently applied mRNA vaccine produces the entire spike protein that
contains the S2 domain, it will have the potential to facilitate fusion of the free-floating SARS-CoV-2 into cells carrying ACE-2 receptors and thus causing infections, as supported by the reference cited. The reference described the role of the S2 domain of the spike protein that has the potential for allowing the entrance of free-floating SARS-CoV-2 if present in the recipient. However, there was no published report from recipients of the mRNA vaccine to that effect, as we predicted in the text. References follow:

50Belouzard S, Millet JK, Licitra BN, et al.: Mechanisms of coronavirus cell entry mediated by the viral spike protein. *Viruses*. 2012;4(6):1011–1033. 22816037 10.3390/v4061011

51Jackson CB, Farzan M, Chen B, et al.: Mechanisms of SARS-Cov-2 entry into cells. *Nat Rev Mol Cell Biol.* 2022;23(1):3–20. 34611326 10.1038/s41580-021-00418-x

Peer question/comment:

"As indicated in the previous round of review, it is still unclear why GFP is required for expressing this antigen. Was it because GFP was included to help the protein folding? Was it used for easy purification? Please note that adding a tag and removing it later on by enzymes adds extra steps in the production process, which is not favorable for vaccine production purposes. Please provide a strong justification for making the GFP fusion RBD."

Answer: In our protocol, we used GFP fused with RBD for easy purification as you rightly indicated above. This procedure is used by many others effectively for the isolation and purification of fusion proteins, and we added citations supporting our proposed protocol in the text. At this time, this is our protocol. In the future, if an alternative to GFP with an additional advantage is available, the use of GFP may be changed by all the users of this protocol.

Peer question/comment:

"The authors add the procedure of “SDS-PAGE” yet did not provide the results. Please run the SDS-PAGE gel and provide it in the results section."

Answer: The “SDS-PAGE” is included in the protocol to identify and confirm the production of RBD protein eluate by size measurement only. SDS-PAGE will not be used to purify the RBD protein for any further application.

Peer question/comment:

"In the section “the modified RBD protein b sheet remains unchanged”:

- Authors claimed that the 3D structure of the expressed protein remained the same and stable, yet, no experiments, e.g., circular dichroism for secondary structure assessment, CryoEM for overall structure, were provided. Simply aligning the protein sequences unfortunately does not prove the structure is intact or properly folded. Please review those paragraphs and rewrite them accordingly."

Answer: Following your suggestion, we have included the following step in our protocol with citations. "Furthermore, the 3D structure of the RBD protein will be generated using a commonly used program, Phyre2,78,79 The sequences of the RBD protein encoded by
Addgene, 141184, with the RBD protein generated by 2019-nCoV, SARS-CoV-2 virus, will be entered into Phyre² to generate their 3D protein structures for comparison. This step will be completed once the protocol is ready for execution. In the meantime, we can share informally, that we have some preliminary and incomplete studies supporting the similarity of RBD structures among the above. ”

Peer question/comment:

"Other comments:

1. No functional study was provided. A good way to demonstrate that the produced RBD is properly folded (either fully or partially) is to examine its function, i.e., to evaluate if the RBD still binds to ACE-2. Authors should conduct such evaluation, either by ELISA, BLI, SPR, or any other binding assay."

Answer: In addition to our other answers to your and other reviewer's questions, we also added in our 2nd and 3rd revisions that “we will be testing the RBD protein to generate antibodies in C57BL mice” to test their efficacy.

“The yield of the purified protein also seemed extremely low (<10 pg/mL out of 1.5mL cell culture). Please address this issue."

Answer: This data only refers to the effectiveness of the BioVision-ELISA test procedure measuring RBD even at a very low level, <10 pg/ml, referring to its sensitivity. It detects <10 pg RBD protein/ml of the sample. However, the total production of the RBD protein will depend on the input to reach a specific production target of the RBD protein.

"After addressing the above comments, please revise the discussion and conclusion sessions accordingly."

Answer: Thank you for your suggestions. We followed your advice and modified the text. Again, we have to thank you for going through our manuscripts and making helpful comments and suggestions. We did our best to answer all your questions. We are looking forward if you have any other questions for us to answer.

Competing Interests: None.
The authors are proposing the use of purified RBD protein as a vaccine formulation. The question that arises here is why are you using the whole RBD protein instead of predicting the most antigenic epitopes and constructing a multi epitope vaccine. Does their whole protein usage have any plus point over that?

In the introduction ‘This suggests that the RBD residue is more conserved and hence, the RBD protein vaccines will be effective against multiple SARS-CoV-2 variants.’ – this statement seems rather contradictory. RBD is a hot spot for mutations of the new variants. Kindly provide supporting references on behalf of your statement.

There have been several variants of SARS-CoV-2 with mutations in the RBD of the spike protein. Will this RBD vaccine be effective against all the variants? Furthermore, what is the significance of this study? The constructed RBD vaccine will serve as a subunit vaccine? Won't the RBD of the spike protein bind with ACE-2? It may serve as a competitive inhibitor.

Administration of this whole protein might induce the innate immune response by producing antibodies. The authors have mentioned in the discussion section “Other authors reported that a single dose of the RBD antigen vaccine delivered to mice has produced a high titer of antibodies effective against both mutant and non-mutant variants of the SARS-CoV-2 virus”

However, it is suggested that authors should perform some analysis that may demonstrate the immune simulation and represent the results to validate the probable effect of their designed vaccine.

Did the authors perform any allergen test for the vaccine?

The authors mentioned “Additionally, the RBD protein alone was found to have effective immunological integration with eight types of ACE-2 variants in human cells” in the discussion section. Although, a detailed population coverage analysis is recommended.

The protein was aligned and compared with the RBD protein generated by 2019- nCoV, SARS-CoV-2 virus (UniProtKB: P00750) only. What about the specific variants? Will the vaccine be effective against all possible variants?

It is suggested to write about any subunit or purified protein vaccine that was subjected to trial against SARS-CoV-2 or other viruses and their outcomes in the introduction. The manuscript is well written. I have no comments regarding the proposed wet lab methodology. Although an extensive immunoinformatics based validation of their proposed outcome must be added in the projected results section. They are suggested to perform protein modeling and docking, in addition to immune simulation analysis to demonstrate the probable outcome of the vaccine. Thus I would suggest a major revision including these proposed analyses.

Is the rationale for, and objectives of, the study clearly described?
Partly

Is the study design appropriate for the research question?
Partly

Are sufficient details of the methods provided to allow replication by others?
Partly

Are the datasets clearly presented in a useable and accessible format?
Not applicable

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Genetics, Immunoinformatics, Biochemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
The introduction section is extremely lengthy with disjointed information. Please consider removing unnecessary paragraphs and making them more concise.

Under “the benefits and importance of SARS-CoV-2 vaccines” section, the fourth paragraph: Authors stated that RBD residue is more conserved, however, based on the mutation maps of the current SARS-CoV-2 variants of concern (VoC; https://asm.org/Articles/2021/December/How-Ominous-is-the-Omicron-Variant-B-1-1-529), one can find that all the VoCs contain mutations within the RBD region, the most recent VoC Omicron even has 15 mutations. Thus, stating RBD is more conserved seems incorrect. Authors, please address accordingly.

Under “Advantages for using the RBD protein vaccine”, the 2nd paragraph: Authors listed several properties of RBD proteins, and stated that all these properties support the RBD protein as an effective antigen; one being the 4 cysteine bridges that RBD possesses. However, it is hard to correlate the effectiveness of this antigen with the Cys bridges. Could the authors clarify? Otherwise, please consider removing such info. Additionally, using RBD as the vaccine antigen against COVID-19 is not a new concept, and one can easily find articles describing similar ideas (e.g., Yang et al., Nature, 2020; Zhang et al., Cell Discov, 2021; Chen et al., BBA-Gen Subj. 2021; Dalvie, PNAS, 2021). This manuscript lacks the description of the advantages, the novelty, or a good rationale over the other published articles. Please consider providing reasons why your RBD design is better than the others to make your manuscript stronger.

Comments on the overall design of the antigen:

The authors designed an antigen that consists of the RBD protein, a linker (FP), and a GFP. However, it is unclear why GFP is required. Also, to remove the GFP, an additional enzyme had to be employed, which adds an extra step to the process and can potentially make it more difficult to purify. Authors should also beware that by using E. coli expression system, a complex protein such as RBD (containing many Cys bridges), may not have a correctly folded structure, as it can easily form an inclusion body instead of a soluble protein, which may explain why the overall yield was so low.

In the whole manuscript, no SDS-PAGE image was presented to demonstrate the expression of the protein actually occurred. The yield of the purified protein also seemed extremely low (<10 pg/mL out of 1.5mL cell culture) when quantified by BioVision ELISA kit. Authors should reconsider the production process, as this process did not seem efficient enough for protein production.

The authors used several in silico analyses to evaluate the structure of the expressed protein. But when the protein is expressed and purified, more sophisticated analyses should be performed. Minimally, a functionality test by evaluating the binding of RBD to ACE-2 should be considered. Additionally, for secondary structure analysis, circular dichroism can be used, and for Cys-bond formation, mass spectrometry should be considered.

Is the rationale for, and objectives of, the study clearly described? 
Partly

Is the study design appropriate for the research question? 
Partly
Are sufficient details of the methods provided to allow replication by others?
Partly

Are the datasets clearly presented in a useable and accessible format?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Recombinant protein production. Vaccines. Protein biophysical characterization. Protein science

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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**Author Response 16 Jan 2022**

**ZMG Sarwar Jahangir**, Kingsborough Community College of The City University of New York, Brooklyn, USA

Professor Wen-Hsiang Chen,
Baylor College of Medicine, Houston, Texas, USA

Dear Professor Chen:
Thank you for reviewing our submission to F1000, “A study protocol to prepare an RBD protein for vaccine against COVID-19”, and for your suggestions. We will address all issues you suggested to enrich the article. However, we will wait to get responses from two additional reviewers before we make the revision.
Yours truly.

ZMG Sarwar Jahangir, Ph.D. Molecular, Cellular and Developmental Biology and Director, AS in Biotechnology Program, Department of Biological Sciences KCC-CUNY and Arleta H. Marnik, M.S. in Biotechnology, GDANSK University of Technology, Poland, Research Scientist, Department of Biological Sciences, KCC-CUNY.

**Competing Interests:** We the authors have no competing interest with the reviewer influencing judgments.
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