

Proof of concept of a virus test that combines DNA and antibodies through the utilization of a cell-free protein expression synthesis

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Abstract

Given the current lack of adequate measures to address newly emerging pandemics, the availability of a rapid and accurate testing method during the early stages can significantly enhance our ability to combat the spread of the virus. In this study, our objective is to explore a new protocol for the development of an *in vitro* test for virus infections, utilizing a combination of nucleic acid and antibody techniques, with the assistance of cell-free protein synthesis (CFPS). In this study, quantitative polymerase chain reaction (qPCR) was employed to amplify 13 virus gene fragments directly from blood samples obtained from a patient with SARS-CoV-2. This was achieved using a set of specifically designed oligonucleotides, which included additional components for transcription, translation, and the incorporation of 6×Hisidine (His6) tags. The linear DNA templates were subsequently transformed into recombinant virus proteins through *E.coli* based CFPS reactions and then immobilized onto pre-treated 96-well plates. Antibodies obtained from blood samples of a patient were utilized to test the immobilized antigens, serving as serological evidence. The findings indicate that among the 13 antigens tested, the N and Orf10 proteins demonstrate the highest potential as candidates for diagnosing SARS-CoV-2 infection in patients. By investigating the advantages of CFPS, this approach offers a robust tool for the identification of suitable antigens in the early stages of developing immunological test products for future pandemics. Additionally, the utilization of a DNA-antibody coupled test facilitated by CFPS presents novel possibilities in the development of more efficacious detection tools for various pathogens and diseases.

1. Introduction

Since December 2019, the global population has been significantly impacted by the coronavirus disease (COVID-19) pandemic, affecting hundreds of millions of individuals worldwide ^[1]. The emergence of the novel coronavirus presents a substantial threat to public health. Although the mortality rate of SARS-CoV-2 in 2019 was lower compared to SARS-CoV in 2002 and Middle East Respiratory Syndrome-CoV in 2012, the transmissibility of SARS-CoV-2 remained significantly high ^[2]. The transmission of SARS-CoV-2 occurs through various routes, including aerosol, contact, fecal, and oral channels, thereby presenting significant challenges for epidemic control. The utilization of rapid and precise diagnostic methods during the initial phases of an outbreak is the most efficient approach to impede the rapid dissemination of epidemics. This strategy also allows for ample time to conduct research on antiviral treatments and vaccines ^[3, 4].

Currently, there exist three predominant types of diagnostic methods for COVID-19: nucleic acid detection, antigen detection, and antibody detection ^[3]. These methods possess distinct advantages and disadvantages on an individual basis ^[4]. Nucleic acid detection plays a crucial role in the early detection of SARS-COV-2 and is widely regarded as the "gold standard" for diagnosis. However, since the SARS-CoV-2 primarily infects the lower respiratory tract, particularly the alveoli, there is a possibility of missed detection when test samples are collected from the nasopharynx, oropharynx, and other upper respiratory tract samples. Additionally, due to the lengthy and complex procedures involved in nucleic acid testing, the issue of "false negatives" is bound to arise ^[5]. Other testing methods are frequently necessary to compensate for the limitations of this method, particularly during the later stages of a pandemic when a significant portion of the population has been vaccinated, resulting in a decreased prevalence of the virus among infected individuals. Antibody detection relies on the immune response triggered by the presence of viral proteins in the bloodstream, serving as a reliable indicator of viral infection. The primary benefit of antibody detection lies in its simplicity and speed, allowing for effective circumvention of limitations associated with alternative detection methods. Unfortunately, the detection of antibodies can yield false positive outcomes due to the phenomenon of cross-reactivity with endogenous disruptors, including rheumatoid factors, xenotropic antibodies, and drug residues ^[6]. The current methods available provide us with tools for virus identification. However, their limitations hinder their ability to yield dependable screening results for epidemiology when utilized in isolation. Combined measurements are frequently utilized to compensate for the limitations of a single method ^[7].

The cell-free protein synthesis (CFPS) system is a rapid and efficient method for producing recombinant proteins. It allows for the simultaneous transcription and translation of DNA in an extracellular environment, facilitating the rapid production and screening of various proteins in a high-throughput manner ^[8, 9]. CFPS systems offer a solution to the constraints imposed by cellular barriers in conventional cell-based protein expression systems. The open nature of the reaction environment facilitates direct utilization of protein expression systems, enabling the synthesis of proteins under controlled chemical conditions. In this manner, the individual design, testing, and optimization of components can be achieved for specific proteins, including toxic proteins and membrane proteins ^[10, 11]. The potential of the CFPS system is being investigated as a novel *in vitro* tool for vaccine discovery and screening functional antibody fragments in various diseases ^[12, 13].

In this study, we have devised a novel approach for the efficient expression of His6-tagged virus antigens. Specifically, we utilized an *E.coli* CFPS system to rapidly express these antigens from qPCR products obtained from a patient. The experimental setup and procedure are illustrated in Fig. 1. We have successfully expressed the 3CL-Mpro, S-RBD, Epro, M, Orf7a, Orf8, N, and Orf10 proteins and detected patient antibodies using an ELISA assay. The study revealed that CFPS possesses the capability to directly generate recombinant virus antigens from blood samples. This finding suggests that CFPS has the potential to facilitate the development of more effective *in vitro* diagnostic (IVD) products by bridging nucleic acid testing and immune diagnostics.

2. Results and Discussion

2.1 Virus gene amplification by qPCR

Primer pairs were specifically designed to amplify 13 randomly selected genes of the SARS-CoV-2 virus. Additionally, His6-tags were incorporated into the corresponding proteins during the downstream CFPS reactions. The gene fragments were amplified using standard dye-based qPCR techniques. The cDNA template was obtained from the blood of a patient diagnosed with COVID-19 at Hwa Mei Hospital, University of Chinese Academy of Sciences (Ningbo No.2 Hospital). The results indicated that all amplified genes successfully migrated to their respective positions in agarose gel electrophoresis, with the exception of the gene encoding the full-length S protein (**Fig. 2**)

The amplified genes were translated into recombinant SARS-CoV-2 antigens with a His6-tag *in vitro*. This was achieved by directly mixing the 13 qPCR products with the CFPS reaction premix. After a 12-hour incubation period, all reactions were subjected to centrifugation, and the resulting supernatants were analyzed using western blotting to determine the expression levels of soluble proteins. The results indicated that out of the 13 proteins examined, 8 (including 3CL-Mpro, S-RBD, Epro, M, Orf7a, Orf8, N and Orf10) were successfully overexpressed, as shown in **Fig.3**. The aforementioned experiments provide the initial evidence that CFPS serves as an effective method for rapidly generating recombinant proteins of RNA viruses directly from blood samples within a timeframe of less than 24 hours. Interestingly, the qPCR kit (TransStart® Top Green qPCR SuperMix kit; Cat. # AQ131-01; Transgen Co., Ltd.) for producing virus DNA templates containing a significant amount of SYBR Green dye. The observation that the direct incorporation of templates into CFPS reactions results in the production of target proteins of the appropriate size indicates that the presence of fluorescent dyes, such as SYBR Green, does not impede DNA transcription and translation in CFPS reactions. The aforementioned experiment significantly expands the utilization of CFPS in high-throughput protein expression, potentially encompassing all types of pathogens.

To investigate the feasibility of conducting a PCR-antibody dual examination linked by CFPS, an ELISA assay was developed to immobilize the His6-tagged antigens and detect antibodies in blood samples obtained from the same patient. The 96-well plate was pre-coated with anti-His6 antibody. 10 µL of the CFPS reaction sample was initially diluted by combining it with 390 µL of PBS (phosphate-buffered saline, pH 7.2) buffer, for each qPCR template. A volume of 250 µL of serum obtained from the patient was diluted by combining it with 9.75 mL of PBS. A volume of 100 µL of CFPS sample was added to each well, and the plate was incubated at 4°C for 10 minutes. The captured antigen was subsequently detected using a commercially available Human IgG (Total) ELISA Kit (Abclonal Co. Ltd.).

Fig. 4 shows among the antigens tested, N (sample 12) and Orf10 (sample 13) elicited significantly higher responses at 450 nm compared to the negative control (NC). The S and N proteins have been extensively utilized in immunological assays for the detection of SARS-CoV-2^[14-16]. However, despite a few publications describing its accessory role in virus invasion^[17-19], there are currently no reports available on the utilization of Orf10 in such assays. On the contrary, it is noteworthy that all truncated S protein elicits significantly lower response comparable to that of the N protein. It appears to be in contradiction with prior research findings. We hypothesize that the disparity in post-translational modifications (PTMs) between the S protein and the N protein may be the reason for this observation. The *E.coli*-based CFPS reaction is unable to generate an S protein that closely resembles the native protein due to the absence of a PTMs pathway^[20,21]. Further investigations utilizing eukaryotic CFPS are imperative for future research endeavors. As a result of the limited accessibility of SARS-Cov-2 positive samples and stringent regulations in China, only one sample was tested in this study. Further experimentation with other pathogens is necessary to explore the robustness of this methodology.

3. Conclusion

We developed a dual PCR-antibody test connected through cell-free protein synthesis (CFPS). Thirteen genes associated with SARS-CoV-2 were amplified from a blood sample of a patient using primers containing transcription and translation components and subsequently converted into recombinant antigens in CFPS reaction mixes. The recombinant antigens were immobilized on a plate coated with an anti-His6 antibody and employed for a serological test using a commercial ELISA kit. The blood of the same patient was tested for the presence of SARS-CoV-2-induced IgG antibodies. The N protein demonstrated the most pronounced response at a wavelength of 450 nm, in line with previous research findings. Notably, the infrequently documented Orf10 also generated a markedly elevated response. Our research has yielded a reliable tool for screening suitable antigens to facilitate the development of immunological test products in the early phases of prospective pandemics. The findings also indicate that nucleic acid intercalators, such as SYBR green, do not impede the translation machinery in *E. coli* cell-free protein synthesis (CFPS) reactions. Furthermore, a novel antigen (Orf10) has been discovered, demonstrating potential utility in SARS-CoV-2 antibody assays.

4. Materials and methods

4.1 Human blood samples

The clinical blood sample utilized in this study was obtained from a 49-year-old male patient who was diagnosed with COVID-19 at Hwa Mei Hospital, University of Chinese Academy of Sciences (Ningbo No.2 Hospital) in March 2020.

4.2 Primer design

Primers for replicating 13 different SARS-COV-2 genes were designed based on the published genome sequence of a virus strain (genbank id: NC_045512.2). To transcribe and translate the genes in CFPS reactions all forward primers were designed to fuse with a T7 promoter, RBS and His6-tag overhang sequence (5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACCAC-3') at their 5'-termini and all reversed primers were designed to fuse with a T7 terminator overhang sequence (5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGTTATGCTAGTTA-3') at their 5'-termini (**Table 1**). All primers were synthesized by Genscript, Inc (Nanjing, China).

Table 1: Primers used for synthesizing SARS-CoV-2 genes in this paper. F and R stand for forward primer and reversed primers respectively.

No	Gene	PCR product	Primer
1	3CL-Mpro	1029	F: 5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACCACATG R: 5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTTATGCTAGTTATTGGAAAGTAACACCTG
2	Orf1ab-tk	684	F: 5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACCACATGTTAA R: 5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTTATGCTAGTTAATTGTTTTGATAATAAAC
3	S	3930	F: 5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACCACATG

R[5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTTATGCTAGTTATGTGTAATGTAATTTGAC

4	S1	2166	F[5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACCACATG
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R5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTTATGCTAGTTAACGTGCCC GCCG-3'

5	S2	1878	F5'-TTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACATG
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R5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTTATGCTAGTTATGTGTAATGTAATTTGAC

6	S-RBD	828	5'- TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACCACATGTTCA 3' 5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTATGCTAGTTAGAAGTTGAAATTGACACA
7	Orf3a	939	5'- TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACCACATGGAT 5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTATGCTAGTTACAAAGGCACGCTAGTAG
8	Epro	336	5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACCACATG 5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTATGCTAGTTAGACCAGAAGATCAGGAA
9	M	777	5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACCACATG 5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTATGCTAGTTACTGTACAAGCAAAGCAA
10	Orf7a	474	5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACCACATG 5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCAAGGGGTATGCTAGTTATTCTGTCTTTCTTTTGAG

11	Orf8	474	F5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACATG 3' R5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATTAGATGAAATCTAAAC
12	N	1377	F5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACATG 3' R5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATGCTAGTTAGGCCTGAG
13	Orf10	225	F5'-TTTTTTTAATACGACTCACTATAGGGTAAGAAGGAGATATACATGCACCACCACCACCACATG 3' R5'-CAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATGTGAGATTAAAGTTAAC

4.3 SARS-CoV-2 nucleic acid amplification using qPCR

SARS-CoV-2 RNA was extracted from 200 µL whole blood samples using a nucleic acid extraction kit (Ex-DNA/RNA, Xi'an Tianlong Science and Technology Co., Ltd.) following the manufacturer's protocol. The cDNA was synthesized in a single reaction using 5 µL of RNA template with the SuperScript™ VILO™ cDNA Synthesis Kit (Cat. # 11754-050, Invitrogen, Life Technologies, Thermo Fisher, USA) according to manufacturer's guideline. qPCR assays were performed using the TransStart® Top Green qPCR SuperMix kit (Cat. # AQ131-01, containing SYBR green dye, Transgen Co., Ltd.) on a SLAN®-96P real-time PCR system manufactured by Shanghai Hongshi Medical Technology Co., Ltd. A total of 5 µL of DNA template and 1 µL of each primer were added to a 20 µL PCR reaction. The cycling conditions were modified as follows: a hot-start step of 5 minutes at 95°C, followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 4 minutes.

4.4 Cell-Free Protein Synthesis (CFPS)

Coupled *in vitro* transcription and translation were conducted using the standard *E.coli* CFPS reactions as previously outlined by Tang *et al* (2023) [22] and Wang *et al* (2024) [23]. Specifically, 40 µL of each qPCR product containing 5'-T7 promoter, RBS sequences and His6-tag, as well as 3'-T7 terminator sequences, was added directly into a 200 µL CFPS reaction (Cat. # CF-EC-1000D; GZL Bioscience Co. Ltd.) The reactions were conducted at a temperature of 25°C for a duration of 12 hours, after which western blotting analysis was performed using anti-His6 monoclonal antibody (Cat. # 0812-1, Huaan Biotech Co. Ltd.).

4.5 Enzyme-Linked Immunosorbent Assay (ELISA)

100 μ L of 2 μ g/mL Anti-His6 Tag monoclonal antibody (Solarbio, K200060M, diluted with 1 $\times$ PBS, PH=7.4) was added to each well of the ELISA plate. The plate was then sealed with film and incubated overnight at room temperature. Subsequently, each well was sealed with 150 μ L of blocking solution at 37°C for 2 hours, followed by air drying at room temperature for 6 hours. Recombinant antigens of the virus were immobilized in individual wells by the addition of 100 μ L of CFPS samples (diluted at 1:40 with 1 $\times$ PBS) containing His6-tagged antigens. The patient's serum (100 μ L) was diluted at a ratio of 1:40 with 1 $\times$ PBS and added to each well. The samples were then incubated at 37°C for 10 minutes, followed by repetitive washing with the same buffer. The antibodies obtained from the serum were identified using the human IgG ELISA Kit (Abclonal, RK00393). The optical density of each well was measured at 450 nm using a microplate reader. The experiment was repeated three times. Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) was employed to graph the results (error bars are standard deviations of the mean).

Declarations

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Authors' contributions

Yajie Tang and Mingming Fei contributed equally to this work. Yao Wang, Shun Zhang, Xin Liu, Lin Wang, and Gang Liu conceived and designed the study. Lei Ma and Zhihui Jiang performed the data analysis. Yajie Tang, Mingming Fei, Shaoting Weng, Noor Muhammad, Zeya Chen, Huiyan Ma, Siyang Chen, and Xiaohu Zhang carried out the experiments and administered the project. Yinrong Wu, Wenhui Zhang, and Sen Lin supervised the study. Yajie Tang and Yao Wang drafted the manuscript.

All authors reviewed, revised, and approved the final manuscript.

Consent to participate

Not applicable.

Consent for publication

Written informed consent for publication was obtained from the patient whose biological sample was used in this study.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Competing interests

The authors declare that they have no competing interests

Ethics Approval

The protocol for the collection and use of human blood samples was approved by the Ethics Committee of Hwa Mei Hospital, University of Chinese Academy of Sciences (Ningbo No.2 Hospital), Ningbo, China. The study was conducted in accordance with the ethical standards of the institutional research committee and in compliance with relevant national and international guidelines and regulations for research involving human participants.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Figures

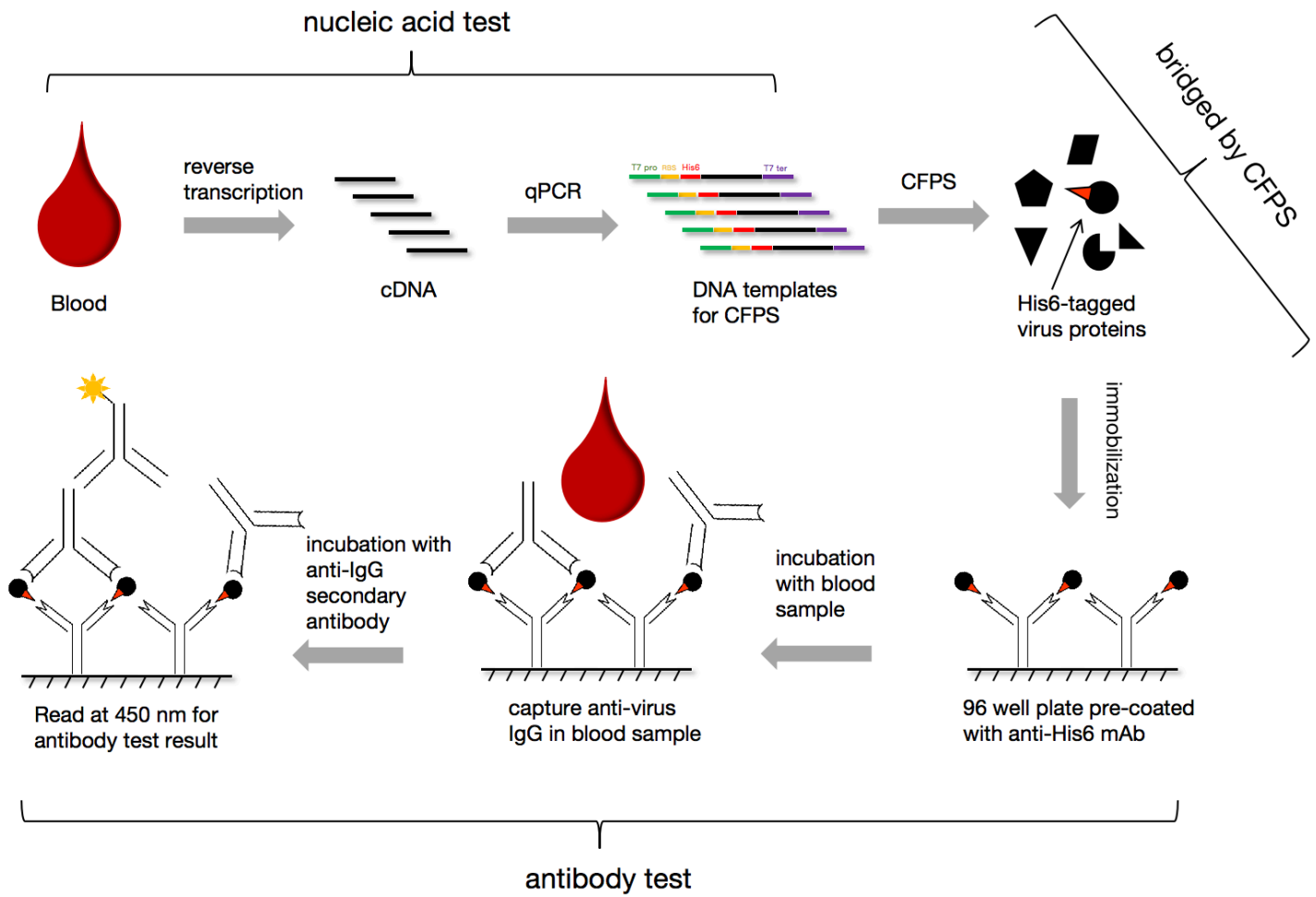


Figure 1

The proposed methodology that involves conducting a dual test of SARS-Cov-2 bridged by CFPS.

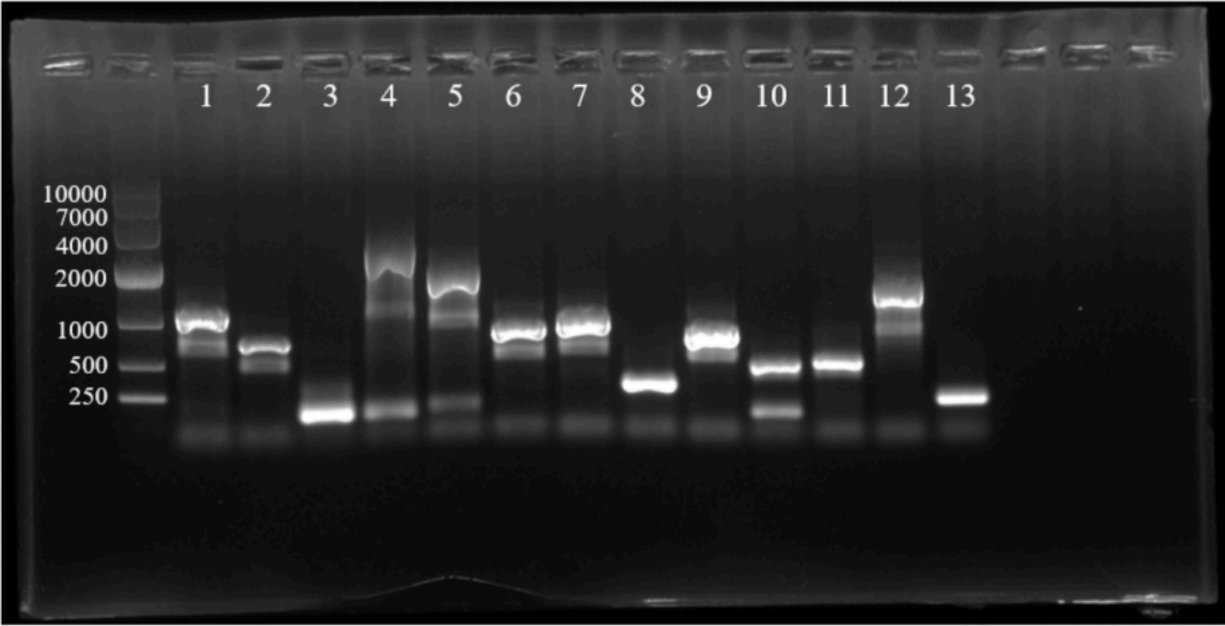


Figure 2

Agarose gel electrophoresis for the qPCR products of 13 randomly selected SARS-CoV-2 genes. Lane 1-13 contained qPCR products corresponding to the following genes: 3CL-Mpro, Orf1ab-tk, S, S1, S2, S-RBD, Orf3a, Epro, M, Orf7a, Orf8, N, and Orf10, respectively. The lane on the left of lane 1 consists of nucleic acid markers, with their respective sizes (in bp) indicated on the side.

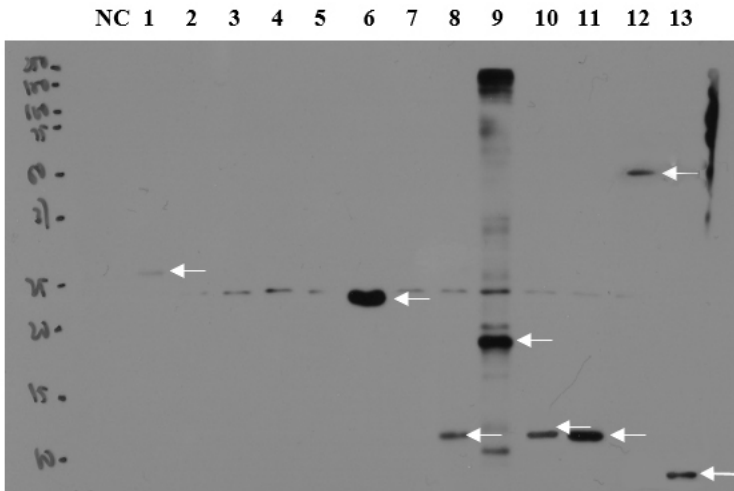


Figure 3

Western blotting for His6-tagged virus proteins produced by CFPS. Lane 1-13 are CFPS samples of 3CL-Mpro (estimated MW: 34 kD), Orf1ab-tk (estimated MW: 25 kD), S (estimated MW: 141 kD), S1 (estimated MW: 75 kD), S2 (estimated MW: 66 kD), S-RBD (estimated MW: 25 kD), Orf3a (estimated MW: 31 kD), Epro (estimated MW: 8 kD), M (estimated MW: 25 kD), Orf7a (estimated MW: 13 kD), Orf8 (estimated MW: 14 kD), N (estimated MW: 45 kD), and Orf10 (estimated MW: 4.7 kD), respectively. The non-template control (NC) is a sample of CFPS without any PCR product added. The target proteins (indicated by the white arrows) were probed using an Anti-His6 tag monoclonal antibody. Eight out of thirteen target proteins were successfully synthesized using this methodology. The protein markers were loaded in the first lane on the left, and the size of each marker was labeled in kilodaltons (kD).

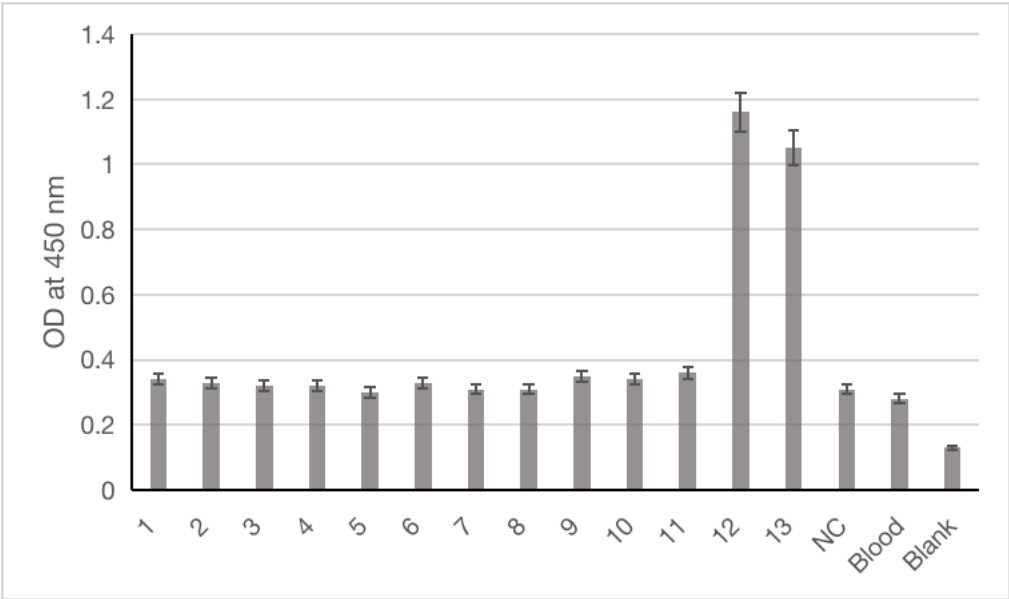


Figure 4

ELISA for detecting antigens generated by CFPS using DNA templates that were amplified from the same patient previously diagnosed with COVID-19. Each well underwent pretreatment with an anti-His6 antibody to capture His6-tagged antigens that were produced by CFPS. Sample 1-13 consists of CFPS samples of 3CL-Mpro, Orf1ab-tk, S, S1, S2, S-RBD, Orf3a, Epro, M, Orf7a, Orf8, N and Orf10,

respectively. The NC is for adding a CFPS reaction sample in the absence of a PCR template, followed by the addition of a blood sample. Blood is for adding a blood sample (from the same patient) only. Blank is for adding PBS buffer only.