

Original Article

Development of a Novel Indirect ELISA Assay for the Detection of SARS-CoV-2 IgG Antibodies Using a Highly Sensitive RBD –Nucleoprotein Fusion Antigen

Sohrab Sam¹ , Behrokh Farahmand² , Hamideh Ofoghi^{1*} 

1. Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran.

2. Department of Influenza and Other Respiratory Viruses. Pasteur Institute of Iran, Tehran, Iran.

Article Info:

Received: 15 April 2024

Revised: 30 July 2025

Accepted: 4 August 2024

Keywords:

COVID-19, ELISA assay,
ELISA sensitivity,
ELISA specificity,
Fusion protein, Nucleoprotein,
Receptor Binding Domain
(RBD), SARS-CoV-2.

Corresponding Author:
ofoghi@irost.ir

<https://orcid.org/0000-0001-5347-3109>

<https://orcid.org/0000-0002-7772-3595>

<https://orcid.org/0000-0003-2163-2448>

ABSTRACT

The early and accurate diagnosis of COVID-19 is essential for efficient disease management. Traditionally, enzyme-linked immunosorbent assays for detecting IgG antibodies to SARS-CoV-2 have relied on single antigens, such as the spike or nucleoprotein. However, the test sensitivity has not been satisfactory. This study diverges from conventional approaches by developing an indirect ELISA assay utilizing a novel, highly sensitive fusion antigen incorporating both the receptor-binding domain (RBD) and nucleoprotein (N). The physicochemical characteristics of this unique antigen were examined and confirmed through experimental validation in our recent research. Our primary objective is to enhance the diagnostic sensitivity and accuracy of the ELISA assay. The indirect ELISA assay was developed using a novel fusion antigen incorporating both the RBD and nucleoprotein (N) of SARS-CoV-2. A cohort of 112 patients presenting with COVID-19 symptoms was evaluated to detect SARS-CoV-2 RNA. Additionally, 25 serum samples from healthy individuals were selected as the negative control group. The study was conducted in Tehran Province, Iran, from March to July 2022. Serum samples from patients who tested positive using the real-time PCR method were collected, and an in-house indirect ELISA assay was developed. The sensitivity and specificity of the assay were evaluated and compared with the results of the commercial Euroimmun anti-SARS-CoV-2 IgG assay, which served as the gold standard. The ROC curve was analyzed using GraphPad Prism to assess the accuracy and reliability of the in-house ELISA assay. The in-house ELISA assay developed in this study demonstrated successful performance and showed 100% sensitivity and 96% specificity in detecting anti-SARS-CoV-2 IgG antibodies. Receiver operating characteristic (ROC) analysis yielded an area under the curve (AUC) of 0.976, indicating high accuracy. Our in-house ELISA assay exhibits high sensitivity and specificity, underscoring its suitability for commercial development as a reliable diagnostic kit for detecting COVID-19 cases.

How to cite this article: Sam S, Farahmand B, Ofoghi H. Development of a Novel Indirect ELISA Assay for the Detection of SARS-CoV-2 IgG Antibodies Using a Highly Sensitive RBD –Nucleoprotein Fusion Antigen. *Archives of Razi Institute*. 2025;80(4):957-968. DOI: 10.32592/ARI.2025.80.4.957

1. Introduction

SARS-CoV-2 is a member of the Coronaviridae family, classified as a positive-sense, single-stranded, enveloped RNA virus. Its genome, spanning 30 kb, encodes four crucial structural proteins—namely, spike (S), envelope (E), membrane (M), and nucleocapsid (NP)—alongside various nonstructural proteins (1,2).

Accurate, prompt, and targeted diagnostic tests for SARS-CoV-2 infection are essential for safeguarding public health, particularly in identifying asymptomatic individuals who may be contagious. Among the various diagnostic methods, serological assays, such as the Enzyme-Linked Immunosorbent Assay (ELISA), have played a significant role in detecting antibodies against SARS-CoV-2 (3,4).

In serological diagnosis using indirect ELISA for SARS-CoV-2, the nucleocapsid (N) and spike (S) proteins are commonly employed as antigens. Notably, selecting the RBD and nucleoprotein as targets in this assay is a critical decision that significantly influences its efficacy (5,6).

The RBD of the spike protein is a critical component in the interaction between the virus and host cell receptors, making it a valuable target for antibody detection in ELISA assays (7).

The specificity of the RBD in targeting antibodies generated against SARS-CoV-2 is crucial for accurately identifying immune responses to the virus. A detailed study involving the design of single-domain antibody (sdAb) libraries and the construction of synthetic sdAbs highlights the importance of antigen-binding affinity and specificity, which are vital for accurately targeting and neutralizing the virus (8,9).

Additionally, utilizing the RBD in ELISA assays enhances the ability of the assay to discriminate between antibodies produced in response to SARS-CoV-2 infection and those from other coronaviruses, thereby improving the accuracy of the assay and reliability in antibody detection. In one study, the sensitivity and specificity of the SARS-CoV-2 S1 subunit, RBD, and native-state S trimer in detecting anti-SARS-CoV-2 antibodies from COVID-19 convalescent patients were compared. The findings revealed that while the S1 subunit exhibited superior sensitivity over the RBD and S trimer, it also showed cross-reactivity with antibodies elicited by other circulating coronaviruses. Therefore, RBD is

considered the best option for achieving high specificity (10).

The unique structure of the RBD and its immunogenicity contribute significantly to its effectiveness as an antigen in ELISA assays, facilitating the specific detection of antibodies directed against SARS-CoV-2. A study on the preclinical immunogenicity and protective efficacy of an RBD-based vaccine against SARS-CoV-2 demonstrated the effectiveness of the RBD as an antigen (11,12).

In contrast to the RBD, the nucleoprotein of SARS-CoV-2 is highly abundant during viral infection and plays a crucial role in viral replication and packaging (13-15). While the nucleoprotein is immunogenic and elicits a robust antibody response, its use as an antigen in ELISA assays may present challenges due to potential cross-reactivity with antibodies from other coronaviruses. Cross-reactivity can lead to false-positive results or reduced assay specificity, impacting the accuracy of antibody detection. Despite these challenges, the nucleoprotein remains a valuable antigen in ELISA assays, particularly for its ability to detect antibodies against a conserved viral component, which could provide valuable insights into the immune response to SARS-CoV-2 over time (16).

Traditional ELISA assays typically rely on single antigens, such as the spike (S) protein or the nucleoprotein (N), which have inherent limitations affecting their diagnostic performance. This means that the assays might not detect low levels of antibodies in individuals who have been exposed to the virus, particularly in the early stages of infection or in mild cases. This limitation can lead to false-negative results, thereby missing potential cases of infection. Relying on a single epitope, such as the RBD of the spike protein, might not capture the full spectrum of the immune response in all individuals. Moreover, variability in individual immune responses and mutations in the virus can affect the binding efficacy of antibodies to these single epitopes, impacting the overall performance of the assay (17,18,19).

Developing fusion protein antigens represents a novel approach for detecting antibodies against SARS-CoV-2. In our previous research, we combined two RBD domains representing the Delta and Omicron variants, along with a C-terminal domain (CTD) from the nucleoprotein. This design aims to capture immune responses directed toward key epitopes on the spike protein variants and the

nucleoprotein for early detection of COVID-19 infection, thereby enhancing the sensitivity and specificity of the assay in distinguishing SARS-CoV-2 Delta and Omicron variants (20).

variations in how patients' immune systems produce antibodies over time complicate standardized measurement, and pre-existing health conditions or medications might interfere with assay results, affecting ELISA reliability. Furthermore, the ongoing evolution of the virus introduces antigenic drift, necessitating continuous updates to the assays to maintain their effectiveness in identifying current strains (21-24, 30,31).

Variations in ELISA assays for detecting COVID-19 antibodies underscore the importance of considering factors such as assay type, antigen selection, sensitivity, specificity, and practicality. Understanding these differences and selecting the most suitable assay based on specific requirements can improve diagnostic capabilities and aid in effective pandemic management (25).

Overall, there is a clear necessity for improved serological assays that offer higher sensitivity and specificity. The ongoing evolution of SARS-CoV-2 and the emergence of new variants further highlight the need for robust diagnostic tools. By utilizing a combination of multiple antigens or epitopes—such as the RBD and the nucleoprotein (N)—these advanced assays can potentially overcome the drawbacks of traditional methods. The fusion of these antigens can provide a broader range of antibody detection, ensuring better diagnostic accuracy and reliability.

This study aims to advance SARS-CoV-2 antibody detection by evaluating the efficacy of novel fusion protein antigens, which combine the RBD from selected variants with the nucleoprotein antigen. The approach is expected to enhance the sensitivity of detecting IgG antibodies in COVID-19 patients, thereby contributing to improved disease management and control efforts. This method will be compared with the Euroimmun Anti-SARS-CoV-2 IgG kit, the gold standard in antibody detection.

2. Materials and Methods

2.1 Molecular Diagnosis of COVID-19

2.1.1 RNA Extraction of SARS-CoV-2

Nasal swab samples were collected from 112 suspected patients referred to the medical laboratory of Tajrish Hospital, Tehran, Iran. The study was conducted in Tehran Province, Iran, from March 2022 to July 2022.

RNA extraction was performed using the QIAcube HT system, which employs the QIAamp 96 Virus QIAcube HT Kit manufactured in China. We carefully followed the instructions provided by the manufacturer to ensure extraction of pure RNA.

2.1.2 One-step Quantitative Real-time PCR

The Real-time RT-PCR method was employed for cDNA synthesis and amplification of genetic material using the 2019-nCoV Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) developed by Sansure Biotech, Changsha, China. This kit targets specific regions of the SARS-CoV-2 genome, including ORF1ab and the conserved coding sequence of the nucleocapsid protein N gene. The process involved a one-step procedure, including cDNA synthesis and amplification, to detect viral RNA in the samples.

The cDNA synthesis and amplification process began with a reverse transcription step at 50°C for 20 minutes, followed by an initial denaturation at 95°C for 2 minutes. Amplification was carried out over 35 cycles, consisting of denaturation at 95°C for 15 seconds, annealing at 65°C for 20 seconds, and extension at 72°C for 1 minute. Finally, there was an extension step at 72°C for 5 minutes. This entire procedure was performed using the ABI 7500 Real-Time PCR System (Table 1 and 2). Furthermore, we employed a real-time PCR setup targeting the Spike and N genes, utilizing specifically designed primers to confirm the kit results (28). The specific primer sequences used in this setup can be found in Table 3. (Table 1,2 and 3).

2.2 ELISA Assay for detecting SARS-CoV-2 IgG

2.2.1 Euroimmun IgG ELISA Assay

The ELISA assay was performed using the Euroimmun Anti-SARS-CoV-2 IgG qualitative indirect ELISA assay from Germany as a gold standard. A total of 76 serum samples were included, comprising 51 samples with confirmed COVID-19 infection and 25 samples from healthy individuals prior to the pandemic. The assay was performed following the instructions of the manufacturer guidelines, incorporating controls and calibrators provided within the kit to ensure precision and reliability.

2.2.2 In-house indirect ELISA assay

2.2.2.1 Antigen preparation

Based on our previous research, using bioinformatic and experimental methods, a multi-domain SARS-CoV-2 antigen (CoV2-Pro) was designed and used in this study.

Table 1. QRT-PCR Reaction Mixture for SARS-CoV-2 RNA Detection.

QRT-PCR Reaction Components and Volumes	
Component	Volume per Test
RNA Sample	20 μ L
PCR Enzyme (RT Enzyme, Taq polymerase)	4 μ L
PCR Mix (PCR Buffer, dNTP, Mgcl2)	26 μ L
Total Volume	50 μ L

Table 2. QRT-PCR Amplification Program for Spike and Nucleocapsid Gene.

QRT-PCR Amplification Program			
Step	Temperature ($^{\circ}$ C)	Time	Number of Cycles
Reverse Transcription	50	20 minutes	1
Initial Denaturation	95	2 minutes	1
Denaturation	95	15 seconds	35
Annealing	65	20 seconds	35
Extension	72	1 minute	35
Final Extension	72	5 minutes	1

Table 3. Primer Sequences for Spike and Nucleocapsid Gene.

Forward and Reverse Primers for Spike and Nucleocapsid Genes of SARS-CoV-2	
Nucleocapsid (N) Gene	F: 5'-GACCCCAAAATCAGCGAAATG-3' R: 5'-GTAGCACGATTGCAGCATTG-3'
Spike gene (S) Gene	F: 5'-TCAGACAAATCGCTCCAGGG-3' R: 5'-AGCAACTGAATTTTCTGCACCA-3'

This antigen encompasses the RBD from both the Omicron and Delta variants of SARS-CoV-2, along with the C-terminal domain of the nucleoprotein. The antigen was synthesized, cloned into a pET-32b(+) vector, expressed in *E. coli* Shuffle T7, and verified and validated via SDS-PAGE and Western blot analysis (20).

2.2.2.2 Optimization of ELISA assay

The main components of the ELISA assay, including antigen concentration, serum dilution, BSA concentration, and anti-human IgG concentration, were optimized.

To determine the optimal antigen concentration for coating, we prepared 5, 2.5, 1.25, and 0.625 μ g/ml of antigen by diluting the stock solution in coating buffer (PBS, phosphate-buffered saline, pH 7.2). Additionally, positive control serum samples were diluted at ratios of 1:100, 1:500, 1:1000, and 1:2000 using PBS buffer to ascertain the most suitable serum dilution. Furthermore, we optimized the blocking step by employing various bovine serum albumin (BSA) concentrations —1%, 2%, and 3% in PBS with 0.05% Tween 20 — to minimize the signal-to-noise ratio.

2.2.2.3 The In-house ELISA Assay Procedure

The in-house ELISA assay was performed on 51 COVID-19-confirmed serum samples and 25 serum samples from the negative control group.

To perform the ELISA assay, 100 μ l of CoV2-Pro antigen at a concentration of 1.25 μ g/ml was added to each well of a 96-well ELISA plate (SPL Life Sciences Co.). The plate was then incubated overnight at 4 $^{\circ}$ C to facilitate the adhesion of the antigen to the well surface. Following incubation, the plate was washed four times with wash buffer (PBS, pH 7.2, containing 0.05% Tween 20) to remove any unbound antigens. Subsequently, the remaining protein-binding sites on the plate were blocked by adding 100 μ l of blocking buffer (3% BSA from Sigma) to each well and incubating for 1 hour at room temperature. After blocking, the plate was washed four times with wash buffer to remove excess blocking buffer. Next, 100 μ l of COVID-19-confirmed patient serum samples, diluted at a ratio of 1:1000, was added to the wells of the ELISA plate. The plate was then incubated

for 2 hours at 37°C to allow the antibodies in the serum to bind to the CoV2-Pro antigen.

Following incubation, the plate was washed four times with wash buffer to remove any unbound antibodies. Subsequently, 100 µl of HRP-conjugated anti-human IgG antibody solution (1:30,000 dilution in PBS with 1% blocking buffer and 0.05% Tween 20) from Sigma was added to each well. The plate was then incubated for 1 hour at 37°C to allow the secondary antibody to bind to any bound primary antibodies.

After the secondary antibody incubation, the plate was washed four times with wash buffer to remove any unbound secondary antibody. A substrate solution (3,3',5,5'-Tetramethylbenzidine, TMB from Sigma) was added to each well and incubated for 20 minutes at room temperature in a dark environment to initiate the colorimetric reaction. The reaction was then stopped by adding 1 M sulfuric acid as the stop solution. Finally, the absorbance of each well was measured at 450 nm against a reference wavelength of 630 nm using a BioTek ELISA reader, USA.

2.3 Statistical Analysis

2.3.1 Determining the Cut-offs Value or ELISA assay

The cut-off value for the ELISA assay was determined based on the optical density (OD) readings of the negative control group. First, the mean OD and standard deviation (SD) were calculated for the negative control samples. The cut-off value was then established as the mean OD of the negative controls plus two standard deviations. Additionally, it is worth noting that the cut-off value determination was confirmed using GraphPad Prism 10.2 software to ensure accuracy and reliability. Formulas 1 and 2 were used to calculate SD and Cut-off value.

$$1. \text{Standard Deviation} = \sqrt{(\sum (x_i - x)^2) / (n-1)}$$

$$2. \text{Cut-off Value} = x + (2 * \text{SD})$$

Σ: sum over all sample points,

x_i : each individual sample point,

x : the mean of the sample points,

n : the number of sample points.

2.3.2 Determining Sensitivity and Specificity of ELISA Assay

In this research, we determined the sensitivity and specificity of our ELISA design by comparing its results with those obtained from the Euroimmun SARS-CoV-2

IgG assay. The sensitivity of a test indicates the conditions under which a test accurately detects a type of disease present in the patient. When the sensitivity of a test is high, the probability of false-negative results is minimal. Specificity in a test refers to the ability of an assay to identify individuals who do not have the disease correctly. In other words, in a test with high specificity, a negative result is truly negative. Conversely, a test with low specificity may yield false-positive results, even in the absence of the disease. Formulas 3 and 4 were used to determine sensitivity and specificity (29).

$$1. \text{Sensitivity} = (\text{True Positives}) / (\text{True Positives} + \text{False Negatives})$$

$$2. \text{Specificity} = (\text{True Negatives}) / (\text{True Negatives} + \text{False Positives})$$

True Positives: Number of individuals correctly identified as having the disease.

False Negatives: Number of individuals incorrectly identified as not having the disease.

True Negatives: Number of individuals correctly identified as not having the disease.

False Positives: Number of individuals incorrectly identified as having the disease when they do not.

2.3.3 Assessment of the Precision of ELISA Assay

To assess the precision of the test, we conducted a receiver operating characteristic (ROC) analysis. ROC is a statistical method used to determine the performance of diagnostic systems in binary classification. It plots the true positive rate (TPR), or sensitivity, against the false positive rate (FPR), or 1 - specificity, across various threshold values. Each point on the ROC curve represents a different threshold value, with the proximity of the curve to the top-left corner and an area under the curve (AUC) approaching 1 indicating superior classifier performance.

TPR and FPR are calculated for every threshold, which is then used to construct the ROC curve with TPR on the y-axis and FPR on the x-axis. The AUC is computed to provide a singular scalar value that encapsulates the overall effectiveness of the classifier. This methodology is particularly valuable when a balance between sensitivity and specificity is crucial, aiding in determining an optimal threshold that maximizes diagnostic accuracy by balancing true positive detection with false positive avoidance. This study used GraphPad Prism 10.2 software to draw the plot.

3. Results

3.1 Real-time qPCR detection of COVID-19

From the 112 patients examined for the presence of viral RNA, 51 samples were found to be positive for SARS-CoV-2 using the Nucleic Acid Diagnostic Kit developed by Sansure Biotech. Furthermore, these positive results were corroborated by the amplification curves observed using our homemade RT-qPCR assay. Additionally, the viral load in each positive sample was quantified using Ct values. Ct values below 35 were considered positive.

3.2 Euroimmun Qualitative IgG ELISA Assay

The results from the Euroimmun ELISA assay demonstrated that all 51 serum samples from COVID-19-confirmed patients tested positive for SARS-CoV-2 antibodies. Furthermore, all 25 samples from the negative control group tested negative, affirming the specificity of the assay in accurately identifying negative samples.

3.3 Antigen Preparation and confirmation

The CoV2-Pro antigen was confirmed successfully through SDS-PAGE and Western blot analyses. Using anti-RBD and anti-His-tag antibodies in Western blotting, we detected the CoV2-Pro protein in *E. coli* cell extracts. The Western blot revealed a distinct band at approximately 83.2 kDa, matching the anticipated size of the antigen. In parallel, the SDS-PAGE analysis also showed a prominent band at 83.2 kDa, indicating the purity of the antigen. These combined results verify that the CoV2-Pro protein was successfully expressed and purified from the cell lysate, ensuring its suitability for subsequent experimental applications (Figure 1 and 2).

3.4. In-house indirect ELISA Assay

To develop an in-house indirect ELISA, we carefully assessed absorbance values to identify robust signals. Through this evaluation, we concluded that an antigen concentration of 1.25 µg/ml and a serum dilution of 1:1000 were optimal for our ELISA setup, as they resulted in a slightly strong OD without saturation (Figure 3). A BSA concentration of 3% and an anti-human IgG antibody dilution of 1:30,000 were also determined to be most suitable (Figure 3).

The in-house ELISA assay was performed on 51 COVID-19-confirmed serum samples and 25 serum samples from the negative control group (Table 4).

Results showed that all 51 PCR-confirmed samples tested positive, indicating the accuracy of the assay in detecting SARS-CoV-2 antibodies. However, among the 25 samples from the negative control group, one sample

unexpectedly yielded a positive result. This positive result in the negative control group warrants further investigation to determine the cause, such as potential cross-reactivity or assay interference. This assay obtained a cut-off value of 0.827. Samples with values exceeding the cut-off were deemed positive (Table 5). (Table 4 and 5).

3.5 Determining Sensitivity and Specificity of in-house ELISA Assay

For assessing sensitivity and specificity, the results of the in-house ELISA assay were compared with those of the Euroimmun kit. The comparison revealed a strong correlation between the two methods, indicating similar performance in terms of sensitivity and specificity for detecting SARS-CoV-2 IgG antibodies. According to the results of ELISA assays using both our in-house method and the Euroimmun kit, we did not observe any false negatives in the assays. However, we did encounter one false positive in our assay. Additionally, using GraphPad Prism 10.2 software, we further analyzed the specificity and sensitivity of our assay. The results showed that the in-house ELISA assay exhibits a sensitivity of 100% and a specificity of 96%. These findings validate the performance of our assay and its suitability for detecting SARS-CoV-2 antibodies in patient samples.

3.6 Precision of In-house ELISA Assay

To evaluate the performance of our diagnostic system, we constructed a Receiver Operating Characteristic (ROC) curve. This graphical representation illustrates the trade-off between sensitivity (true positive rate) and 1 - specificity (false positive rate) across various threshold values. Utilizing GraphPad Prism, we computed the AUC using the Wilson-Brown method.

The resulting AUC value was calculated to be 0.9765, with a p-value below 0.0001. This indicates a statistically significant deviation from the null hypothesis of no discrimination (AUC = 0.5). Therefore, our diagnostic system demonstrates exceptional discriminatory power in distinguishing between positive and negative samples with a high level of accuracy (Figure 4).

4. Discussion

Early and precise detection of COVID-19 facilitates timely isolation and treatment of infected individuals, thereby reducing the risk of transmission and preventing virus spread within communities.

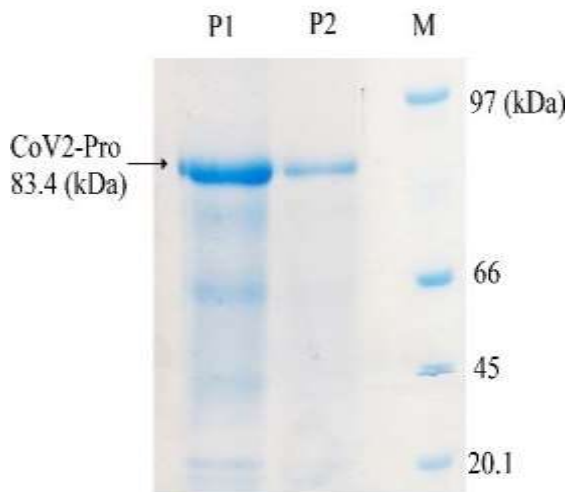


Figure 1. SDS-PAGE Analysis of CoV2-Pro Antigen. The SDS-PAGE gel shows a prominent band at 83.2 kDa, indicating the successful expression and purification of the CoV2-Pro protein from E. coli cell extracts. Lane P1 shows the lysis extract, and lane P2 shows the purified antigen. Lane M contains the molecular weight markers, which are indicated on the left side of the gel.

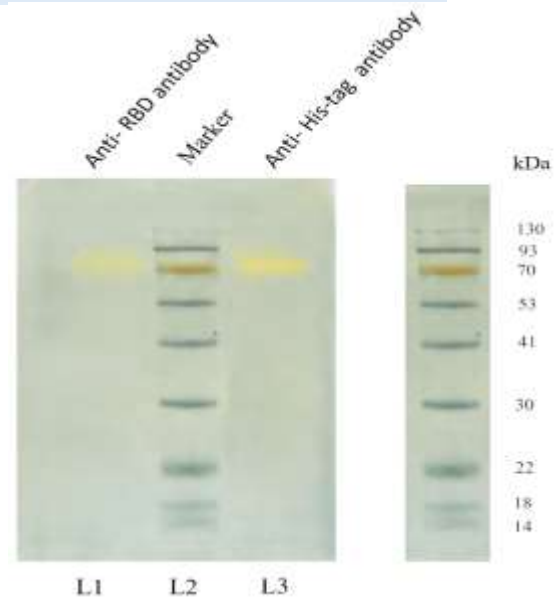


Figure 2. Western Blot Analysis of CoV2-Pro Antigen. The Western blot shows a specific band at approximately 83.2 kDa, Lane L1 shows the detection with anti-RBD antibody, Lane L2 shows the molecular weight markers, and Lane L3 contains the detection with anti-His-tag antibody

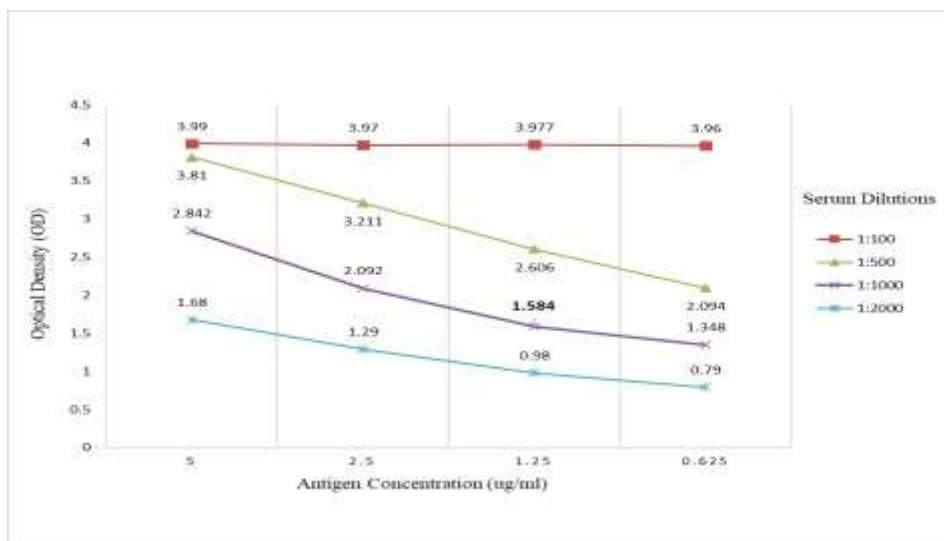


Figure 3. Displays the plot of optimization for an in-house ELISA assay. The experiment evaluated four different coating antigen concentrations (5, 2.5, 1.25, and 0.625 µg/ml) along with four different positive serum dilutions (1:100, 1:500, 1:1000, and 1:2000). The OD obtained from the assay is plotted on the vertical axis, while the antigen concentration is shown on the horizontal axis. Each line represents a specific serum dilution. The chart shows that for all serum dilutions used, the OD decreases as the antigen concentration decreases, except for the 1:100 dilution line (red line). The objective is to select an antigen concentration and serum dilution that yields a relatively strong positive OD and also not being saturated. The optimal combination identified was an antigen concentration of 1.25 µg/ml with a serum dilution of 1:1000 (purple line), achieving an OD of 1.584.

Table 4. Table displaying the number of patient samples and their respective OD values obtained from an in-house ELISA assay, alongside the number of negative control samples and their corresponding OD values.

Patients No.	Patient Samples OD	Patients No.	Patient Samples OD	Negative Control No.	Negative Control OD
1	3.807	27	1.393	1	0.68
2	1.783	28	2.213	2	0.758
3	2.236	29	2.755	3	0.345
4	1.815	30	1.733	4	0.726
5	2.973	31	2.926	5	0.239
6	1.656	32	2.474	6	0.519
7	1.956	33	2.794	7	0.293
8	1.847	34	1.103	8	0.662
9	1.695	35	2.622	9	0.372
10	0.98	36	1.352	10	0.641
11	1.653	37	1.411	11	0.48
12	3.747	38	1.42	12	0.561
13	2.057	39	3.043	13	0.237
14	0.907	40	2.083	14	0.199
15	0.864	41	2.982	15	0.745
16	3.793	42	1.002	16	0.328
17	3.806	43	3	17	0.29
18	3.021	44	2.784	18	0.671
19	1.708	45	3.163	19	2.436
20	2.098	46	1.391	20	0.35
21	3.103	47	1.685	21	0.643
22	0.944	48	1.663	22	0.297
23	3.342	49	2.422	23	0.382
24	1.975	50	1.934	24	0.791
25	3.19	51	2.765	25	0.332
26	2.787				

Table 5. Summarizing diagnostic performance metrics at various cut-off values from GraphPad Prism analysis. The optimal cut-off identified is 0.82, achieving a sensitivity of 100% and specificity of 96%. The highest likelihood ratio observed is 25, indicating a strong association between test results and the presence of the condition.

Cutt-Off Values	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 0.2180	100.0	93.00% to 100.0%	4.000	0.2052% to 19.54%	1.042
> 0.2380	100.0	93.00% to 100.0%	8.000	1.421% to 24.97%	1.087
> 0.2645	100.0	93.00% to 100.0%	12.00	4.167% to 29.96%	1.136
> 0.2915	100.0	93.00% to 100.0%	16.00	6.403% to 34.65%	1.190
> 0.2950	100.0	93.00% to 100.0%	20.00	8.861% to 39.13%	1.250
> 0.3125	100.0	93.00% to 100.0%	24.00	11.50% to 43.43%	1.316
> 0.3300	100.0	93.00% to 100.0%	28.00	14.28% to 47.58%	1.389
> 0.3385	100.0	93.00% to 100.0%	32.00	17.21% to 51.59%	1.471
> 0.3475	100.0	93.00% to 100.0%	36.00	20.25% to 55.48%	1.563
> 0.3610	100.0	93.00% to 100.0%	40.00	23.40% to 59.26%	1.667
> 0.3770	100.0	93.00% to 100.0%	44.00	26.67% to 62.93%	1.786
> 0.4310	100.0	93.00% to 100.0%	48.00	30.03% to 66.50%	1.923
> 0.4995	100.0	93.00% to 100.0%	52.00	33.50% to 69.97%	2.083
> 0.5400	100.0	93.00% to 100.0%	56.00	37.07% to 73.33%	2.273
> 0.6010	100.0	93.00% to 100.0%	60.00	40.74% to 76.60%	2.500
> 0.6420	100.0	93.00% to 100.0%	64.00	44.52% to 79.75%	2.778
> 0.6525	100.0	93.00% to 100.0%	68.00	48.41% to 82.79%	3.125
> 0.6665	100.0	93.00% to 100.0%	72.00	52.42% to 85.72%	3.571
> 0.6755	100.0	93.00% to 100.0%	76.00	56.57% to 88.50%	4.167
> 0.7030	100.0	93.00% to 100.0%	80.00	60.87% to 91.14%	5.000

> 0.7355	100.0	93.00% to 100.0%	84.00	65.35% to 93.60%	6.250
> 0.7515	100.0	93.00% to 100.0%	88.00	70.04% to 95.83%	8.333
> 0.7745	100.0	93.00% to 100.0%	92.00	75.03% to 98.58%	12.50
> 0.8275	100.0	93.00% to 100.0%	96.00	80.46% to 99.79%	25.00
> 0.8855	98.04	89.70% to 99.90%	96.00	80.46% to 99.79%	24.51
> 0.9255	96.08	86.78% to 99.30%	96.00	80.46% to 99.79%	24.02
> 0.9620	94.12	84.08% to 98.40%	96.00	80.46% to 99.79%	23.53
> 0.9910	92.16	81.50% to 96.91%	96.00	80.46% to 99.79%	23.04
> 1.053	90.20	79.02% to 95.74%	96.00	80.46% to 99.79%	22.55
> 1.228	88.24	76.62% to 94.49%	96.00	80.46% to 99.79%	22.06
> 1.372	86.27	74.28% to 93.19%	96.00	80.46% to 99.79%	21.57
> 1.392	84.31	71.99% to 91.83%	96.00	80.46% to 99.79%	21.08
> 1.402	82.35	69.75% to 90.43%	96.00	80.46% to 99.79%	20.59
> 1.416	80.39	67.54% to 88.98%	96.00	80.46% to 99.79%	20.10
> 1.537	78.43	65.37% to 87.51%	96.00	80.46% to 99.79%	19.61
> 1.655	76.47	63.24% to 86.00%	96.00	80.46% to 99.79%	19.12
> 1.660	74.51	61.13% to 84.45%	96.00	80.46% to 99.79%	18.63
> 1.674	72.55	59.05% to 82.89%	96.00	80.46% to 99.79%	18.14
> 1.690	70.59	57.00% to 81.29%	96.00	80.46% to 99.79%	17.65
> 1.702	68.63	54.97% to 79.67%	96.00	80.46% to 99.79%	17.16
> 1.721	66.67	52.97% to 78.03%	96.00	80.46% to 99.79%	16.67
> 1.758	64.71	50.99% to 76.37%	96.00	80.46% to 99.79%	16.18
> 1.799	62.75	49.03% to 74.68%	96.00	80.46% to 99.79%	15.69
> 1.831	60.78	47.09% to 72.97%	96.00	80.46% to 99.79%	15.20
> 1.891	58.82	45.17% to 71.25%	96.00	80.46% to 99.79%	14.71
> 1.945	56.86	43.27% to 69.50%	96.00	80.46% to 99.79%	14.22
> 1.966	54.90	41.38% to 67.73%	96.00	80.46% to 99.79%	13.73
> 2.016	52.94	39.52% to 65.95%	96.00	80.46% to 99.79%	13.24
> 2.070	50.98	37.68% to 64.14%	96.00	80.46% to 99.79%	12.75
> 2.091	49.02	35.86% to 62.32%	96.00	80.46% to 99.79%	12.25
> 2.156	47.06	34.05% to 60.48%	96.00	80.46% to 99.79%	11.76
> 2.225	45.10	32.27% to 58.62%	96.00	80.46% to 99.79%	11.27
> 2.329	43.14	30.50% to 56.73%	96.00	80.46% to 99.79%	10.78
> 2.429	41.18	28.75% to 54.83%	96.00	80.46% to 99.79%	10.29
> 2.455	41.18	28.75% to 54.83%	100.0	86.68% to 100.0%	
> 2.548	39.22	27.03% to 52.91%	100.0	86.68% to 100.0%	
> 2.689	37.25	25.32% to 50.97%	100.0	86.68% to 100.0%	
> 2.760	35.29	23.63% to 49.01%	100.0	86.68% to 100.0%	
> 2.775	33.33	21.97% to 47.03%	100.0	86.68% to 100.0%	
> 2.786	31.37	20.33% to 45.03%	100.0	86.68% to 100.0%	
> 2.791	29.41	18.71% to 43.00%	100.0	86.68% to 100.0%	
> 2.860	27.45	17.11% to 40.95%	100.0	86.68% to 100.0%	
> 2.950	25.49	15.55% to 38.87%	100.0	86.68% to 100.0%	
> 2.978	23.53	14.00% to 36.76%	100.0	86.68% to 100.0%	
> 2.991	21.57	12.49% to 34.63%	100.0	86.68% to 100.0%	
> 3.011	19.61	11.02% to 32.46%	100.0	86.68% to 100.0%	
> 3.032	17.65	9.572% to 30.25%	100.0	86.68% to 100.0%	
> 3.073	15.69	8.169% to 28.01%	100.0	86.68% to 100.0%	
> 3.133	13.73	6.811% to 25.72%	100.0	86.68% to 100.0%	
> 3.177	11.76	5.505% to 23.38%	100.0	86.68% to 100.0%	
> 3.266	9.804	4.261% to 20.98%	100.0	86.68% to 100.0%	
> 3.545	7.843	3.092% to 18.50%	100.0	86.68% to 100.0%	
> 3.770	5.882	1.603% to 15.92%	100.0	86.68% to 100.0%	
> 3.800	3.922	0.6968% to 13.22%	100.0	86.68% to 100.0%	
> 3.807	1.961	0.1006% to 10.30%	100.0	86.68% to 100.0%	

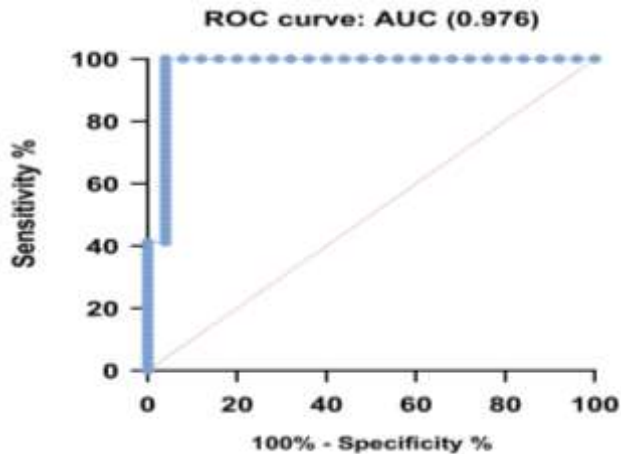


Figure 4. Displays an ROC curve with high diagnostic performance. An AUC of 0.976 indicates excellent sensitivity and specificity in the binary classification task. This plot illustrates the trade-off between the true positive rate (100% sensitivity) and the false positive rate (96% specificity), with the curve closely approaching the top-left corner, signaling a high true positive rate, combined with a low false positive rate across various threshold levels. The near-perfect AUC underscores the exceptional capability of the model to differentiate between the two classes accurately.

Indirect ELISA assays present several advantages, including high sensitivity, versatility in detecting various antibodies, cost-effectiveness, and the ability to perform qualitative or quantitative analysis. This makes them a valuable tool for both research and clinical diagnostics.

In this study, we developed an indirect qualitative ELISA assay to detect IgG antibodies specific to COVID-19. This study was based on our previous research, where we successfully engineered a multi-domain SARS-CoV-2 fusion antigen for use in ELISA assays. The primary objective was to enhance sensitivity and facilitate early detection of COVID-19. In previous investigations, we extensively analyzed the structural characteristics of this antigen, the results of which were published in a reputable journal (20).

The key difference between our ELISA assay and conventional ELISA assays lies in the composition of the antigens used. While conventional ELISA assays typically employ a single protein or fragment, such as spike (S1), spike trimer, or nucleocapsid protein (N Protein), our assay utilizes a multi-epitope antigen. This multi-epitope feature provides several advantages, including cost-effectiveness and increased sensitivity. Furthermore, the RBD domain not only facilitates the identification of active SARS-CoV-2 infections but also enables the detection of neutralizing antibodies, as most SARS-CoV-2 neutralizing antibodies are made against RBD domains.

In this study, we focused on investigating the early stages of the disease while ensuring the specificity of our test. The study samples were gathered through the medical laboratory of Tajrish Hospital in Tehran. Additionally, we incorporated patients who tested positive by real-time PCR, as this method is recognized for its ability to detect the virus in the early stages of the disease and confirm COVID-19 cases. To ensure the reliability of our findings, we performed real-time PCR using a commercial QIAamp 96 Virus QIAcube HT Kit and a homemade real-time PCR method. This approach allowed us to validate the results of our tests and enhance the robustness of our findings.

In the present assessment, we validated our findings by comparing the results of our ELISA assay with those obtained from the Euroimmun qualitative IgG kit, which is considered a gold standard for COVID-19 detection. Notably, we observed a high degree of agreement between the outcomes obtained from these two assays, confirming the reliability and accuracy of the in-house ELISA assay.

The sensitivity of our in-house ELISA assay was determined to be 100%, indicating its capability to identify true positive cases precisely. Additionally, the specificity was calculated at 96%, indicating a minimal occurrence of false-positive results. These values were derived using GraphPad Prism 10.2 software, which suggests a score allowing for a balanced selection between sensitivity and specificity.

Regarding the acquired specificity, we believe that the single false positive in the in-house ELISA assay may be attributed to the antigen used, which contains a nucleoprotein domain. This domain exhibits conserved regions among various coronaviruses, potentially leading to the persistence of antibodies against it in the body for several years (26,27). As a result, cross-reactivity with antibodies from previous infections might lead to false-positive results in our assay.

While this study provides valuable insights, certain aspects warrant consideration. The data was collected from a small sample size; therefore, future studies with a broader range of participants would be beneficial. Additionally, as this is a qualitative study, incorporating quantitative assessments in future research could enhance the evaluation of antibody levels and their correlation with clinical outcomes. Future research should prioritize developing quantitative ELISA assays to evaluate the dynamic antibody response to COVID-19. Furthermore, strategies for validating and optimizing ELISA protocols across diverse populations and varying disease severities are crucial for ensuring accurate and reliable results. Assessing neutralizing antibodies in future studies could also provide valuable insights into vaccine seroprevalence and immune response evaluation.

In conclusion, our ELISA design effectively diagnoses COVID-19 cases with high sensitivity. It is also well suited for various applications, including large-scale seroprevalence studies and point-of-care testing. Its affordability, simplicity, and high sensitivity make it ideal for population-wide screening and monitoring of COVID-19 immunity. Moreover, this robust design is particularly suitable for commercial kit development, ensuring widespread accessibility and utility.

Acknowledgment

The Authors are grateful for the valuable support of the Pasteur Institute of Iran, which greatly contributed to the successful implementation of this study, also thanks to Zohre Amidi, Parisa Rooshani, Zahra Barghi, Azita Eshratkhah and Dr. Vahideh Mazaheri for their assistance and support throughout this research.

Authors' Contribution

Study concept and design: H. O, B. F, S. S.

Acquisition of data: S. S.

Analysis and interpretation of data: H. O, B. F.

Drafting of the manuscript: S. S.

Critical revision of the manuscript for important intellectual content: H. O, B. F.

Statistical analysis: S. S.

Administrative, technical, and material support: B. F, H. O.

Study supervision: H. O, B. F.

Ethics

This study involved routine diagnostic practices without any additional interventions, it was conducted in accordance with ethical standards. Blood samples were collected from patients who visited the laboratory for COVID-19 testing as part of standard medical procedures.

Conflict of Interest

The authors declare that they have no financial or personal relationships that could inappropriately influence or bias the content of the paper. Dr. Hamideh Ofoghi and Dr. Behrokh Farahmand have no relevant financial interests related to the material in the manuscript. Sohrab Sam has no financial interests to disclose.

Data Availability

The data that underpin the findings of this study are comprehensively available in the article. This accessibility is intended to facilitate transparency, enable independent

verification, and support additional research and analysis by providing all necessary datasets and documentation. By making the data openly accessible, we aim to contribute to the broader scientific community and encourage further exploration and innovation in this field.

References

1. Gorbalenya A, Baker S, Baric RS, de Groot R, Drosten C, Gulyaeva AA, Haagmans B, Lauber C, Leontovich AM, Neuman BW, Penzar D. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nature microbiology*. 2020;5(4):536-44.
2. V'kovski P, Kratzel A, Steiner S, Stalder H, Thiel V. Coronavirus biology and replication: implications for SARS-CoV-2. *Nature Reviews Microbiology*. 2021;19(3):155-70.
3. Luo J, Brakel A, Krizsan A, Ludwig T, Mötzing M, Volke D, et al. Sensitive and specific serological ELISA for the detection of SARS-CoV-2 infections. *Virology journal*. 2022;19(1):50.
4. de la Guardia C, Rangel G, Villarreal A, Goodridge A, Fernández PL, Leonart R. Development of in-house, indirect ELISAs for the detection of SARS-CoV-2 spike protein-associated serology in COVID-19 patients in Panama. *PloS one*. 2021;16(9):e0257351.
5. Liao WC, Adame M, Webb J, Nguyen D, Ni J. Development of Quantitative Spike and Nucleocapsid Protein-Based Enzyme-Linked Immunosorbent Assays (ELISAs) for Measurement of Antibodies against SARS-CoV-2. *The Journal of Immunology*. 2022;208(1):125-23.
6. Liu PP, Zong Y, Jiang SP, Jiao YJ, Yu XJ. Development of a nucleocapsid protein-based ELISA for detection of human IgM and IgG antibodies to SARS-CoV-2. *ACS omega*. 2021;6(14):9667-71.
7. Premkumar L, Segovia-Chumbez B, Jadi R, Martinez DR, Raut R, et al. The receptor-binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Science immunology*. 2020;5(48):eabc8413.
8. Chen F, Liu Z, Kang W, Jiang F, Yang X, Yin F, et al. Single-domain antibodies against SARS-CoV-2 RBD from a two-stage phage screening of universal and focused synthetic libraries. *BMC Infectious Diseases*. 2024;24(1):199.
9. Barnes CO, Jette CA, Abernathy ME, Dam KM, Esswein SR, Gristick HB, et al. SARS-CoV-2 neutralizing antibody structures inform therapeutic strategies. *Nature*. 2020;588(7839):682-7.
10. Tian Y, Lian C, Chen Y, Wei D, Zhang X, Ling Y, et al. Sensitivity and specificity of SARS-CoV-2 S1 subunit in COVID-19 serology assays. *Cell discovery*. 2020;6(1):75.

11. Gonzalez-Hernandez M, Kaiser FK, Steffen I, Ciurkiewicz M, van Amerongen G, Tchelet R, et al. Preclinical immunogenicity and protective efficacy of a SARS-CoV-2 RBD-based vaccine produced with the thermophilic filamentous fungal expression system *Thermothelomyces heterothallica* C1. *Frontiers in Immunology*. 2023;14:1204834.
12. Li Q, Wu J, Nie J, Zhang L, Hao H, Liu S, et al. The impact of mutations in SARS-CoV-2 spike on viral infectivity and antigenicity. *Cell*. 2020;182(5):1284-94.
13. Malone B, Urakova N, Snijder EJ, Campbell EA. Structures and functions of coronavirus replication-transcription complexes and their relevance for SARS-CoV-2 drug design. *Nature Reviews Molecular Cell Biology*. 2022;23(1):21-39.
14. Wu W, Cheng Y, Zhou H, Sun C, Zhang S. The SARS-CoV-2 nucleocapsid protein: its role in the viral life cycle, structure and functions, and use as a potential target in the development of vaccines and diagnostics. *Virology journal*. 2023;20(1):6.
15. Huang JC, Chang YF, Chen KH, Su LC, Lee CW, Chen CC, et al. Detection of severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein in human serum using a localized surface plasmon coupled fluorescence fiber-optic biosensor. *Biosensors and Bioelectronics*. 2009;25(2):320-5.
16. Ladner JT, Henson SN, Boyle AS, Engelbrektson AL, Fink ZW, Rahee F, et al. Epitope-resolved profiling of the SARS-CoV-2 antibody response identifies cross-reactivity with endemic human coronaviruses. *Cell Reports Medicine*. 2021;2(1).
17. Liu W, Liu L, Kou G, Zheng Y, Ding Y, Ni W, et al. Evaluation of nucleocapsid and spike protein-based enzyme-linked immunosorbent assays for detecting antibodies against SARS-CoV-2. *Journal of clinical microbiology*. 2020;58(6):10-128.
18. Verissimo CD, O'Brien C, Corrales JL, Dorey A, Cwiklinski K, Lalor R, Doyle JM, Field S, Masterson C, Martinez ER, Hughes G. Improved diagnosis of SARS-CoV-2 by using nucleoprotein and spike protein fragment 2 in quantitative dual ELISA tests. *Epidemiology & Infection*. 2021;149:e140.
19. Reiners N, Schnurra C, Trawinski H, Kannenberg J, Hermsdorf T, Aebischer A, Schöneberg T, Reiche S, Jassoy C. Performance of a SARS CoV-2 antibody ELISA based on simultaneous measurement of antibodies against the viral nucleoprotein and receptor-binding domain. *European Journal of Clinical Microbiology & Infectious Diseases*. 2021;40(12):2645-9.
20. Sam S, Ofoghi H, Farahmand B. Developing of SARS-CoV-2 fusion protein expressed in *E. coli* Shuffle T7 for enhanced ELISA detection sensitivity—an integrated experimental and bioinformatic approach. *Journal of Biomolecular Structure and Dynamics*. 2024;6:1-6.
21. Egger AE, Sahanic S, Gleiss A, Ratzinger F, Holzer B, Irsara C, et al. One-year follow-up of COVID-19 patients indicates substantial assay-dependent differences in the kinetics of SARS-CoV-2 antibodies. *Microbiology Spectrum*. 2022;10(6):e00597-22.
22. Pallett SJ, Jones R, Abdulaal A, Pallett MA, Rayment M, Patel A, et al. Variability in detection of SARS-CoV-2-specific antibody responses following mild infection: a prospective multicentre cross-sectional study, London, United Kingdom, 17 April to 17 July 2020. *Eurosurveillance*. 2022;27(4):2002076.
23. Lakshmikanth T, Muhammad SA, Olin A, Chen Y, Mikes J, Fagerberg L, et al, Brodin P. Human immune system variation during 1 year. *Cell reports*. 2020;32(3).
24. Bei Y, Pinet K, Vrtis KB, Borgaro JG, Sun L, Campbell M, et al. Overcoming variant mutation-related impacts on viral sequencing and detection methodologies. *Frontiers in Medicine*. 2022;9:989913.
25. Taffertshofer K, Walter M, Mackeben P, Kraemer J, Potapov S, Jochum S. Design and performance characteristics of the Elecsys anti-SARS-CoV-2 S assay. *Frontiers in Immunology*. 2022;13:1002576.
26. Rodrigues-da-Silva RN, Conte FP, da Silva G, Carneiro-Alencar AL, Gomes PR, Kuriyama SN, Neto AA, Lima-Junior JC. Identification of B-cell linear epitopes in the nucleocapsid (N) protein B-cell linear epitopes conserved among the main SARS-CoV-2 variants. *Viruses*. 2023;15(4):923.
27. Thura M, Sng JX, Ang KH, Li J, Gupta A, Hong JM, Hong CW, Zeng Q. Targeting intra-viral conserved nucleocapsid (N) proteins as novel vaccines against SARS-CoVs. *Bioscience reports*. 2021;41(9):BSR20211491.
28. Fotouhi F, Salehi-Vaziri M, Farahmand B, Mostafavi E, Pouriayeali MH, Jalali T, Mazaheri V, Larijani MS, Tavakoli M, Afzali N, Zokaei A. Prolonged viral shedding and antibody persistence in patients with COVID-19. *Microbes and Infection*. 2021;23(4-5):104810.
29. Sil BK, Jamiruddin MR, Haq MA, Khondoker MU, Jahan N, Khandker SS, Ali T, Oishee MJ, Kaitsuka T, Mie M, Tomizawa K. AuNP coupled rapid flow-through dot-blot immuno-assay for enhanced detection of SARS-CoV-2 specific nucleocapsid and receptor binding domain IgG. *International journal of nanomedicine*. 2021;4739-53.
30. Nikbakht, G. Novel Insights into Infection and immunity. *Iranian Journal of Veterinary Medicine*, 2022; 16(2): 99-100.
31. Motamed, N. An Overview of Future Development Methods of Infectious Bronchitis Vaccines. *Iranian Journal of Veterinary Medicine*, 2000; 18(1): 1-12.