Heterologous Expression, Purification, and Immune-Based Assay Application of Soluble SARS-CoV-2 Nucleocapsid Protein

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The serological monitoring of SARS-CoV-2 infection using the measurement of IgG is an important tool to determine the number of infected people in a population, and the disease spreading. One of the most important proteins from SARS-CoV-2 applied to immune-based assays is the nucleocapsid protein, also known as N protein. This is an abundant protein in the pathogen and exerts important functions in viral viability and replication. Moreover, protein N is highly immunogenic. The use of N protein as a recombinant protein is difficult since this biomolecule is mainly produced inside inclusion bodies in *Escherichia coli*. In this work, we present a simple and easy method for the expression and purification of soluble his-tagged N protein, suitable for serological assays, such as ELISA. ELISA assays using this recombinant protein presented 100% of specificity and 86% of sensitivity. The protocol for expression and purification of this recombinant protein can be applied in low infrastructure laboratories, without equipment dedicated to protein expression. This work may help other research groups to develop serological assays to monitor antibody production against SARS-CoV-2.

Keywords: SARS-CoV-2. ELISA. Protein N. Recombinant Protein. Purification.

Abbreviations: COVID-19: Corona Virus Disease-19; Protein N: Nucleocapsid protein; IgG: Immunoglobulin G; *E. coli*: *Escherichia coli*; ELISA: Enzyme-Linked ImmunoSorbent Assay; RT-qPCR: Reverse transcriptase-quantitative polymerase chain reaction; CLIA: Chemiluminescent Immunoassay; SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2; WHO: World Health Organization; LB: Luria-Bertani; TB: Terrific Broth; IPTG: Isopropyl β-D-1-thiogalactopyranoside; RPM: Rotation per minute; PBS: Phosphate buffer saline; pH: Hydrogen potential; DTT: Ditiotreitol; EDTA: Ethylenediaminetetraacetic acid; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HRP: Horseradish peroxidase; TBS: Tris-buffered saline; TMB: tetramethylbenzidine; ROC: Receiver Operator Characteristic; kDa: kilodaltons; GFP-6His: 6-histidine tagged green fluorescent protein; OD: Optical density; IMAC: Immobilized metal affinity chromatography.

The novel coronavirus (SARS-CoV-2), is one of the highly pathogenic β-coronaviruses [1] and causes COVID-19. It was initially identified in Wuhan province, China, in December 2019, and declared an international health emergency on January 30, 2020, by the World Health Organization (WHO) [2]. Since its emergence in Asia, it has spread to all continents, affecting countries around the world. Moreover, it has led well-structured health systems to collapse due to the exponential increase in hospitalizations as a result of its high transmissibility potential [3].

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Given the accelerated spread and symptoms common to other diseases, early diagnosis of COVID-19 has become a critical step to treat infected patients, control and monitor disease transmission, and practice social isolation [1]. The direct detection of the virus is possible by molecular diagnostic performed through reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) [4]. However, this diagnostic method is not compatible with mass testing of the population and does not match the point of care features. To solve this issue and provide a mass population diagnostic screening, serological tests are being used due to their easy manipulation and lower cost. Thus, the need for studies about the coronavirus and its markers grew so that there was development, production, and application of mass tests for disease detection as a strategy to prevent the further spread of the virus [5, 6].

The main component of a serological test kit is the target for antibody recognition during the assay, usually being a recombinant protein or fragments of the full-length polypeptide. One of the main proteins encoded in the SARS-CoV-2 genome and one of the most conserved among coronaviruses is the nucleocapsid protein, or N protein [7, 8]. This protein participates directly in infection in the cell by playing a key role during viral self-assembly and viral genome packaging [7, 9]. Its participation during infection and its high expression makes the N protein immunogenic and highly abundant in the infected cell, turning it into an important disease marker antigen [7, 8, 10].

SARS-CoV-2 N protein is an important target to pursue and has become a major ally of researchers in the development of diagnostic tests and the rapid, accurate, and simple screening for coronavirus contamination, either through antigen or specific antibody detection [9]. The N protein recombinant production and its antigenic potential for application in serological diagnostics have been previously explored [11].

The N protein produced in *E. coli* has been applied in serological diagnostics. However, such recombinant productions are either from inclusion bodies [12], as a fragment [11] or expressed using robust approaches to increase protein solubility [13]. Thus, the present work aims to express and purify the recombinant native and soluble N protein for use in serological tests, such as ELISA (enzyme-Linked ImmunoSorbent Assay) and CLIA (Chemiluminescent Immunoassay), to effectively and accurately detect the presence of antibodies against SARS-CoV-2. To accomplish this goal, we used the pET21a vector and a codon-optimized sequence for *E. coli* expression, producing a recombinant protein fused to a 6-histidine tag at the C-terminal domain. Using this construct, we set up a protocol for protein expression and purification using current reagents and avoiding the requirement of robust equipment and chromatographic columns.

The method applied in this work does not require expensive and robust equipment and can be easily replicated in laboratories with minimal infrastructure to produce recombinant proteins. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the integrated campus of manufacturing and technology (CIMATEC – Salvador, BA, Brazil) (approval number 4.334.505). This research received funding from SENAI National Department (SENAI DN – Grant Number 329266).

The *E. coli* soluble fraction lysate was incubated with the Ni-NTA agarose beads, the material was collected in an empty column to retain the immobilized phase. The beads were exhaustively washed with 50 mM imidazole to remove the low-biding contaminants, followed by serial elution with imidazole at 250 and 500 mM. Figure 1A shows the SDS-PAGE containing the diluted soluble fraction (1) and elution of soluble N protein of SARS-CoV-2 (2-5). The first incubation of the beads with the 250 mM imidazole solution gave us a small amount of recombinant protein (2) around 48 kDa. However, most of the N protein was eluted in the second wash with 250 mM imidazole solution (3). The elution with 500 mM of imidazole removed the remaining protein still adhered to the surface of the beads (4 and 5). All the aliquots were pooled and submitted to concentration and buffer exchange (6), followed by storage at -80° C. It is noteworthy that buffer exchange is pivotal for protein stabilization in solution since freeze and thawing in absence of glycerol rendered low-quality protein, based on downstream immunoassay performed (data not shown).

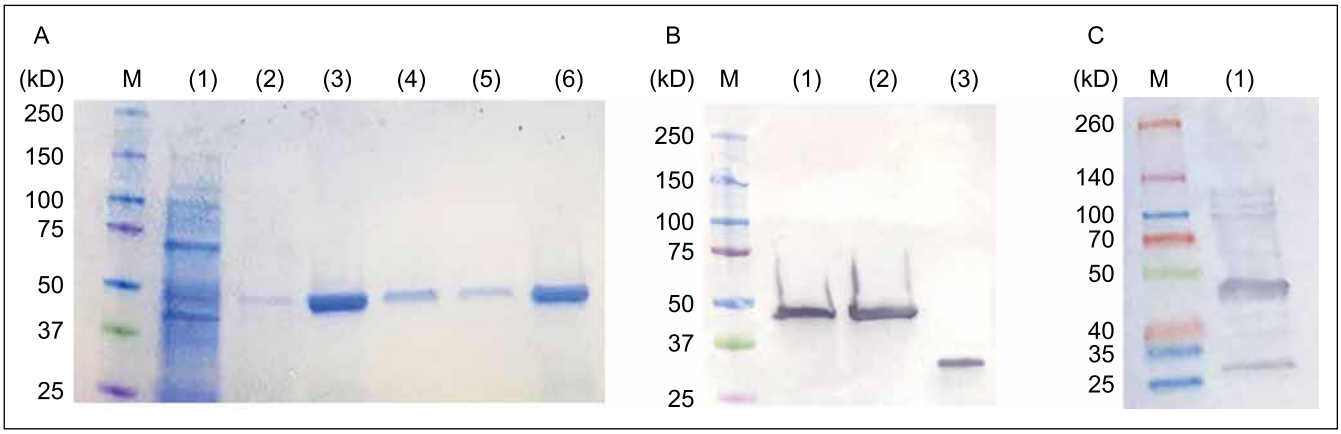
To confirm the presence of a recombinant 6-histidine tagged protein, we performed western blotting targeting the histidine tag in the C-terminal end. The eluted samples (1 and 2) were detected at around 48 kDa. As a positive control, we used a 6-histidine tagged green fluorescent protein (GFP-6His) detected at 30 kDa (3) (Figure 1B). To confirm if the soluble recombinant protein would be suitable for immune-based assays, we performed western blotting using convalescent sera collected

fourteen days after SARS-CoV-2 RNA detection by RT-qPCR. The convalescent sera were able to recognize the 48 kDa recombinant protein, corresponding to the viral N protein (Figure 1C).

An ELISA assay was standardized using the soluble recombinant N protein, based on the need to develop a specific and sensitive immune-based assay to detect antibodies as earlier as possible in the sera of patients. The astringent cut-off was derived from the mean plus three standard deviations of the OD values detected in 63 negative serum samples. The mean OD values from positive samples collected either 7 or 14 days after positive RT-qPCR results, were significantly higher than OD from negative samples (Figure 2). Using the cut-off value of 0.11, it was possible to detect anti-N protein IgG in 25 from 29 positive samples selected after 14 days of positive PCR diagnostic, resulting in a sensitivity of 86% (Figure 3). Regarding the positive samples selected from patients after 7 days of positive RT-qPCR results, IgG was detected in 29 out of 49 patients, rendering a sensitivity of 59%. The assay presented a specificity of 100% since any negative serum samples generated OD values above 0.11. The calculated ROC curve presented the AUC equal to 0.9637 (95% confidence interval 0.9329 to 0.9945) and 0.9918 (95% confidence interval 0.9794 to 1.000) for patient samples collected 7 and 14 days after positive RT-qPCR result, respectively. Moreover, the p values found were below 0.0001 for both RT-qPCR positive sample groups. For comparison, most of the samples were also submitted to ELISA using a commercial N protein as a coating antigen. Thirty-three from 35 samples collected after 14 days of symptoms onset was positive rendering a sensitivity of 95%. All the 48 samples collected from patients after 7 days of symptoms onset were detected above the cut-off (0.07), rendering a sensitivity of 100%. None of the negative samples were detected as positive (100% specificity).

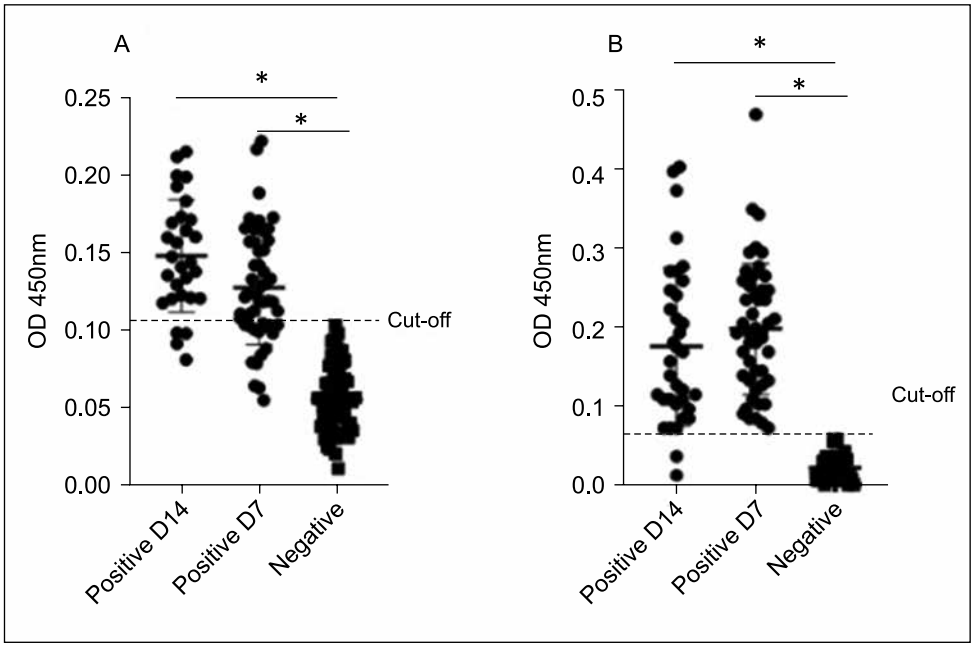
Indirect diagnosis by antibody detection is important to understand the immunological response against the pathogen and the spread of COVID-19. In addition, it is an important tool to measure the pandemic control by vaccination. Moreover, serological diagnostics are essential to identify individuals who are immune and theoretically protected from infection of severe disease, either after natural infection or by vaccination [14-16].

Figure 1. Analysis of recombinant protein by SDS-PAGE and western blotting.



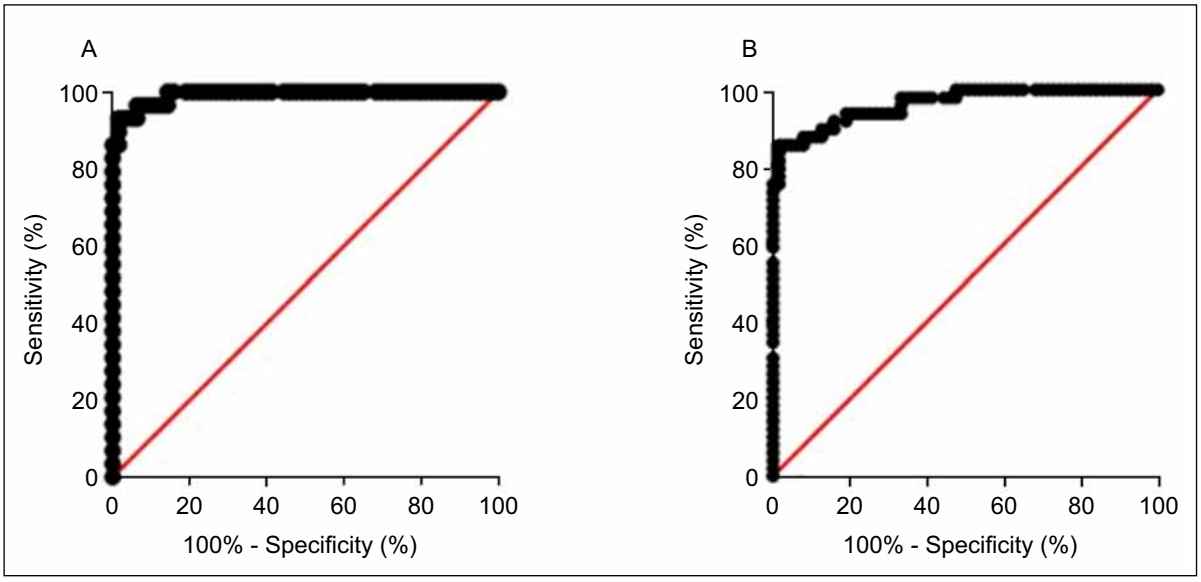
Soluble recombinant N protein from SARS-CoV-2 was expressed in *E. coli* and purified from the soluble fraction by IMAC. (A) SDS-PAGE showing the soluble fraction (1) and eluted protein at 48kDa (2-5) and pooled elutions. (B) western blotting targeting the his-tagged recombinant protein (1-2) at 48kDa and his-tagged GFP as positive control (3) at 30kDa. (C) western blotting targeting the recombinant N protein by convalescent serum 14 days after symptoms onset (1).kDa- kilodalton; M-Modecular weight marker.

Figure 2. ELISA to detect antibodies against N protein from SARS-CoV-2.



An ELISA assay was designed to search antibodies in convalescent serum from patients after 7 or 14 days after symptoms onset. The recombinant soluble N protein was used as coating antigen (A), and a commercial one as a quality control (B), both at 2μg/mL and 100ηg/well. The negative samples were used to calculate de cut-off as the mean OD plus three times the standard deviation (mean OD+3SD). OD - Optical density.

Figure 3. ROC curve generated with positive and negative samples determined by the ELISA.



ROC curve was calculated using Graph Pad Prims version 8.0.1. The AUC determined was 0.9637 (95% confidence interval 0.9329 to 0.9945) and 0.9918 (95% confidence interval 0.9794 to 1.000) for patient samples collected 7 (A) and 14 (B) days after positive RT-qPCR result, respectively. The *p* values found were 0.0001.

In this study, we described the expression and purification of soluble N protein from SARS-CoV-2, as well as the validation of the recombinant protein in immune-based assays. Protein purification by immobilized metal affinity chromatography (IMAC) using nickel was standardized most entirely, making it possible to be performed in low infrastructure laboratories. The purified soluble protein was able to be recognized by convalescent serum and has been applied in ELISA assays to detect antibodies against SARS-CoV-2. It is noteworthy that commercial N recombinant protein used as quality control generates an ELISA assay which has better performance. However, the purchased protein is expensive and is sold in low amounts, meaning that not all laboratories will be able to buy and apply the protein in the routine serological assay. Moreover, the estimated time to import the reagent is too long and expensive, as well. The ELISAs performed in this work demonstrated that the antibodies produced by patients infected with SARS-CoV-2 showed reactivity against the recombinant N protein produced in *E. coli*. This data suggests that the antigenic conformation of the recombinant protein expressed is maintained as being suitable for antibody recognition. During the performance of the serological assay, this similarity is crucial to obtaining reliable results, since it not only confirms the immunogenic potential of N protein but also reaffirms the application of the recombinant protein in other serological tests. Moreover, this recombinant protein presented 100% of specificity and did not cross-react with human sera collected before and during the pandemic, in SARS-CoV-2 negative individuals. It also reinforces the fact that the expressed soluble protein keeps its native conformation after storage and manipulation during the assay.

The sensitivity of the ELISA using convalescent serum from patients 7 and 14 days after symptoms onset agrees with data reported by meta-analysis studies and similar assays in the literature [17-19]. The sensitivity of the ELISA for the detection of anti-SARS-CoV-2 antibodies depends on the time of blood taken after the beginning of the symptoms, the immunocompetence of the individual, and the severity of the clinical case [19]. The immune response and the production of antibodies may not be sufficient to be detected in the early stages of infection, decreasing the sensitivity of the test, which limits its applicability at this time point [19]. According to a previous report, the titer of specific IgG and the switch from IgM to IgG increases from the second or third week of infection [15]. This may explain the low sensitivity in the detection of IgG antibodies from samples after 7 days compared to the greater sensitivity observed in the detection of antibodies using convalescent sera collected after 14 days of infection.

In addition, studies have shown that individuals who had mild symptoms or were asymptomatic produce little or no antibodies to the disease. It has been suggested that before the adaptive immune system produces antibodies, the innate immune system acts by eliminating the virus [16, 19, 20]. These data demonstrate that the use of serological methods for the diagnosis of COVID-19 has limitations and requires proper interpretation of the results. The final result of serological diagnostics will depend on factors inherent to the protein used, disease severity, and the time of symptoms onset.

However, it is important to note that studies have shown that IgM antibody levels throughout infection are not well described, and are unreliable for detecting the acute phase of the disease [16]. Furthermore, meta-analysis studies of serologic diagnostics have concluded that immunoglobulin type (IgM/ IgG) was not associated with diagnostic accuracy. In the same study, comparisons of sensitivity in ELISA tests using IgM and IgG, demonstrated a higher sensitivity, in general, using IgG [17]. The limitation of this approach relies on the kinetics of antibody production during the infection.

Overall, this work demonstrated that SARS-CoV-2 N protein can be produced and purified from the soluble fraction of *E. coli*, rendering a tool for serological assays. The use of the soluble

protein may be useful for crystallography studies, antibody development, phage display, and other techniques that can improve the fight against COVID-19.

The non-detection of IgM antibodies is one of the limitations of the study. The variations of antibodies in individuals upon infection become another limiting factor. In this case, it is not possible to make sure that the measurement of IgG levels is related to a past or recent infection. However, the fact that IgG can become detectable three days after the onset of symptoms or at least 7 to 10 days after infection suggests both situations are possible even without the determination of IgM in the samples [16].

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Materials and Methods

Cloning of the Nucleocapsid Protein Gene

The SARS-CoV-2 N protein was cloned by inserting the optimized coding sequence in the pET21a(+) vector (FastBio, São Paulo, Brazil). *E. coli* (DE3) pRARE2 were transformed by heat shock with the recombinant plasmid and the transformants were selected in Luria-Bertani (LB) Agar (Sigma Aldrich, St. Louis, MO, USA) plates containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol (both from Sigma Aldrich). The recombinant protein was produced fused to a C-terminal histidine tag.

Induction of Recombinant N Protein Expression in *E. coli*

The selected *E. coli* clones were cultured in 5 mL of LB medium (Tryptone 10 g/L, Yeast extract 5 g/L, NaCl 10 g/L) containing ampicillin at 100 µg/mL and 34 µg/mL chloramphenicol under 150 rpm orbital shaking at 37°C for 16 hours (Jeio Tech - Shaker incubated, SIF-500R, Yuseong-gu, Korea). Subsequently, 50 mL of phosphate buffer (0.17 M KH2PO4, 0.72 M K2HPO4) was poured into 450 mL of TB (Terrific Broth) medium (Yeast Extract 24 g/L, Tryptone 20 g/L, Glycerol 4 mL/L), containing the abovementioned antibiotics. Then, the entire volume of pre-inoculum (5 mL) was transferred to the solution and incubated at 37°C under 150 rpm agitation until it reached an optical density at 600 ηm of 1.5. Upon reaching the optical density, IPTG (Isopropyl β-D-1-thiogalactopyranoside – Invitrogen, Waltham, MA, USA) was added at a final concentration of 0.5 mM for the induction of recombinant protein expression. After the addition of IPTG, the culture was incubated at 18° C under 150 rpm orbital shaking for 16 hours.

Purification of Soluble Recombinant N Protein by Affinity Chromatography

After protein expression, the culture was centrifuged at 4° C, 4000 rpm, for 20 minutes. The cells were then resuspended and lysed in lysis buffer (PBS buffer with dithiothreitol 1 mM, protease inhibitor cocktail, lysozyme 200 µg/mL, DNAse 2 mM – all reagents from Sigma Aldrich), followed by sonication. The sonication was performed at 40% amplitude in 4 cycles of 15-second pulse and 30-second rest and repeated three times separately, 12 cycles in total (Qsonica LLC, Newtown, CT, USA). After sonication, 0.01% triton X-100 (Sigma-Aldrich) was added and the sample was submitted to the vortex for 5 minutes. Then, the lysate was centrifuged at 4°C, 18,000 g for 10 minutes.

Subsequently, the lysate was incubated with the Ni-NTA Agarose (Qiagen, Hilden, Germany) beads at 4°C, 60 rpm, for 2 h for protein binding. After this period, the sample with the beads was placed into the column for packing. After this step, the column was washed thoroughly with 20 column volumes (40 mL) with wash buffer (25 mM Tris (pH 7.4), 500 mM NaCl, 1 mM DTT, 5% Glycerol, 50 mM Imidazole), followed by elution with 1mL of elution buffer 1 (25 mM Tris pH 7.4, 200 mM NaCl, 1 mM DTT, 5% glycerol, 250 mM imidazole), twice. Subsequently, the remaining protein was eluted again with elution buffer 2 (25 mM Tris pH 7.4, 200 mM NaCl, 1 mM DTT, 5% glycerol, 500 mM imidazole), twice. The concentration of the purified protein solution was determined using the NanoDrop and Qubit protein BR assay kit (Invitrogen).

The collected fractions were mixed and submitted to a centrifugal ultrafiltration concentrator (10 kDa, Merck, Darmestadium, Germany) with storage buffer (200 mM Tris pH 8.0, 0.1 mM EDTA, 500 mM NaCl, 10% glycerol) at 4° C, 4,000 rpm for 20 minutes, twice, to achieve the highest concentration of purified recombinant protein, obtaining about 3 mL of protein in storage buffer.

Analysis by SDS-PAGE and Western Blotting

The eluted fractions and the concentrated protein were analyzed by SDS-PAGE and the sample was separated by electrophoresis in a 10% gel stained by Coomassie Brilliant Blue R-250 (Bio-Rad; Hercules, CA, USA). The presence of recombinant protein was analyzed by Western blotting, using rabbit anti-histidine polyclonal and anti-rabbit IgG-HRP antibodies (Santa Cruz, Dallas, TX, USA). In addition, a western blotting assay with the protein was performed with convalescent sera from COVID-19 positive and negative patients.

After electrophoresis, the separated samples on the gel were transferred to a nitrocellulose membrane under constant amperage of 250 mA for 1 hour. After transfer, the membrane was blocked with Tris-buffered saline (TBS) containing 5% skim milk and incubated for 1 hour at room temperature. The membrane was washed three times with TBS 0.1% tween-20 (Sigma Aldrich) (TBS-T) and TBS respectively. After washing, the primary rabbit anti-histidine polyclonal antibody (Santa Cruz) was diluted 1:500 in 2% skim milk in TBS solution and added to the membrane, followed by overnight incubation and three washes with TBS-T and TBS 1X. Next, the presence of a recombinant

his-tagged protein was revealed by incubation with an anti-rabbit IgG-HRP conjugated antibody (1:5000; Santa Cruz) and Opti-4CN Substrate Kit (Bio-Rad).

In another assay, the anti-histidine polyclonal antibody was replaced by COVID-19 convalescent human serum (1:2000) and a negative serum sample. The membranes were incubated overnight at 4°C, followed by 3 times washes with TBS-T and TBS 1X. The material was incubated with secondary anti-human IgG-HRP antibody (Santa Cruz) 1:5000 diluted in TBS plus 2% skim milk and incubated for 1 hour at room temperature. After the period, the membrane was washed again and incubated with Opti-4CN Substrate Kit (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA)

To perform the ELISA, a 96-well plate was sensitized with 50 µL of a 2 µg/mL recombinant N protein solution (100 ηg/well) diluted in phosphate-buffered saline (PBS) and incubated at 4° C overnight. For quality control, a commercially available N recombinant protein (Invitrogen) was also used. The plate was washed three times with PBS containing 0.05% Tween 20 (wash solution) and blocked with 200 µL per well of the blocking solution of PBS containing 3% skim milk and incubated at room temperature for 1 hour. Serum samples from RT-qPCR positive and negative patients were diluted at a ratio of 1:50 in a blocking solution with 1% skim milk. After the incubation time, the diluted samples were added to the plate and incubated at room temperature for 2 hours. After that, the plate was washed and the secondary anti-human-HRP (peroxidase) IgG antibody (Bio-Rad) diluted at a 1:5000 ratio was added and incubated for 1 hour at room temperature. Finally, TMB substrate was added (Scienco, Santa Catarina, Brazil) for 2 minutes, followed by phosphoric acid (1M) to stop the reaction. The plate was read on the (Multiskan FC; Skanit Software 6.0.2 for Microplate readers, Thermo Scientific, Waltham, MA, EUA), at 450 nm.

Statistical Analysis

The statistical analysis was performed in Graph Pad Prism version 8.0.1 (San Diego, CA, USA). To test the normal distribution of data, the Kolmogorov-Smirnov test was applied. Statistical difference between controls and positive samples with normal distribution was determined by the T-test ($p< 0.05$). The Receiver Operator Characteristic (ROC) curve and area under the curve were also calculated based on positive and negative samples. Sensitivity was calculated based on positive samples diagnosed by RT-qPCR and positive samples determined by the developed assay. Specificity was calculated based on negative samples collected before and during the COVID-19 pandemic, also diagnosed by RT-qPCR, and the negatives samples determined by the developed assay.