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RESEARCH

Validation of an ELISA for the quantification of the SARS-CoV-2 nucleocapsid protein

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ABSTRACT

SARS-CoV-2 virus has a capsid made up of the nucleocapsid protein (N-protein) that binds to viral genome to participate in the viral genetic material replication. The N-protein is also an immunodominant antigen, and thus it can be used as a suitable reagent for COVID-19 diagnosis and therapy. In this work, an enzyme-linked immunosorbent assay (ELISA) was validated, to accurately quantify the N-protein in human serum and research samples. For that purpose, experimental parameters were analyzed, including: the standard curve linear range, detection and quantification limits, specificity, accuracy, edge-effect and robustness. The standard curve linear range showed an r^2 of 99.54 % (n = 9) from 29.6 to 150 ng/mL, a detection limit equal to 19 ng/mL, a quantification limit equal to 29.6 ng/mL and a recovery ranged 93.50 ± 1.55 - 109.43 ± 1.71 %. The ELISA was specific, since it did not recognize non-related proteins: HBcAg, Vip3A, KLH and the monoclonal antibody CB.SCoV2-M20P19.24. The accuracy test average recovery ranged 93.50 ± 0.02 - 109.54 ± 0.29 %. Nevertheless, ELISA did not maintain its performance in the event of some analytical condition changes. As conclusions, the 4 hours-ELISA validated in this study is capable of quantify SARS-CoV-2 N-protein with high specificity and accuracy; and without interferences in phosphate buffered solution, human serum and cell culture medium samples (up to 29.6 ng/mL), although due to aggregation and degradation of N-protein, the ELISA is not able to quantify N-protein in HEK-293 cells bioreactor supernatant.

Keywords: Immunoassay, SARS-CoV-2, nucleocapsid protein, COVID-19

RESUMEN

Validación de un ELISA para la cuantificación de la proteína de la nucleocápsida del SARS-CoV-2. La proteína de la nucleocápsida (proteína N) del SARS-CoV-2 forma la cápsida viral, y se une al genoma viral e interviene en su replicación. Al ser un antígeno inmunodominante, puede usarse como blanco para el diagnóstico y la terapia contra la COVID-19. En este trabajo se validó un ensayo inmunoenzimático (ELISA) para cuantificar con precisión la proteína N en el suero y en muestras de investigación. Se analizó el rango lineal de la curva estándar, los límites de detección y cuantificación, la especificidad, la precisión, el efecto borde y la robustez. El rango lineal de la curva estándar mostró un r^2 = 99.54 % (n = 9) de 29.6 a 150 ng/mL, un límite de detección de 19 ng/mL, un límite de cuantificación de 29.6 ng/mL y un rango de recuperación de 93.5 ± 1,55-109,43 ± 1,71 %. El ELISA es específico, pues no reconoció proteínas no relacionadas: HBcAg, Vip3A, KLH, ni el anticuerpo monoclonal CB.SCoV2-M20P19.24. El recobrado promedio del ensayo de precisión osciló entre 93,50 ± 0,02 % y 109,54 ± 0,29 %. Sin embargo, el ELISA no mantuvo su rendimiento ante algunos cambios de condiciones experimentales. Este ELISA, validado y con una duración de 4 h, permite cuantificar la proteína N del SARS-CoV-2 con alta especificidad y precisión; y sin interferencias de muestras tales como solución tamponada con fosfato, suero humano y medio de cultivo celular (hasta 29,6 ng/mL). Debido a la agregación y la degradación de la proteína N, este ensayo no permite cuantificar la proteína N en el sobrenadante de células KEK-293 cultivadas en biorreactor.

Palabras clave: Inmunoensayo, SARS-CoV-2, proteína de la nucleocápsida, COVID-19

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Introduction

In December, 2019, cases of pneumonia of unknown etiology were detected in Wuhan, Hubei province, China. In January, 2020, the SARS-CoV-2 virus was identified as the causing agent and the disease named COVID-19. This disease spread out throughout the world rapidly, causing global instability and millions of diseased and dead persons [1-4].

SARS-CoV-2 is a virus encapsulated with a single-stranded ribonucleic acid (RNA) in positive sense, with a genome of approximately 30 kb. The genome encodes sixteen non-structural proteins, nine accessory proteins and four structural proteins: the spike-like (S), the envelope (E), the membrane (M) and the nucleocapsid (N) [5-7].

The N-protein is highly conserved among the Coronavirus genus, and it is one of the most abundant structural proteins found in virus-infected cells [8]. The fundamental function of N-protein is packaging viral RNA in a long-helical ribonucleocapsid complex through its interactions with viral genome and M-protein during virion assembly. Furthermore, the N-protein has been shown to be involved in host

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cellular machinery, such as interferon inhibition, RNA interference, and apoptosis playing an important regulatory role in the virus life cycle [9].

Since SARS-CoV-2 is highly contagious and the disease is severe with a relative high mortality rate, diagnostic testing for SARS-CoV-2 is an essential component of the overall strategy for preventing and controlling COVID-19. In this sense, the N-protein is an immunodominant antigen, supporting its use in COVID-19 diagnosis and as experimental and vaccine immunogen [10-13]. There is a wide information about methods used to diagnose COVID-19, among them, there are serology detection tests that help detecting, if a person has been infected by the virus, if has developed defenses, and whether or not infection is still active [14]. Within these diagnose tests, Enzyme-Linked Immunosorbent Assays (ELISA) allows analyzing samples usually within 24 hours with high specificity, sensitivity and accurate [15]. Therefore, attempts to establish an assay that would serve to quantify the N-protein in human serum and research samples have followed. In this line, a N-protein quantification ELISA was initially standardized based on a double-monoclonal antibody (mAb) sandwich-type ELISA in which N-protein is captured by the CB.SCoV-2.PN14 mAb and subsequently detected by the CB.SCoV-2.PN14 mAb conjugated to a horseradish peroxidase enzyme. However, this ELISA has not been validated yet.

Therefore, in order to verify the standardized test veracity, a validation study was carried out gathering evidences of compliance with regulatory guidelines. The assay was validated following the specifications of the Resolution No. 41/2007 of the Center for the State Control of Medicines, Equipment and Medical Devices (CECMED), as the Cuban national regulatory agency, and complying with the ICH guidelines requirements for the specific use [16-18].

Materials and methods

Biological reagents

The biological material used to make the standard curve was a N-protein reference material expressed in *E. coli* (code: R02512; SARS-CoV-2 Genbank accession No. MN908947.3), CB.ScoV-2PN14 mAb was used to coating the ELISA-plate. The CB.ScoV-2PN14 mAb-horseradish peroxidase conjugate was used as labelled antibody preparation. All biologicals were produced and supplied by the Center for Genetic Engineering and Biotechnology, Havana, Cuba.

Total protein quantification and purity determination

Total proteins were quantified by the Lowry method [19], and by 280 nm-optical densitometry method. The identity pattern and the purity of the N-protein and mAb CB.ScoV-2PN14 mAb were determined following the procedure described by Laemli [20].

CB.SCoV-2PN14 mAb production

The CB.SCoV-2PN14 mAb was produced using hybridoma cells seeded in spinner-flasks at a 0.4×10^6 cells/mL, and further incubated in an incubator (ASSAAB, Stockholm, Sweden), under a humid, 5 % CO_2 atmosphere, at 37 °C for 72 h. Next, the culture was inoculated into a Lambda MINIFOR bioreactor (LAMBDA Laboratory Instruments, Baar, Switzerland), and the culture let to proceed for 13 days. The protein free medium PFHM-II (Life Technologies, UK) was used, supplemented with 1 g/L of Pluronic F-68 (Sigma-Aldrich, St. Louis, USA), 2 mM L-glutamine, 1 mM pyruvate, 2.5 g/L HEPES, and phenol red. The bioreactor was operated in a fed-batch mode and maintained at 37 °C. All key parameters were automatically controlled, the amount of fish-tails submerged into the medium volume was 4 (5 cm of distance), and the fish-tail hardness used was 20 [21]. Supply of antibiotic, dissolved oxygen, antifoam, CO_2, base and acid were not required during the experiment. Cell concentration and viability were progressively estimated by the cell counting method, using a Neubauer chamber [22].

Culture of HEK-293 cells in the CelCradle™ Benchtop bioreactor

A CelCradle™-500 bioreactor bottle was filled with 450 mL of DMEN medium preheated at 37 °C, and supplemented with 10 % fetal bovine serum. Next, approximately 200×10^6 HEK-293 cells were resuspended in 50 mL of medium and added to the bioreactor. The CelCradleTM-500 bottle was subsequently transferred to an incubator, at 37 °C and under a 5 % CO_2 atmosphere. Cell anchorage to the BioNOC™ II macrocarrier was facilitated by adjusting its operational parameters as follows: rising rate (2 mm/s), top holding time (20 s), down rate (2 mm/s) and bottom holding time (0 s). After 5 h, the bioreactor parameters were changed to rising rate (1 mm/s), top holding time (30 s), down rate (1 mm/s) and bottom holding time (20 s).

Purification of the CB.SCoV-2PN14 mAb

The Lambda MINIFOR bioreactor supernatant was harvested, centrifuged at 4800 *g* for 20 min at 4 °C, and filtered through 0.22 µm. Next, the supernatant was applied to a Cytiva Protein A MabSelect™ SuRe™ matrix (Uppsala, Sweden), using an adsorption buffer (150 mM phosphate buffered solution (PBS), pH 8.0) and elution buffer (100 mM citric acid, pH 3.0). The matrix was loaded into a PD10 column (10 × 1.6 cm, i.d.; Amersham-Pharmacia Biotech, GE Healthcare Biosciences AB, USA), and the column was operated at a linear flow rate of 88 cm/h. Following three washings with 150 mM PBS; pH 8.0, the captured CB.ScoV-2PN14 mAb was eluted from the column with 100 mM citric acid, pH 6.0 (IgG1). Then, the sample buffer was changed to 20 mM Tris/150 mM NaCl, pH 7.6 and the sample processed by size-exclusion chromatography, using Sephadex G-25 coarse in a XK 16 column (Amersham-Pharmacia Biotech, GE Healthcare Biosciences AB, USA) operated at 130 cm/h. The eluted fractions were collected and filtered by 0.22 µm under sterile conditions [23].

Conjugation of the CB.SCoV-2PN14 mAb to the horseradish peroxidase

The anti-N-protein IgG was conjugated to the horseradish peroxidase according to Nakane and Kawaoi [24]. A proportion of 2 mg of CB.ScoV-2PN14 mAb per 1 mg of horseradish peroxidase was used. The

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conjugate was dialyzed in 150 mM PBS, pH 7.6 and further preserved with glycerol (50/50, v/v) and thimerosal 0.01 %, and stored at –20 °C until use.

Standardization of the ELISA procedure for N-protein quantification

Briefly, a high-binding (3590) flat-bottom 96-well plate (Costar, USA) was coated with 10 µg/mL CB.ScoV-2PN14 mAb, applying 100 µL/well of coating buffer (100 mM Na_2CO_3/100 mM $NaHCO_3$ mM/500 mM NaCl, pH 8.5), followed by incubation at 37 °C for 1 h. Next, the plate was washed five times with 150 mM PBS/Tween-20 0.05 %, pH 7.6. All washes were the same as described. Afterwards, 200 µL/well of blocking solution (150 mM PBS/Tween-20 0.05 %/skimmed milk 5 %, pH 7.6) was added and to avoid unspecific binding, and the plate was incubated at 37 °C for 1 h.

A standard curve of N-Protein was prepared, from a 0.97 mg/mL reference material solution, with dilutions ranging 29.6-150 ng/mL (29.6, 44.4, 66.7, 100 and 150 ng/mL) with 150 mM PBS/Tween-20 0.05%, pH 7.6. The standard curve was added to the plate, 100 µL/well, and incubated at 37 °C for 1 h. The 150 mM PBS/Tween-20 0.05% (pH 7.6) solution was used as blank. The plate was further washed five times, and the CB.SCoV-2PN14 mAb-horseradish peroxidase conjugate was applied at the optimal dilution in 150 mM PBS/Tween-20 0.05 %, pH 7.6, and incubated at 37 °C for 1 h. After washings, TMB (3, 3', 5, 5;-tetramethylbenzidine) was applied in substrate buffer, and 0.015 % hydrogen peroxide as chromogenic agent for the development of the assay. Absorbance was measured at 450 nm in Multiskan reader (LabSystem Multiskan MS type 352, Finland).

Validation of the standardized ELISA for N-protein quantification

Linear range

Nine N-protein concentrations ranging from 19.8 to 506 ng/mL were initially assessed to determine the linear range. Next, curve parameters (relationship between analyte concentration and response, expressed as absorbance at 450 nm) were determined with the curve prepared at concentrations of 19.8, 29.6, 44.4, 66.7, 100, 150, 225, 337, and 506 ng/mL; with three replicates per point, and running a minimum of three independent assays.

Specificity

Specificity was determined by comparing the results of the standard curves prepared by diluting the N-protein reference material and four non-related proteins (hepatitis B virus core protein, Vip3A, KLH and CB.SCoV2-M20P19.24 mAb). The same concentrations of the ELISA linear range (n = 3) were applied.

Accuracy

The test accuracy was estimated by calculating the recovery for each sample at three different concentrations of the N-protein reference material (29.6, 66.7 and 150 ng/mL). The edge-effect was evaluated measuring optical density variability between peripheral and central plate-wells, for the same concentrations of the standard curve.

Robustness

The assay robustness was studied at two 37 °C and 50 °C, for three incubation times (30, 45 and 60 min) and four coating concentrations (1.0, 2.5, 5.0, 10 µg/mL). The resulting validated standard curve was compared in different analytical matrices: 150 mM PBS (pH 7.6), human serum, cell culture medium (DMEN) and HEK-293 cell bioreactor supernatants.

Statistical analysis

Mathematical analyzes were performed using the software (EXCEL) and statistical analyzes were done using the Statgrahic Centurion XV Version 15.2.06 (Statistical Graphics Corp., USA). The standard curve was obtained with different standard concentrations of N-protein. Linearity was determined using the least-squares method, regression coefficient (r^2), y-intercept, slope of the regression line, and residual sum of squares were analyzed to stablish the working range between the highest and lowest concentration values with satisfactory accuracy (n = 3).

The linear range was established as the widest range with accuracy (recovery) in the range 80-120 %. The slopes and intercepts of the standard curves obtained in the specificity assay were compared by analysis of variance (ANOVA), also examining their regression coefficient (r^2). The accuracy was calculating using the Student's-t test with a confidence of 95 % for each sample. Sensitivity was defined as the capacity of the method to quantify the smallest amount of the analyte with acceptable accuracy and precision. The edge-effect data was statistically analyzed to determine the coefficient of variation (CV) and the differences of the mean OD of the outer wells versus that of the inner wells. In this case, recovery was tested for 29.6 ng/mL (low), 66.7 ng/mL (medium) and 150 ng/mL (high) values of concentration of the standard curve and confirmed with the Student's t-test. Robustness was measured by comparing the recovery of the selected points in experimental variables (80-120 %).

Results and discussion

COVID-19 pandemic may continue having a great impact on societies and economies worldwide [25]. Thus, the need for high-performance SARS-CoV-2 tests is still ongoing. Furthermore, SARS-CoV-2 detection systems can be used for quantification of SARS-CoV-2 proteins for research, which are expressed in different expression systems and bioreactors samples. Therefore, a standardized ELISA to quantify the SARS-CoV-2 N-protein was validated. The ELISA was designed for N-protein quantification, due to the large number of protein copies (~1000) per viral particle, the amount of mutations described in the SARS-CoV-2 S-protein and the need for N-protein quantification in bioreactor samples [26].

In order to validate the quantification of the N-protein in human serum and research samples, the N-protein reference material concentration was first corroborated at 280 nm in a spectrophotometer, with a 0.954 molar extinction coefficient. Next, the linear range was established, known as the ability of a test to generate results directly proportional to the properties of the analyte. The linear range was obtained from

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results of three standard curves with three replicates each, generated from a curve in the range of 19.8-506 ng/mL (19.8, 29.6, 44.4, 66.7, 100, 150, 225, 337, and 506). The statistical analysis performed on these values provided a probability value associated with a F-Snedecord of 0.0020, for a P value of the F-ratio ≥ 0.05, indicating the lack of statistically significant differences among means of three replicates, with a 95 % confidence level. Therefore, the linear range of the curve was set to 29.6-150 µg/mL, with an average regression coefficient of 0.9981 (Figure 1). Consequently, the lowest concentration of N-protein detected in the standard curve was 19.8 ng/mL, and a quantitation limit of 29.6 ng/mL with an acceptable accuracy (109.54 ± 0.292 and a CV of 5.29 %). Consequently, this ELISA is less sensitive than others developed for SARS-CoV-2 nucleocapsid detection, based on human IgM and IgG antibodies [27, 28]. This restrains its application to human serum containing high concentrations of nucleocapsid protein and research samples.

Regarding the test specificity, this important parameter is known as the ability of the assay to identify analytes in the presence of other predictable components of the target product. This parameter is more relevant for mAb-based assays used for protein quantification, because proteins are highly immunogenic and cross-reactions among antibodies and non-related proteins are largely common. In this study, the specificity was evaluated by applying samples of four non-related proteins: HBcAg, Vip3A, KLH and the CB.SCoV2-M20P19.24 mAb, under the conditions of the previously validated standard curve. These proteins were selected due to their unrelated biological origins, other than SARS-CoV-2, and, therefore, they will never induce a mAb that could recognize the SARS-CoV-2 N-protein. Also, their relatively large molecular sizes make available multiples epitopes on their respective surfaces, facilitating the assessment of any potential unspecific antibody binding.

Accordingly, none of the non-related proteins were recognized by the CB.SCoV-2PN14 mAb, specific for the SARS-CoV-2 N-protein, demonstrating the high specificity of ELISA (Figure 1). The use of this mAb is relevant, since the quantification of N-protein in human serum and plasma of infected patients can occur in the presence of N-protein-specific antibodies, at late stages of SARS-CoV-2 infection. At the same time, this statement is partially supported, because the CB.SCoV-2PN14 mAb is a murine rather than a human mAb. Therefore, other tests need to be done with human antibodies, to gather more evidences on the potential interference of N-protein specific human antibodies in the N-protein quantification by our test.

Another property of the assay is accuracy, intended as the ability of a given quantification method to provide results close to true values. For this aim, recovery values of the N-protein standard curve were analyzed in three concentration points within the validated linear range: 29.6, 66.7 and 150 ng/mL (Figure 1). As shown in Table 1, a general 108.54 ± 0.20 % recovery was obtained at the three concentration points of the standard curve, which is a permissible value according to both the CECMED No. 41/2007 and the ICH regulations [16-18]. Both regulatory agencies recommend recovery values in the

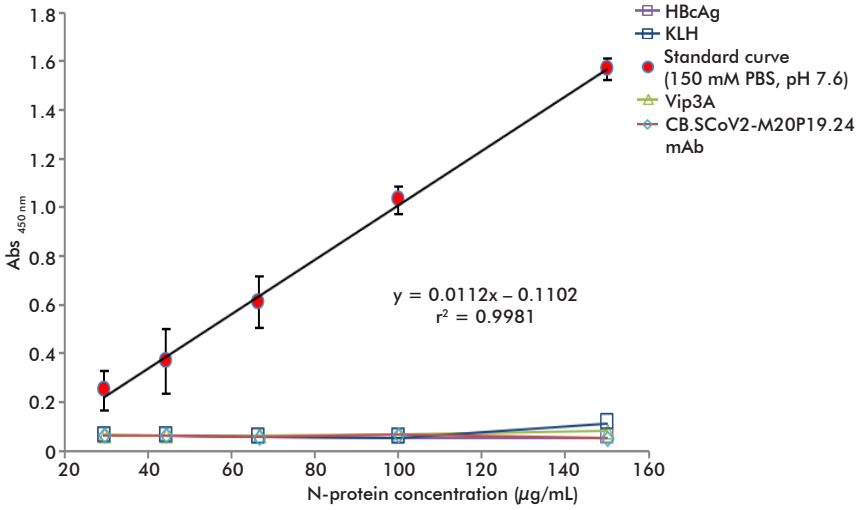

Figure 1. Standard curve of the ELISA validated to quantify the SARS-CoV-2 N-protein, in the linearity range 29.6-150 µg/mL (n = 3). The test stands for the specificity test against the N-protein (standard curve) and four unrelated antigens: hepatitis B core antigen (HBsAg), Keyhole limpet hemocyanin (KLH), *Bacillus thuringiensis* Vip3A protein, and the CB.SCoV-2.PN14 mAb against the SARS-CoV-2. Data is presented as values ± SD.

Table 1. Recovery of the validated ELISA test for the quantification of the SARS-CoV-2 N-protein in the accuracy test

Standard curve points (ng/mL)	Replicates			Average	Revovery (%)	CV (%)	T-calculated
	1	2	3				
Low (29.6)	34.37	31.79	31.10	32.42 ± 1.71	109.54 ± 0.292	5.29	4.88
Medium (66.7)	60.76	62.55	63.85	62.39 ± 1.55	93.5 ± 0.023	2.49	–7.47
High (150.0)	149.08	151.23	154.62	151.64 ± 2.79	101.10 ± 0.019	1.84	42.84

The t-critic was calculated for the three standard curve points, showing the same value (2.92).

80-120 % range. Furthermore, the lack of significant differences between expected concentration values and true values was also statistically verified, for a 0.05 significance level. The standard curve 66.7 ng/mL point showed the lowest recovery values (93.5 ± 0.023 %). Hence, the immunoassay is accurate to quantify N-protein in the validated range.

On the other hand, it is well known that the edge-effect causes variations in the absorbance values at the edges of the ELISA plates during incubation, affecting the homogeneity of results. To evaluate this effect, the assay was assessed by preparing three samples of N-protein with different concentrations of the validated standard curve: low (29.6 ng/mL), medium (66.7 ng/mL) and high (150 ng/mL). The respective samples were applied in different positions of ELISA-plate. Subsequently, the average and standard deviation (SD) of absorbance values were calculated for each testing point, located in the center of ELISA-plate. The upper and lower limits were defined from consecutive points applied to the edges of the ELISA-plate represented as the mean ± SD. The absorbance values obtained at the center were plotted, falling all between the lower and upper limits (–0.278 and 1.065, respectively) for the three concentration points: 150 ng/mL (0.85 ± 0.062, central/0.70 ± 0.052 edge), 66.7 ng/mL (0.22 ± 0.017, central/0.18 ± 0.0012 edge), 29.6 ng/mL (0.11 ± 0.004, central/0.10 ± 0.009 edge). This demonstrated the absence of an edge-effect in the immunoassay

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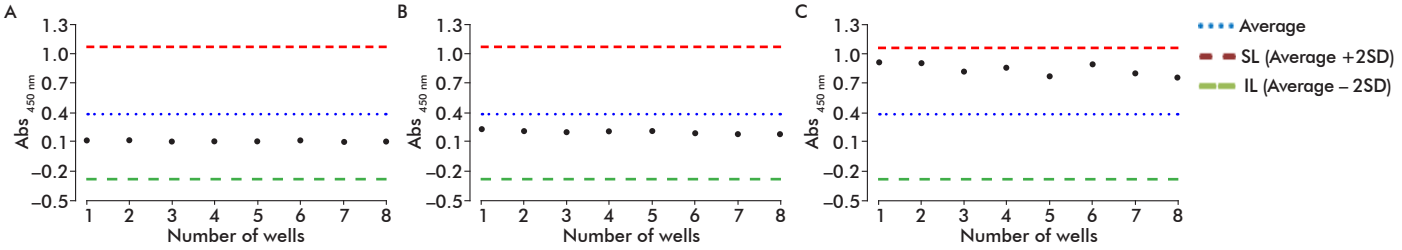

Figure 2. Performance of the edge-effect among wells of the plate of the ELISA assay for the quantification of the SARS-CoV-2 N-protein. The edge effect was determined, including the upper and lower limits (UL and LL, respectively), at three antigen concentrations: A) Low (29.6 ng/mL); B) Medium (66.7 ng/mL); C) High (150.0 ng/mL).

standardized for N-protein quantification, under the incubation conditions used (Figure 2).

Regarding the robustness, it is defined as the ability of assays to maintain its performance in the event of analytical condition changes, where factors cause minor fluctuations and significant variations of the assay, which could theoretically affect the reliability of results. In this sense, analytical conditions were subjected to experimental changes, aimed to validate this parameter in the studied ELISA, at different mAb coating concentration (1.0 2.5, 5.0, 10 µg/mL), sample application temperature (37 and 50 °C), and incubation time (30 and 45 min, and 1 h). The main criteria used to accept ELISA robustness were $r^2 > 0.9800$ and 80-120 % recovery. In each variant, the N-protein was quantified, with three replicates for each point of the standard curve. Results are shown in table 2. This analysis also revealed a decrease in the slopes of standard curves associated to the increase of the temperature in the coating step from 0.0111 to 0.0029. A similar phenomenon was observed when sample temperature and incubation time were modified in the sample application step. The slopes of the standard curve also decreased from 0.0111 to 0.005, with changes done in the sample's application step. Robustness was also tested at different mAb coating concentrations (Table 3). As results, the r^2 of all assays fulfilled the approval pre-established criterion ($r^2 \geq 98.00$). However, a very high average CV was calculated at the lowest mAb coating concentrations, 58.97 % (2.5 µg/mL) and 56.85 (1.0 µg/mL). Therefore, these two coating conditions cannot be used in the validated ELISA.

Finally, an ELISA applicability study was additionally done, to determine the influence of the analytical matrices on the N-protein quantification (150 mM PBS, pH 7.2; human serum of a healthy individual; DMEN culture medium, pH 7.9; HEK-293 cell bioreactor supernatant, pH 7.2; HEK-293 cell bioreactor supernatant, pH 7.2 after exchange in gel-filtration chromatography; HEK-293 cell bioreactor supernatant, pH 7.2/Tween-20 0.05%; HEK-293 cell bioreactor supernatant, pH 7.2, heated 5 min at 100 °C; HEK-293 cell bioreactor supernatant, pH 6.0. All these sample matrices were spiked with N-protein in the range from 29.6 to 150 ng/mL. Three N-protein standard curves from each analytical matrix were performed, to calculate the mean and SD of each standard curve point. The absorbance values obtained at 450 nm for each evaluated sample are shown in Figure 3. The standard curves of N-protein diluted in serum and DMEN culture medium, pH 7.9, allowed to estimate

Table 2. Results of the robustness analysis of the ELISA to detect the SARS-CoV-2 N-protein at two different temperatures and storage times during the coating and sample application steps

Step	T (°C)	Time (h)	Slope	Intercept	r^2	Revovery (%)	F-Snedecor (0.005)	P
Control Coating	37	1:00	0.0111	–0.0931	0.9954	101.00 ± 0.052	0.0021	0.9979
	50	0:30	0.0029	–0.0523	0.9614	104.00 ± 0.215	0.0245	0.9759
		0:45	0.0070	–0.1363	0.9733	100.00 ± 0.111	0.4947	0.5547
		1:00	0.0042	–0.1387	0.8349	107.00 ± 0.366	0.1100	0.7717
Sample application	50	0:30	0.0021	0.0110	0.9650	104.00 ± 0.178	1.1700	0.3711
		0:45	0.0005	0.0773	0.8659	93.00 ± 0.391	2.1300	0.2005
		1:00	0.0006	0.0874	0.8559	96.00 ± 0.243	1.4900	0.2976

Table 3. Results of the robustness analysis of the ELISA to detect the SARS-CoV-2 N-protein at different coating concentrations

MAb concentration (ng/mL)	Expected values (ng/mL)	True values (ng/mL)	SD	Recovery (%)	Average recovery (%)	CV (%)	Average CV (%)
1.0	150.0	142.35	15.38	95.00		59.37	
	100.0	113.62	7.89	114.00		48.25	
	66.7	66.11	18.08	99.00	98.24	56.46	56.89
	44.4	42.79	2.79	96.00		57.69	
	29.6	25.81	14.85	87.00		62.68	
2.5	150.0	132.17	3.34	88.11		63.64	
	100.0	126.85	8.25	126.85		41.36	
	66.7	73.55	30.28	110.27	95.91	50.11	58.97
	44.4	33.33	7.71	75.07		71.54	
	29.6	23.45	12.79	79.23		68.20	
5.0	150.0	174.08	15.14	115.00		10.51	
	100.0	111.71	10.22	108.00		7.82	
	66.7	60.03	3.29	96.00	103.86	7.44	6.78
	44.4	42.05	4.60	104.00		3.84	
	29.6	31.46	3.69	97.00		4.31	
10.0	150.0	151.64	2.79	101.10		0.77	
	100.0	103.70	3.32	103.70		2.57	
	66.7	62.39	1.55	93.54	100.89	4.72	3.39
	44.4	42.88	2.14	96.58		2.46	
	29.6	32.42	1.71	109.54		6.43	

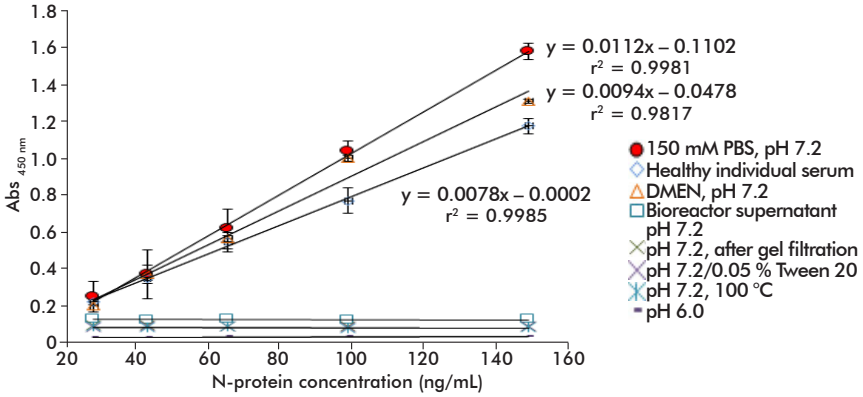

Figure 3. Standard curve of the ELISA validated to quantify the SARS-CoV-2 N-protein in different analytical matrices.

their r^2 values, 0.9985 and 0.9981, respectively. Nevertheless, quantification of N-protein diluted in HEK-293 cell bioreactor supernatants could not be quantified when the bioreactor supernatant was heated ($r^2 = 0.005$), by adding 0.05 % Tween-20 to the sample ($r^2 = 0.3014$) or when pH of the bioreactor supernatant was changed ($r^2 = 0.1635$), after gel filtration chromatography in Sephadex-25 ($r^2 = 0.0608$), or in the bioreactor supernatant, pH 7.2 ($r^2 = 0.1191$).

This phenomenon excludes the use of the validated ELISA to quantify the N-protein in HEK-293 cell bioreactor supernatants. This could be related to the aggregation and degradation of the N-protein, as detected by GF-HPLC in bioreactor supernatant samples (Figure 4).

Conclusions

The validated 4-h ELISA supports the assessment of the SARS-CoV-2 N-protein to a concentration of up to 29.6 ng/mL with specificity, high accuracy and no interferences due to the phosphate buffer, human serum or cell culture medium components. The validated 4-h ELISA status is only valid under the conditions tested, while not changing the coating temperature, sample incubation time and mAb coating concentration as established. Particularly, the 4-h ELISA cannot be used to quantify the N-protein in HEK-293 cell culture supernatants, due to the high aggregation and degradation seen for this protein in that type of sample.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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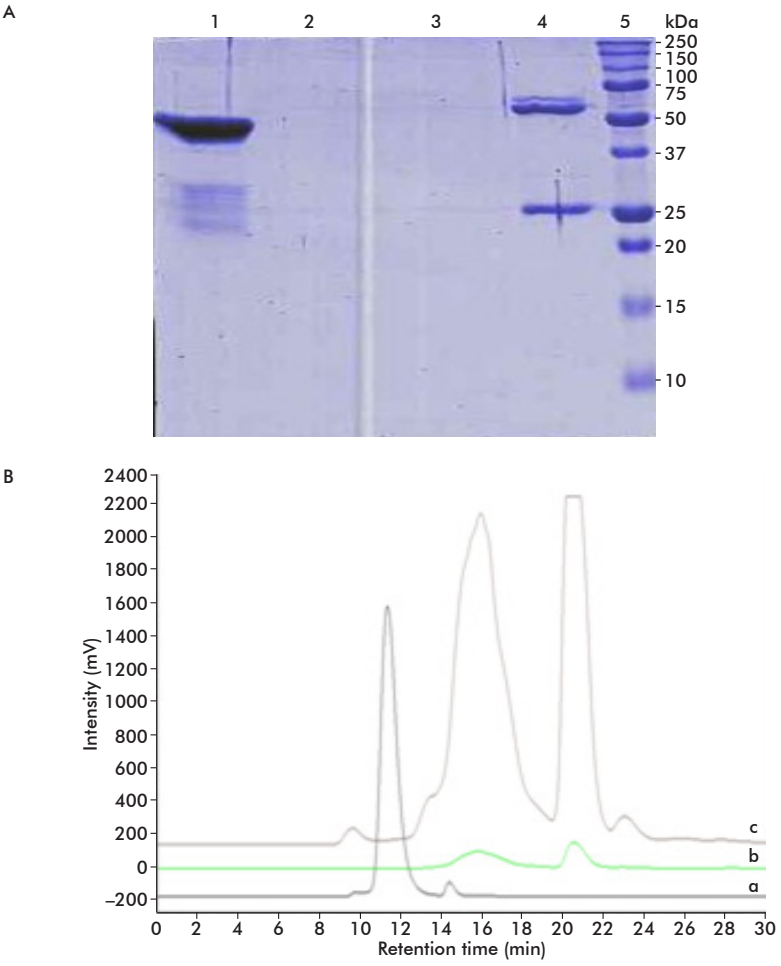

Figure 4. SDS-PAGE and GF-HPLC of proteins used in the ELISA against the SARS-CoV-2 N-protein. A) SDS PAGE. Lanes: 1, N-protein used as reference material; 2, Cell culture medium (DMEN); 3, N-Protein in HEK-293 cell bioreactor supernatant (aggregated), pH 7.2; 4, Purified CB.ScoV-2PN14 mAb; 5, Molecular weight marker (BIO-RAD, USA, Catalog # 161-0373). B). GF-HPLC profiles: a) CB.ScoV-2PN14 mAb; b) HEK-293 culture supernatant; c) SARS-CoV-2 N-protein diluted in HEK-293 culture supernatant