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Development and Analytical Validation of an In-House Quantitative Indirect ELISA for Detection of IgG Antibodies Against SARS-CoV-2 Spike Protein: A Pilot Study

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Abstract

The COVID-19 pandemic highlighted critical gaps in serological diagnostic capacity in low- and middle-income countries, particularly the strategic dependence on imported commercial kits. Here we report the development, optimisation, and analytical validation of an in-house quantitative indirect enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of IgG antibodies against the SARS-CoV-2 Spike protein (S1-S2). Four antigen coating concentrations (1, 2, 3 and 5 µg/mL) and three serum dilutions (1:50, 1:100 and 1:1000) were systematically evaluated. An internal calibration standard was prepared from a pool of six convalescent sera. Analytical validation followed CLSI EP17-A criteria and included linearity assessment, intra- and inter-assay precision (coefficient of variation, CV%), and analytical limits (limit of blank [LOB], limit of detection [LOD], and limit of quantification [LOQ]). Preliminary diagnostic performance was evaluated on an independent pilot cohort of 20 sera (10 RT-PCR-confirmed COVID-19, 10 pre-pandemic). The optimal conditions were antigen coating at 2 µg/mL and serum dilution 1:100. The calibration curve showed strong linearity (R^2 = 0.996; y = 0.0050x + 0.0793). Intra-assay CVs ranged from 4.1% to 6.6% and inter-assay CVs from 2.7% to 8.8%, both within acceptance criteria (≤10% and ≤15%, respectively). All analytical limits were well below positive signals. On the pilot cohort, the assay achieved sensitivity of 90.0% (95% CI: 59.6–98.2%), specificity of 90.0% (95% CI: 59.6–98.2%), and diagnostic accuracy of 90.0% (95% CI: 69.9–97.2%), with an area under the receiver operating characteristic curve (AUC-ROC) of 0.92. These preliminary results suggest the feasibility of locally developing a quantitative ELISA platform applicable to serological surveillance of SARS-CoV-2 and, more broadly, to future in-house immunoassay development for emerging infectious diseases.

Introduction

The emergence of SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) in late 2019 and the ensuing COVID-19 pandemic resulted in over 770 million confirmed cases and 6.9 million deaths globally as of 2023[1]. While real-time reverse transcription PCR (RT-PCR) rapidly became the gold standard for acute infection diagnosis[2], serological assays proved essential for understanding humoral immune dynamics, conducting seroprevalence studies, assessing vaccine-induced immunity, and supporting long-term epidemiological surveillance[3–5].

Antibody responses to SARS-CoV-2 are directed primarily against the Spike glycoprotein (S), which mediates viral entry through binding to the angiotensin-converting enzyme 2 (ACE2) receptor[6,7]. IgG antibodies against the S1 and S2 Spike subdomains are detectable from 2–3 weeks post-infection, persist for months, and represent reliable serological markers for both infection history and vaccine response assessment[8,9]. The Spike protein is therefore considered the antigen of choice for ELISA-based serological assays[7,10,11].

In Morocco, as in many low- and middle-income countries (LMICs), the COVID-19 pandemic exposed a critical strategic vulnerability: dependence on imported commercial diagnostic kits, which were subject

to supply disruptions, prohibitive costs, and logistical delays during the most critical phases of the pandemic[12,13]. This dependence highlighted the urgent need to develop national immunodiagnostic platforms capable of supporting research, seroepidemiological surveillance, and pandemic preparedness independently. The development of locally validated quantitative ELISA assays represents a strategic response to this challenge, as demonstrated by comparable initiatives in Ethiopia[14], Uganda[15], and Ghana[16].

Although numerous commercial SARS-CoV-2 serological assays are available[17,18], in-house ELISA development offers critical advantages in LMIC settings: reduced reagent costs, adaptability to locally available equipment, flexibility for antigen modification in response to emerging variants, and capacity building for future immunoassay development[15,16]. The reference protocols established by Stadlbauer et al.[19], Bittaye et al.[20] and Amanat et al.[21] have provided robust methodological frameworks that have been adapted across multiple laboratories worldwide.

Here we report the development, systematic optimisation, and complete analytical validation of an in-house quantitative indirect ELISA for the detection and quantification of IgG antibodies against the SARS-CoV-2 Spike protein (S1-S2 subdomains). Specifically, we describe the establishment of an internal calibration curve enabling antibody quantification in arbitrary units per millilitre (AU/mL), the systematic optimisation of critical experimental parameters including antigen coating concentration and serum working dilution, and the comprehensive analytical validation of the method covering linearity, intra- and inter-assay precision, and analytical limits (LOB, LOD, and LOQ) according to CLSI EP17-A criteria. Preliminary diagnostic performance was assessed on an independent pilot cohort of 20 sera using RT-PCR as the reference method, with sensitivity, specificity, and discriminant capacity evaluated by ROC analysis. Beyond its immediate application to SARS-CoV-2 serology, this work establishes a complete, reproducible methodological platform for in-house quantitative immunoassay development that is directly applicable to future emerging and re-emerging infectious disease threats, a strategic priority for national diagnostic capacity building in Morocco and comparable resource-limited settings.

Results

1. Calibration curve and linearity

The internal calibration curve constructed from serial dilutions of the convalescent serum pool demonstrated strong linearity across the full quantification range (0–400 AU/mL), with the regression equation OD_{405} = 0.0050 × [AU/mL] + 0.0793 and a coefficient of determination R^2 = 0.996 (Table 1, Fig. 1). CVs at individual calibration points decreased progressively with increasing concentration, ranging from 6.3% at the zero standard to 1.5% at 400 AU/mL, all within the 10% acceptance criterion.

Table 1
Calibration curve parameters of the in-house anti-Spike SARS-CoV-2 ELISA.

Concentration (AU/mL)	OD_{405} mean ± SD	CV (%)	Calculated OD (regression)
0	0.079 ± 0.005	6.3	0.079
25	0.205 ± 0.008	3.9	0.204
50	0.329 ± 0.010	3.0	0.329
100	0.581 ± 0.015	2.6	0.579
200	1.081 ± 0.021	1.9	1.079
400	2.079 ± 0.032	1.5	2.079
R^2 (linear regression)	0.996	—	y = 0.0050x + 0.0793

Values are mean ± SD of three independent measurements. AU/mL, arbitrary units per millilitre. R^2, coefficient of determination.

2. Dose-response evaluation and optimal serum dilution

Dose-response curves generated from five RT-PCR-confirmed COVID-19 sera across three serum dilutions (1:50, 1:100, 1:1000) and four coating concentrations (1, 2, 3, 5 µg/mL) revealed a consistent proportional relationship between antibody concentration and OD_{405} signal (Fig. 2). Three analytical zones were identified: a saturation zone at 1:50 where signals for highly reactive sera (P2, P5) approached the OD ceiling, compromising quantification precision; a linear zone at 1:100 where the response was proportional to antibody concentration; and a low-response zone at 1:1000 where sensitivity was insufficient for weakly positive sera. The 1:100 dilution was therefore selected as the working serum dilution for all subsequent assays (Fig. 3).

3. Antigen coating concentration optimisation

Comparison of IgG concentrations interpolated from the calibration curve for five COVID-19-positive sera at the optimal 1:100 serum dilution across four coating concentrations showed that 2 µg/mL provided the highest mean maximum antibody concentration (AUmax: 132.0 ± 70.6 AU/mL), while 1 µg/mL gave substantially lower responses (30.6 ± 9.4 AU/mL). Concentrations of 3 µg/mL (101.4 ± 39.9 AU/mL) and 5 µg/mL (83.4 ± 30.5 AU/mL) did not improve upon 2 µg/mL, suggesting saturation of plate-binding sites (Table 2, Fig. 4). The 2 µg/mL concentration was adopted for all subsequent analyses.

Table 2
Anti-Spike IgG concentrations (AU/mL ± SD) by antigen coating concentration at the optimal 1:100 serum dilution.

Serum	1 µg/mL	2 µg/mL	3 µg/mL	5 µg/mL	AUmax (AU/mL)
P1	25 ± 2	88 ± 4	78 ± 3	62 ± 3	88 (at 2 µg/mL)
P2	42 ± 3	225 ± 8	145 ± 6	115 ± 5	225 (at 2 µg/mL)
P3	30 ± 2	105 ± 5	102 ± 4	85 ± 4	105 (at 2 µg/mL)
P4	18 ± 1	57 ± 3	50 ± 2	45 ± 2	57 (at 2 µg/mL)
P5	38 ± 2	185 ± 7	132 ± 5	110 ± 5	185 (at 2 µg/mL)
Mean ± SD	30.6 ± 9.4	132.0 ± 70.6	101.4 ± 39.9	83.4 ± 30.5	—

Values are mean ± SD of independent triplicates. AUmax, maximum antibody concentration obtained across the four coating concentrations for each serum.

4. Analytical limits: LOB, LOD and LOQ

Blank measurements (assay wells containing all reagents except anti-Spike antibodies) performed in triplicate from five pre-pandemic sera at all four coating concentrations yielded low, homogeneous OD_{405} values (0.057 ± 0.002 to 0.088 ± 0.004). Analytical limits calculated according to CLSI EP17-A guidelines[21] were: LOB 0.060–0.095, LOD 0.063–0.102, and LOQ 0.077–0.128 OD_{405} (Table 3, Fig. 5). All limits were well below the minimum positive signal observed (COV9: OD_{405} = 0.17), confirming adequate analytical sensitivity across all coating concentrations tested.

Table 3
Analytical limits (LOB, LOD, LOQ) of the in-house anti-Spike SARS-CoV-2 ELISA (OD_{405}).

Parameter	Formula (CLSI EP17-A)	1 µg/mL	2 µg/mL	3 µg/mL	5 µg/mL
Blank (mean ± SD)	—	0.058 ± 0.003	0.066 ± 0.003	0.076 ± 0.004	0.087 ± 0.004
LOB	Mean + 1.645 × SD	0.063	0.071	0.082	0.093
LOD	LOB + 1.645 × SD	0.068	0.076	0.089	0.100
LOQ	Mean + 10 × SD	0.088	0.096	0.115	0.126

LOB, limit of blank; LOD, limit of detection; LOQ, limit of quantification. All values in OD_{405}. Individual blank OD_{405} triplicates and calculated analytical limits per serum are provided in Supplementary Table S1

5. Analytical precision

Intra-assay CVs calculated from triplicate measurements across twelve combinations of coating concentration and serum dilution ranged from 4.1% to 6.6%, all below the 10% acceptance criterion (Table 4). The lowest intra-assay CV (4.1%) was obtained at 2 µg/mL/1:50 and 5 µg/mL/1:50, and the highest (6.6%) at 3 µg/mL/1:1000.

Inter-assay CVs evaluated from three independent analytical series performed on different days under optimal conditions (2 µg/mL, 1:100) ranged from 2.7% to 3.8% between individual patient sera, and from 6.6% to 8.8% across three response levels (low, intermediate, and high responders), all within the 15% acceptance criterion (Tables 5–6, Fig. 6).

Table 4
Intra-assay precision (repeatability) of the in-house anti-Spike SARS-CoV-2 ELISA.

Ag (µg/mL)	Dilution	Replicate 1	Replicate 2	Replicate 3	Mean ± SD (OD_{405})	CV (%)
1	1:50	0.42	0.44	0.46	0.44 ± 0.02	4.5
1	1:100	0.30	0.31	0.33	0.31 ± 0.02	4.9
1	1:1000	0.11	0.12	0.12	0.12 ± 0.01	4.8
2	1:50	1.05	1.10	1.14	1.10 ± 0.05	4.1
2	1:100 *	0.84	0.88	0.92	0.88 ± 0.04	4.5
2	1:1000	0.24	0.25	0.27	0.25 ± 0.02	6.0
3	1:50	1.12	1.17	1.22	1.17 ± 0.05	4.3
3	1:100	0.78	0.81	0.85	0.81 ± 0.04	4.4
3	1:1000	0.22	0.23	0.25	0.23 ± 0.02	6.6
5	1:50	1.18	1.23	1.28	1.23 ± 0.05	4.1
5	1:100	0.70	0.73	0.77	0.73 ± 0.04	4.8
5	1:1000	0.20	0.21	0.22	0.21 ± 0.01	4.8

** Optimal condition selected. All intra-assay CVs (4.1–6.6%) are within the ≤ 10% acceptance criterion.*

Table 5
Inter-assay precision per patient (optimal conditions: 2 µg/mL, 1:100, n = 3 independent series).

Serum	Series 1 (AU/mL)	Series 2 (AU/mL)	Series 3 (AU/mL)	Mean ± SD	CV (%)
P1	85	88	91	88 ± 3	3.4
P2	220	225	231	225 ± 6	2.7
P3	102	105	109	105 ± 4	3.8
P4	55	57	59	57 ± 2	3.5
P5	181	185	190	185 ± 5	2.7

Table 6
Inter-assay precision by response level (n = 3 independent series).

Response level	Series 1 (AU/mL)	Series 2 (AU/mL)	Series 3 (AU/mL)	Mean ± SD	CV (%)
Low responder	68	81	74	74.3 ± 6.5	8.8
Intermediate responder	148	169	158	158.3 ± 10.5	6.6
High responder	272	318	289	293.0 ± 23.2	7.9

All inter-assay CVs (2.7–8.8%) within the ≤ 15% acceptance criterion.

6. Preliminary diagnostic performance

The positivity threshold (cut-off) was established from a set of ten pre-pandemic sera (PP1–PP10; mean OD_{405} = 0.097 ± 0.018, range 0.071–0.126) that were designated exclusively for cut-off determination and were independent of the ten pre-pandemic sera used in the diagnostic validation cohort. The derived cut-off was 0.151 OD_{405} (mean + 3 × SD). None of the ten pre-pandemic cut-off sera exceeded this threshold (Table 7, Fig. 7).

Table 7
Reactivity of pre-pandemic sera used for cut-off determination.

Sample	Replicate 1	Replicate 2	Replicate 3	Mean ± SD (OD_{405})	Classification
PP1	0.065	0.072	0.076	0.071 ± 0.006	TN
PP2	0.075	0.083	0.091	0.083 ± 0.008	TN
PP3	0.073	0.079	0.082	0.078 ± 0.005	TN
PP4	0.092	0.102	0.112	0.102 ± 0.010	TN
PP5	0.062	0.069	0.076	0.069 ± 0.007	TN
PP6	0.078	0.087	0.096	0.087 ± 0.009	TN
PP7	0.109	0.121	0.133	0.121 ± 0.012	TN
PP8	0.070	0.076	0.082	0.076 ± 0.006	TN
PP9	0.073	0.081	0.089	0.081 ± 0.008	TN
PP10	0.084	0.094	0.104	0.094 ± 0.010	TN
Overall mean	—	—	—	0.097 ± 0.018	—
Cut-off (mean + 3×SD)	—	—	—	0.151 OD_{405}	0/10 above threshold

TN, true negative. PP, pre-pandemic serum. Cut-off = 0.097 + (3 × 0.018) = 0.151 OD_{405}. Individual OD_{405} triplicates per serum are provided in Supplementary Table S2.

7. Diagnostic performance on the pilot cohort

On the independent validation cohort of 20 sera, the assay correctly identified 9 of 10 RT-PCR-confirmed COVID-19 positive sera and 9 of 10 pre-pandemic negative sera (Table 8). One false negative (COV10; OD_{405} = 0.12 ± 0.02, below cut-off) was observed in a serum with very low reactivity, possibly reflecting a weak or early humoral response. One false positive (PP7; OD_{405} = 0.128 ± 0.014, borderline) was observed in the pre-pandemic group, potentially attributable to low-level cross-reactivity with endemic coronaviruses[22][23] (Fig. 8).

Table 8
Contingency table and diagnostic performance indicators (pilot cohort, n = 20).

Indicator	Formula	Value (95% CI)
True positives (TP)	—	9
False negatives (FN)	—	1 (COV10)
True negatives (TN)	—	9
False positives (FP)	—	1 (PP7)
Sensitivity	TP/(TP + FN)	90.0% (95% CI: 59.6–98.2%)
Specificity	TN/(TN + FP)	90.0% (95% CI: 59.6–98.2%)
Positive predictive value	TP/(TP + FP)	90.0% (95% CI: 59.6–98.2%)
Negative predictive value	TN/(TN + FN)	90.0% (95% CI: 59.6–98.2%)
Diagnostic accuracy	(TP + TN)/N	90.0% (95% CI: 69.9–97.2%)
AUC-ROC	ROC analysis	0.92

95% CIs calculated using the Wilson score method. AUC-ROC, area under the receiver operating characteristic curve. Individual OD_{405} values for each COVID-19 validation serum are provided in Supplementary Table S3

8. Receiver Operating Characteristic (ROC) Analysis

ROC curve analysis on the full pilot cohort (n = 20) yielded an AUC of 0.92 (Fig. 9), indicating strong discriminant capacity between RT-PCR-positive COVID-19 and pre-pandemic negative sera, exceeding the threshold of 0.90 for a test with enhanced diagnostic performance[24].

Discussion

In this study we developed, optimized, and analytically validated an in-house quantitative indirect ELISA for the detection of IgG antibodies targeting the SARS-CoV-2 Spike protein (S1-S2) in human serum. The assay demonstrated strong analytical performance across all parameters evaluated, and produced promising preliminary diagnostic results on a pilot cohort, supporting its potential utility for serological research and epidemiological surveillance in resource-limited settings.

The calibration curve (R^2 = 0.996) established using a convalescent serum pool as internal standard is consistent with those reported by Bittaye et al.[20], Stadlbauer *et al.*[19] and Vernet *et al.*[25], confirming the robustness of the linear quantification approach over the 0–400 AU/mL range. The use of an internal standard with results expressed in arbitrary units (AU/mL) is a widely adopted strategy for in-house

ELISA quantification[19,25], though harmonisation with the WHO International Standard expressed in Binding Antibody Units (BAU/mL) represents a relevant future step to enable inter-laboratory comparability[26,27].

The systematic optimisation identified 2 µg/mL antigen coating and 1:100 serum dilution as optimal parameters, consistent with values reported in comparable in-house ELISA protocols[19,25,28]. The plateau observed at concentrations above 2 µg/mL suggests saturation of plate-binding sites, as previously described for microplate-based immunoassays[19]. The selection of the 1:100 dilution maintained signal responses within the linear dynamic range, avoiding the saturation effects observed at 1:50 and the sensitivity loss at 1:1000.

Analytical precision was robust, with intra-assay CVs of 4.1–6.6% and inter-assay CVs of 2.7–8.8%, both well within standard acceptance thresholds for quantitative immunoassays (≤ 10% and ≤ 15%, respectively) [21,29]. These values are comparable to, or better than, those reported for similar in-house ELISA platforms[25,28]. The low LOQ values (maximum 0.128 OD_{405}), well below the minimum positive signal observed (0.17 OD_{405}), confirm that the assay has sufficient sensitivity to detect even low-level antibody responses, which is critical for seroprevalence studies in populations with heterogeneous immune responses.

Preliminary diagnostic validation on the pilot cohort yielded sensitivity and specificity of 90.0% (95% CI: 59.6–98.2%) and diagnostic accuracy of 90.0% (95% CI: 69.9–97.2%), with an AUC-ROC of 0.92. These figures are consistent with performance metrics reported for comparable in-house ELISA assays developed in LMICs[14,15,16]. The single false negative (COV10) likely reflects an attenuated humoral response, a phenomenon well documented in patients with mild COVID-19 or early-stage infection[30,31]. The single false positive (PP7) may be attributable to low-level cross-reactivity with endemic human coronaviruses, a known limitation of assays using full-length Spike protein as the capture antigen[22,23].

The most important limitation of this study is the small pilot cohort size (n = 20 for diagnostic validation), which yields wide confidence intervals for sensitivity and specificity estimates. This prevents definitive characterisation of the diagnostic performance of the assay, which will require validation on a substantially larger, clinically diverse cohort. Additionally, the diagnostic cohort did not include subjects vaccinated against COVID-19, patients infected with SARS-CoV-2 variants, or individuals with other respiratory infections that may cause cross-reactive responses. The cohort size limitation is, however, inherent to the staged design of diagnostic assay development. At this phase, the primary objective was analytical validation, not clinical validation; a cohort of 20 well-characterised samples is sufficient to demonstrate proof-of-concept discriminant capacity and to identify gross assay failures, consistent with established frameworks for staged immunoassay development and with comparable in-house ELISA studies from LMIC settings. Large-scale clinical validation on a diverse cohort of ≥ 200 samples constitutes the immediate next step of this research programme. The use of arbitrary units rather than WHO-harmonised BAU/mL units limits inter-laboratory comparison. Additionally, inter-assay precision was evaluated from only three independent analytical series; although all CVs were within acceptance

criteria, a larger number of series (as recommended by CLSI EP05-A3) would provide a more robust estimate of reproducibility. Finally, single-site, single-operator evaluation precludes assessment of inter-operator and inter-laboratory reproducibility.

The absence of a head-to-head comparison with a commercial assay reflects the developmental stage of this work rather than a conceptual limitation. Comparative evaluation is methodologically meaningful only once the in-house assay has been analytically characterised and shown to meet predefined performance criteria which the present study indicates. Furthermore, the use of arbitrary units (AU/mL) rather than WHO-harmonised BAU/mL precludes direct quantitative comparison without a prior harmonisation step. Benchmarking against validated commercial platforms (e.g. EUROIMMUN Anti-SARS-CoV-2 IgG, Roche Elecsys Anti-SARS-CoV-2 S) is planned as an integral component of the clinical validation phase.

Beyond its immediate application to SARS-CoV-2 serology, this study suggests a complete and reproducible methodological workflow for in-house quantitative ELISA development, covering systematic optimisation, full analytical validation according to CLSI EP17-A criteria, and preliminary diagnostic performance assessment. This workflow is directly transferable to the development of serological assays targeting other emerging or re-emerging infectious agents, representing a strategic contribution to national diagnostic sovereignty and pandemic preparedness capacity in Morocco and comparable settings in the African and MENA regions[12,13]. Future priorities include large-scale clinical validation on a diverse cohort encompassing vaccinated subjects, infections caused by SARS-CoV-2 variants, and conditions liable to generate cross-reactive responses; inter-operator and inter-laboratory reproducibility assessment; harmonisation against the WHO First International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC 20/136) to enable expression of results in Binding Antibody Units per millilitre (BAU/mL)[26,27]; and longitudinal evaluation of antibody persistence following natural infection or vaccination. In conclusion, this work provides preliminary evidence that a quantitative immunoassay can be developed, optimised, and analytically validated within a national research institution in a resource-limited setting, and that the methodological platform established here constitutes a durable scientific asset for infectious disease diagnostic research and outbreak response preparedness.

Material and Methods

1. Study design and biological samples

This was a single-centre, laboratory methodological study conducted at Institut Pasteur du Maroc (Casablanca, Morocco), designed to develop and analytically validate an in-house quantitative indirect ELISA for the detection of IgG antibodies against the SARS-CoV-2 Spike protein. The study followed a two-phase design. The first phase (development and analytical validation) used 16 sera to systematically optimise assay parameters and assess analytical performance. The second, independent phase (preliminary diagnostic validation) used 20 sera to generate a pilot estimate of diagnostic sensitivity, specificity, and discriminant capacity relative to RT-PCR as the reference method.

A total of 36 residual human serum samples were provided by Laboratoire d'Analyses Médicales Mohamed Amine (Casablanca, Morocco). Samples were classified into three groups according to their intended use. Pre-pandemic negative controls (n = 15; 5 for analytical validation, 10 for diagnostic validation) were collected before December 2019 and had no documented exposure to SARS-CoV-2, serving as the reference negative population for both specificity assessment and positivity cut-off determination. RT-PCR-confirmed COVID-19 positive sera (n = 15; 5 for analytical validation, 10 for diagnostic validation) were obtained from patients with virologically confirmed SARS-CoV-2 infection and used to assess assay sensitivity and positive signal characteristics. Convalescent COVID-19 sera (n = 6) were obtained from patients who had recovered from documented COVID-19 infection with high anti-Spike reactivity; these were combined to prepare the internal calibration standard.

All samples were residual sera from routine diagnostic activities at the source laboratory. They were irreversibly anonymised prior to transfer to Institut Pasteur du Maroc, and no nominative, clinical, or personal identifiable data were accessible to investigators at any stage of the study. No specific patient recruitment was performed and no additional biological material was collected for the purposes of this research. Samples were aliquoted and stored at − 20°C immediately upon receipt and maintained at this temperature until use to prevent freeze-thaw degradation of immunoglobulins.

2. Antigens, reagents, and consumables

The ELISA was developed using a recombinant SARS-CoV-2 Spike protein encompassing the S1 and S2 subdomains (Atheris Laboratories, Geneva, Switzerland) as the solid-phase capture antigen. This protein was selected because the S1-S2 Spike region is the primary target of the neutralising humoral response to SARS-CoV-2 and constitutes the antigenic reference for serological assay development[7–10]. Detection of antigen-bound IgG was achieved using a goat anti-human IgG secondary antibody (γ-chain-specific) conjugated to alkaline phosphatase (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA), selected for its high sensitivity and compatibility with colorimetric pNPP-based revelation.

Immunoassays were performed in 96-well flat-bottom polystyrene microplates with high protein-binding capacity (NUNC MaxiSorp™, Thermo Fisher Scientific, Roskilde, Denmark), which provide a consistent hydrophobic surface for passive antigen adsorption. All aqueous solutions were prepared in phosphate-buffered saline (PBS 1×, pH 7.4; Gibco™, Thermo Fisher Scientific, Waltham, MA, USA), used as the coating and dilution buffer. Interwell washing was performed with PBS supplemented with 0.05% (v/v) Tween® 20 (PBS-T; Sigma-Aldrich, USA) to remove non-specifically bound proteins while preserving specific antigen–antibody interactions. Non-specific binding sites were saturated using Blocker™ Casein in PBS (Thermo Fisher Scientific, USA), a casein-based blocking agent selected for its low background performance with alkaline phosphatase-conjugated detection systems.

Enzymatic signal revelation was carried out using 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP; Sigma-Aldrich, USA) as the chromogenic substrate for alkaline phosphatase. Hydrolysis of pNPP by the enzyme generates para-nitrophenol, a yellow product whose absorbance at 405 nm is directly

proportional to the quantity of bound IgG. Enzymatic reactions were terminated by addition of 1 M sulphuric acid (H_2SO_4; prepared in-house from analytical-grade reagent), which shifts the product absorbance and stops further enzymatic activity.

3. Equipment

Absorbance readings at 405 nm were obtained using an ELx800™ absorbance microplate reader (BioTek Instruments, Winooski, VT, USA) equipped with a 405/10 nm bandpass filter, providing a dynamic OD range of 0.000–3.999. Interwell plate washing was automated using an ELx50™ microstrip washer (BioTek Instruments, USA), configured to perform three aspiration–dispensing cycles per wash step, ensuring consistent removal of unbound reagents and minimising inter-well variability attributable to manual washing. All liquid dispensing was performed using calibrated Eppendorf® single-channel and multi-channel micropipettes (Eppendorf SE, Hamburg, Germany), verified against gravimetric standards prior to use. Reagents and sera were maintained at 4°C and − 20°C, respectively, between experiments. All statistical analyses, ROC curve analysis, AUC calculation, and figure generation were performed using Python (version 3.11) with the matplotlib (version 3.8), scipy (version 1.11), numpy (version 1.26), and scikit-learn (version 1.3) libraries."

4. ELISA protocol and parameter optimisation strategy

The ELISA protocol was adapted from the reference procedure described by Bittaye et al.[19], with systematic optimisation of the two parameters most critical to assay performance: antigen coating concentration and serum working dilution. Four coating concentrations (1, 2, 3, and 5 µg/mL) and three serum dilutions (1:50, 1:100, and 1:1000) were evaluated in a factorial design across all five COVID-19-positive and five pre-pandemic development sera, in independent triplicates. This approach allowed simultaneous assessment of signal intensity, linearity, signal-to-noise ratio, and inter-serum discrimination across a full matrix of conditions.

All assay steps were performed at room temperature (20–25°C) unless otherwise indicated, and each incubation step was followed by three automated washing cycles with PBS-T. Microplates were coated by adding 100 µL per well of recombinant Spike protein (S1-S2) diluted in PBS 1× at the concentration under evaluation, followed by overnight incubation at 4°C to allow passive adsorption of the antigen to the microplate surface. After washing, non-specific binding sites were blocked by incubation with 200 µL per well of Blocker™ Casein for 1 hour, a step essential for minimising background absorbance and ensuring the specificity of subsequent antibody detection.

Serum samples, diluted in PBS-T at 1:50, 1:100, or 1:1000 as appropriate, were added at 100 µL per well and incubated for 1 hour to allow specific IgG binding to the immobilised Spike antigen. Following three wash cycles to remove unbound immunoglobulins, bound anti-Spike IgG were detected by adding 100 µL per well of the alkaline phosphatase-conjugated anti-human IgG secondary antibody diluted 1:10,000 in

PBS-T, followed by 1 hour incubation. After a final wash series, enzymatic revelation was initiated by adding 100 µL per well of pNPP substrate solution and incubating for exactly 15 minutes at room temperature, protected from direct light exposure. Reactions were stopped by addition of 50 µL per well of 1 M H_2SO_4, and OD_{405} was measured within 10 minutes of stopping to prevent signal drift. All samples were processed in independent triplicates. Blank control wells, containing all assay components with the exception of anti-Spike antibodies, were included in each run and used for analytical limit determination.

5. Internal calibration standard and antibody quantification

To enable quantitative expression of anti-Spike IgG concentrations, an internal calibration standard was prepared from a pool of six convalescent COVID-19 sera, selected on the basis of their high reactivity against the Spike protein, as previously recommended for in-house ELISA standardisation[19–25]. The six sera were mixed in equal volumes, thoroughly homogenised, aliquoted into single-use volumes, and stored at − 80°C to prevent protein degradation and ensure batch-to-batch stability across analytical series.

The pooled standard was subjected to twofold serial dilution in PBS-T to generate a six-point calibration curve spanning 0 to 400 AU/mL (arbitrary units per millilitre). The highest standard concentration was assigned an arbitrary value of 400 AU/mL based on empirical signal intensity observed during preliminary optimisation, with subsequent dilution points set at 200, 100, 50, 25, and 0 AU/mL. Although expressed in arbitrary units rather than WHO-harmonised Binding Antibody Units (BAU/mL), this approach provides a reproducible internal quantification framework consistent with comparable in-house ELISA studies[19–25]. Harmonisation with the WHO First International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC 20/136) is planned as a future step to enable inter-laboratory comparability[26–27]. All calibration points were assayed in independent triplicates within each analytical series. A linear regression model (OD_{405} as a function of AU/mL concentration) was fitted to the mean calibration values using least-squares regression, and IgG concentrations in test samples were determined by interpolation from this curve.

6. Analytical validation

Analytical validation was conducted according to the criteria defined by the Clinical and Laboratory Standards Institute (CLSI, guideline EP17-A)[21] and the formulas described in the CLSI EP17-A[21] guideline. Four performance parameters were evaluated:

- Linearity was assessed by fitting a linear regression model to the calibration curve OD_{405} values across the full 0–400 AU/mL quantification range. The coefficient of determination R^2 was used as the primary indicator of linear fit quality, with $R^2 \geq 0.99$ set as the acceptance criterion, consistent with standard practice for quantitative immunoassays.

- Intra-assay precision (repeatability) was evaluated by calculating the coefficient of variation (CV%) from triplicate OD_{405} measurements obtained within a single analytical run for each experimental condition. CV% was calculated as (SD/mean) × 100. The acceptance criterion was CV ≤ 10%, in accordance with established guidelines for quantitative ELISA methods
- Inter-assay precision (reproducibility) was assessed from three independent analytical series, each conducted on a different day, under the optimal conditions identified during the optimisation phase (antigen coating 2 µg/mL; serum dilution 1:100). CV% was calculated from the three series for each serum, and separately for three response-level categories (low, intermediate, and high responders) to characterise precision across the full dynamic range of the assay. The acceptance criterion was CV ≤ 15%.
- **Analytical limits** : limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ), were determined from blank well OD_{405} values measured in triplicate from five pre-pandemic sera across all four coating concentrations, using the following formulas: LOB = mean(blanks) + 1.645 × SD(blanks); LOD = LOB + 1.645 × SD(blanks); LOQ = mean(blanks) + 10 × SD(blanks). These formulas, derived from parametric tolerance intervals, quantify the analytical noise floor of the assay and define the minimum signal distinguishable from background with defined statistical confidence.

7. Preliminary diagnostic performance evaluation

Diagnostic performance was evaluated on the independent pilot cohort of 20 sera under the optimised assay conditions. The positivity threshold (cut-off) was established empirically from the ten pre-pandemic negative sera as: cut-off = mean (OD_{405} of negative sera) + 3 × SD. This approach, widely adopted for indirect ELISA serology[19–28], sets a threshold above which the probability of a signal arising from background noise alone is less than 0.1%, thereby minimising false-positive classification in populations without prior SARS-CoV-2 exposure. Test results were classified as positive (OD_{405} > cut-off) or negative (OD_{405} ≤ cut-off) and compared against RT-PCR status as the reference diagnostic standard.

The following performance metrics were calculated from the resulting 2 × 2 contingency table: sensitivity = TP/(TP + FN); specificity = TN/(TN + FP); positive predictive value (PPV) = TP/(TP + FP); negative predictive value (NPV) = TN/(TN + FN); and diagnostic accuracy = (TP + TN)/N total, where TP, FP, TN, and FN denote true positives, false positives, true negatives, and false negatives, respectively. Ninety-five percent confidence intervals (CIs) for all proportional metrics were computed using the Wilson score method, which provides more accurate coverage probability than the standard Wald interval, particularly for small samples or extreme proportions[32]. Global discriminant capacity was quantified by receiver operating characteristic (ROC) analysis, with the area under the curve (AUC) calculated and interpreted according to Zweig and Campbell[24], using Python (scikit-learn library, version 1.3). An AUC > 0.90 was considered indicative of good diagnostic performance.

Declarations

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Competing Interests

The authors declare no competing interests.

Ethical Statement

This study was conducted exclusively on residual, irreversibly anonymised human serum samples collected during routine diagnostic activities at Laboratoire d'Analyses Médicales Mohamed Amine (Casablanca, Morocco). All samples were fully anonymised prior to transfer to Institut Pasteur du Maroc, and no nominative, clinical, or personal identifiable data were accessible to the investigators at any stage of the study. No specific patient recruitment was performed, no additional biological samples were collected, and no intervention was conducted on any participant. Institutional authorisation for the use of these anonymised residual samples for research purposes was granted by the Director of Laboratoire d'Analyses Médicales Mohamed Amine (written authorisation on file). Under Moroccan regulations governing the use of residual anonymised biological materials in non-interventional methodological research, formal ethics committee review and individual informed consent were not required for this study. All procedures involving human samples were conducted in accordance with the ethical principles of the Declaration of Helsinki (2013 revision) and with applicable national regulations. All methods were performed in accordance with relevant institutional guidelines and regulations.

Data Availability Statement

The datasets generated and analysed during the current study are not publicly available due to ethical restrictions governing the use of anonymised residual human biological samples under the institutional data sharing policy of Laboratoire d'Analyses Médicales Mohamed Amine (Casablanca, Morocco), but are available from the corresponding author upon reasonable request. A summary of all numerical data underlying the figures and tables in this article is provided as Supplementary Information (Supplementary Tables S1–S4).

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Figures

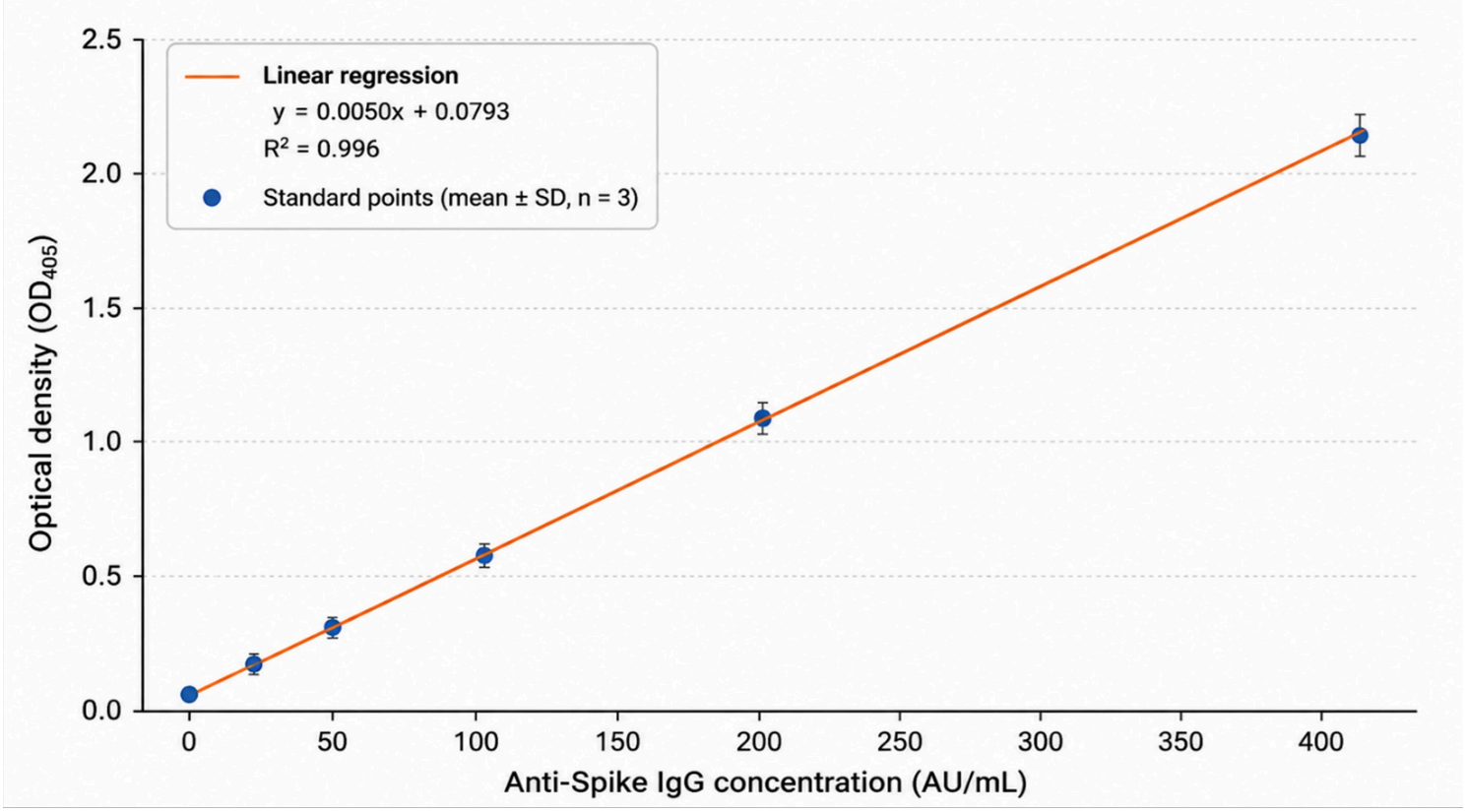


Figure 1

Internal calibration curve of the in-house anti-Spike SARS-CoV-2 ELISA. Linear relationship between OD_{405} and anti-Spike IgG concentration (AU/mL) over the range 0–400 AU/mL. Points represent mean ± SD of triplicate measurements from serial dilutions of the convalescent serum pool. The regression line ($R^2 = 0.996$; $y = 0.0050x + 0.0793$) and 95% confidence interval (shaded area) confirm excellent method linearity throughout the quantification range.

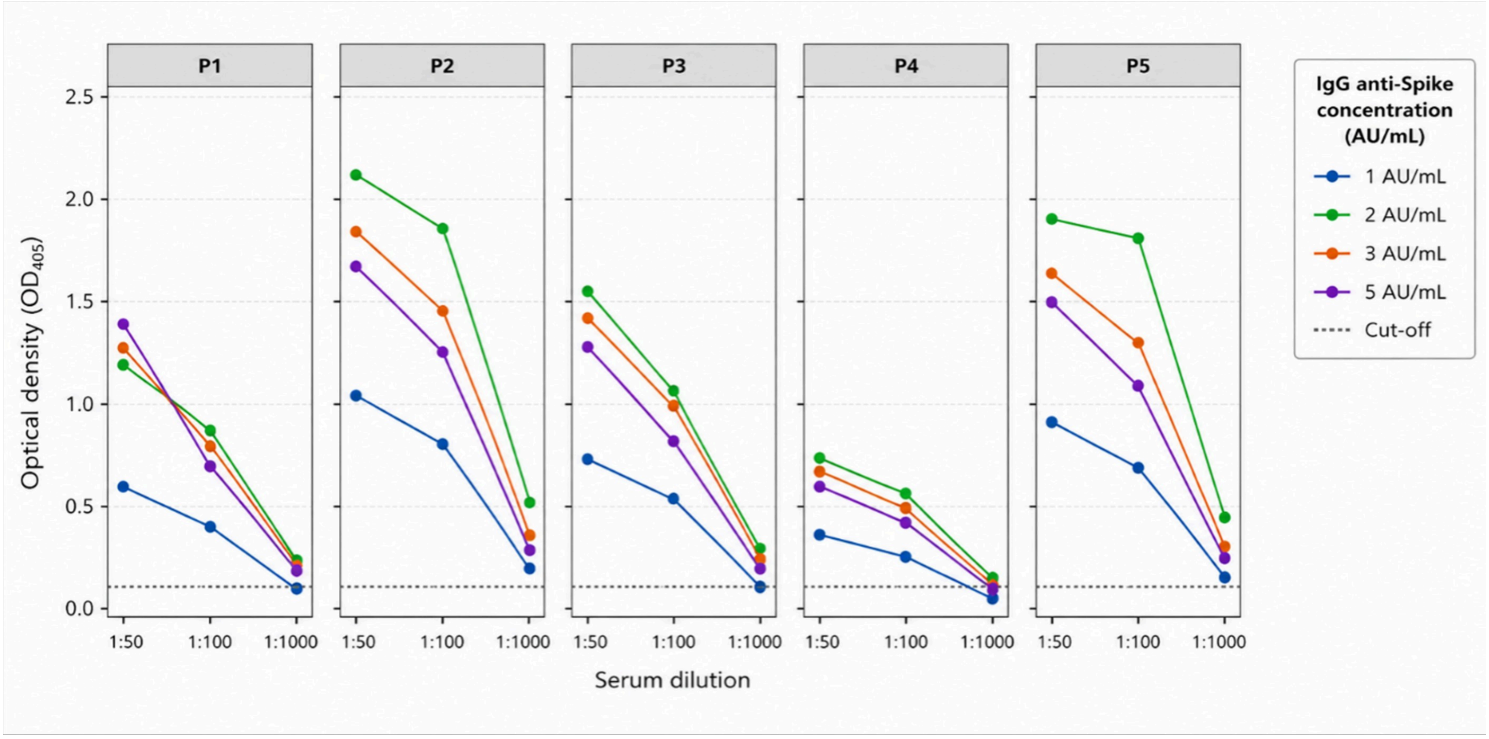


Figure 2

Dose-response curves of the anti-Spike SARS-CoV-2 ELISA. OD_{405} values for five COVID-19-positive sera (P1–P5) at dilutions 1:50, 1:100, and 1:1000 under four antigen coating concentrations (1, 2, 3, 5 µg/mL). Data are mean of independent triplicates. The dashed horizontal line indicates the positivity threshold (cut-off = 0.151 OD_{405}). Three analytical zones are defined: saturation (1:50), optimal linear range (1:100), and low-response (1:1000).

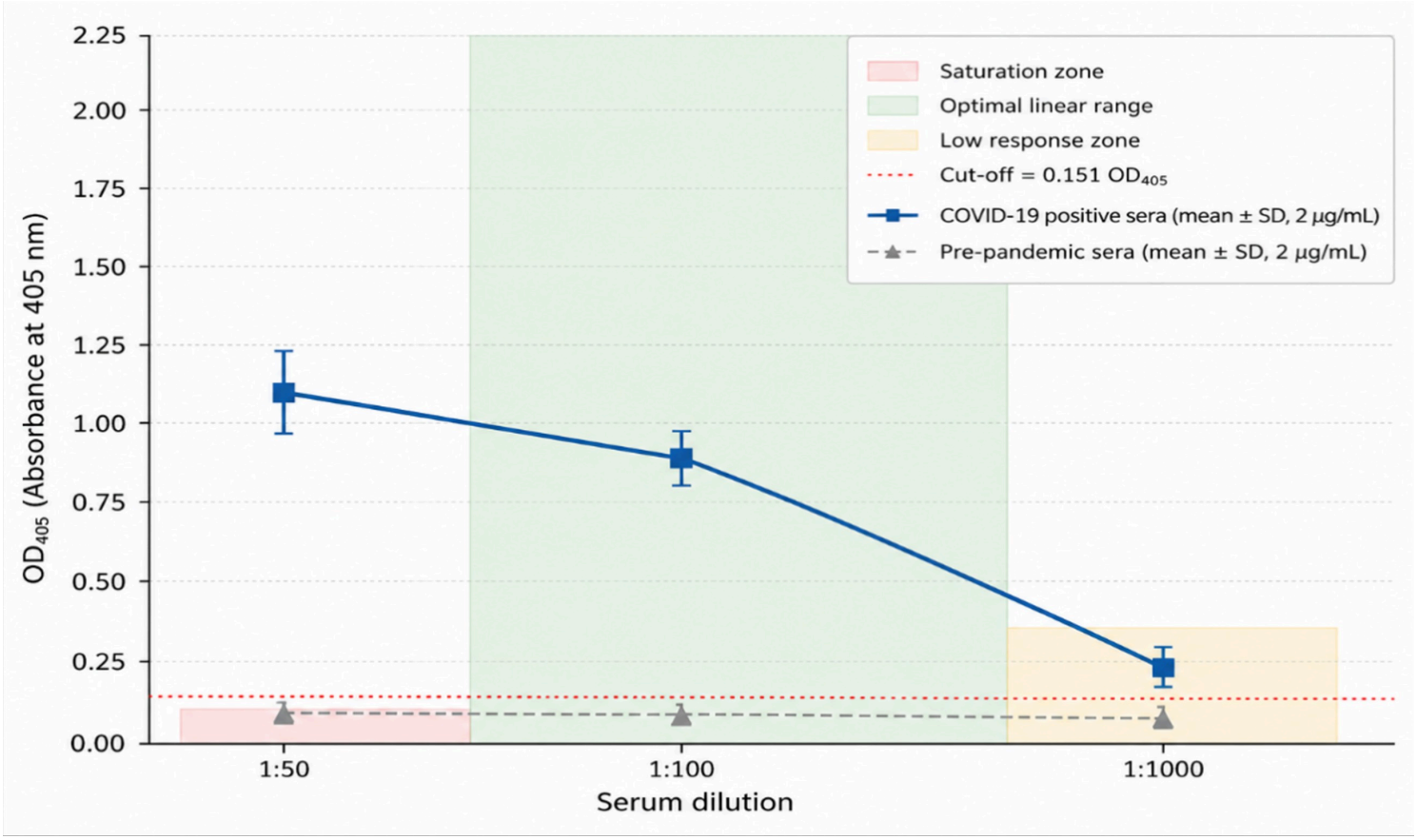


Figure 3

Determination of the optimal serum dilution. OD_{405} as a function of serum dilution illustrating the three analytical zones. The 1:100 dilution was selected as the working dilution based on optimal signal intensity, linearity, and discrimination between positive and negative sera. Error bars represent SD.

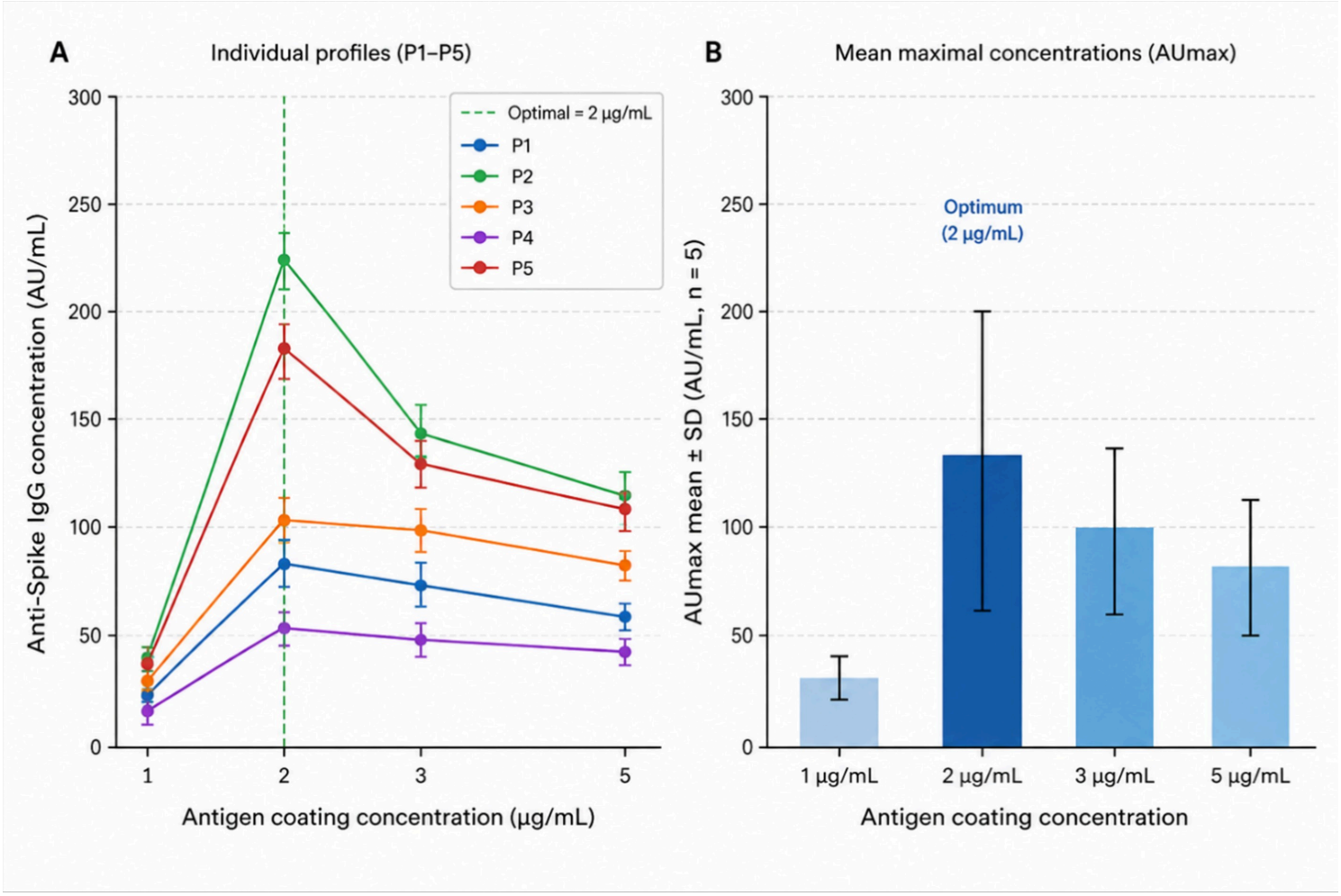


Figure 4

Optimisation of the recombinant Spike protein (S1-S2) coating concentration. (A) Individual anti-Spike IgG concentration profiles (AU/mL) for five COVID-19-positive sera (P1–P5) across four coating concentrations at the 1:100 serum dilution. (B) Mean AUmax ± SD (n = 5). The 2 µg/mL condition provided the highest mean quantitative response for all individual sera and was selected as the optimal coating concentration.

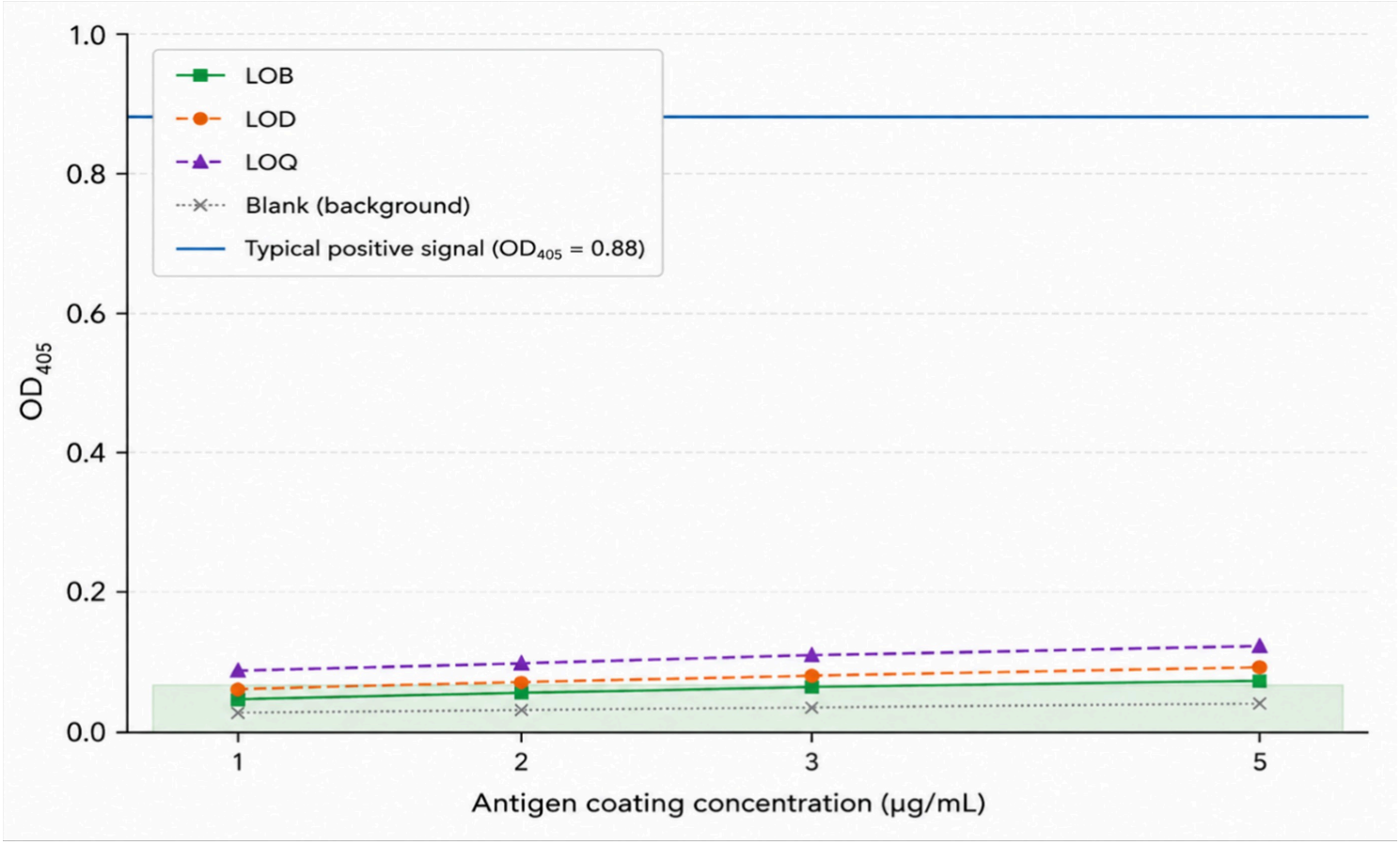


Figure 5

Analytical limits (LOB, LOD, LOQ) of the in-house ELISA across coating concentrations. All limits remained well below the minimum positive signal observed (OD_{405} = 0.17 for the weakest positive serum at 2 µg/mL/1:100), confirming adequate analytical sensitivity. The horizontal blue dashed line represents the typical positive signal at optimal conditions (OD_{405} = 0.88).

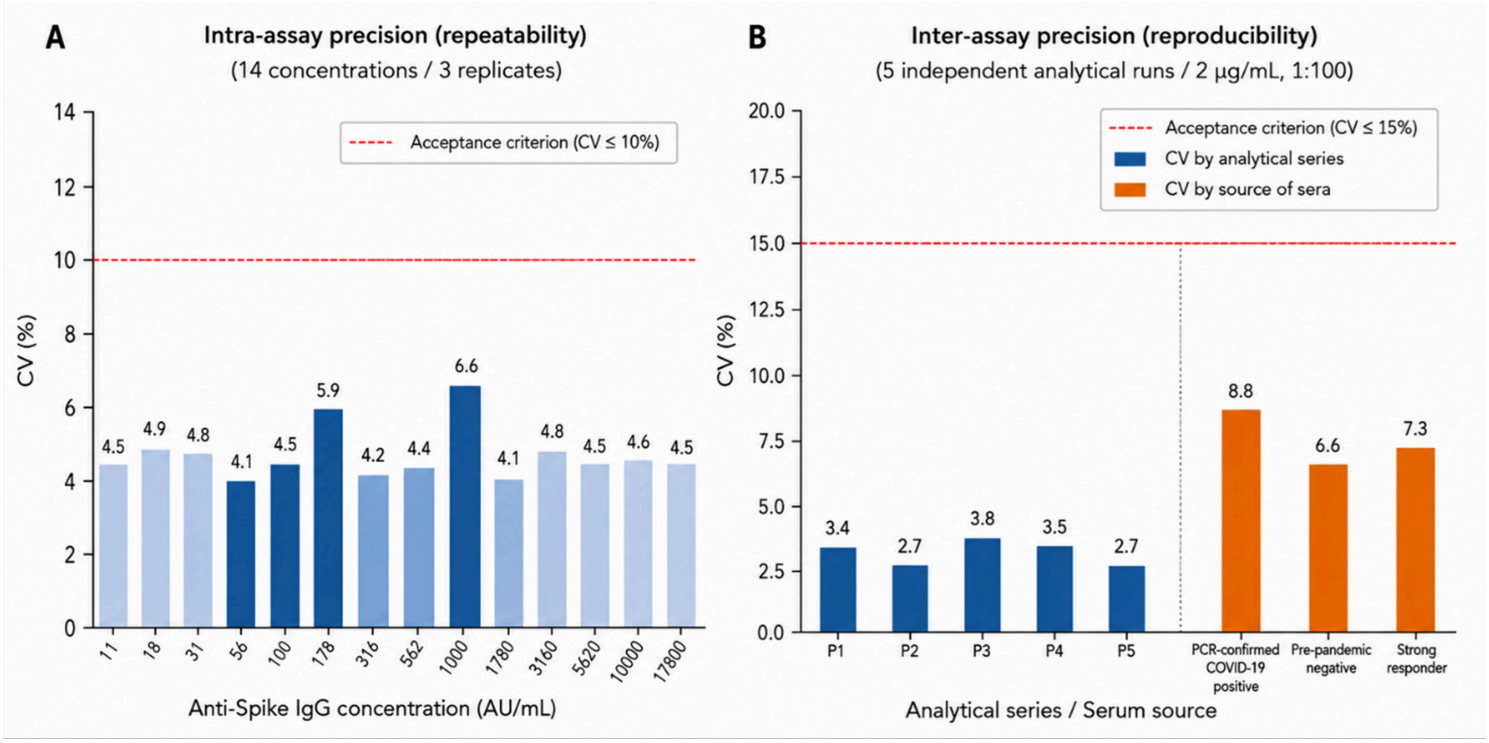


Figure 6

Analytical precision of the in-house anti-Spike SARS-CoV-2 ELISA. (A) Intra-assay CVs (%) across all coating concentration and serum dilution combinations (4.1–6.6%). (B) Inter-assay CVs by individual serum (2.7–3.8%) and by response level (6.6–8.8%). Dashed red lines indicate acceptance thresholds (10% for intra-assay; 15% for inter-assay). All criteria were met.

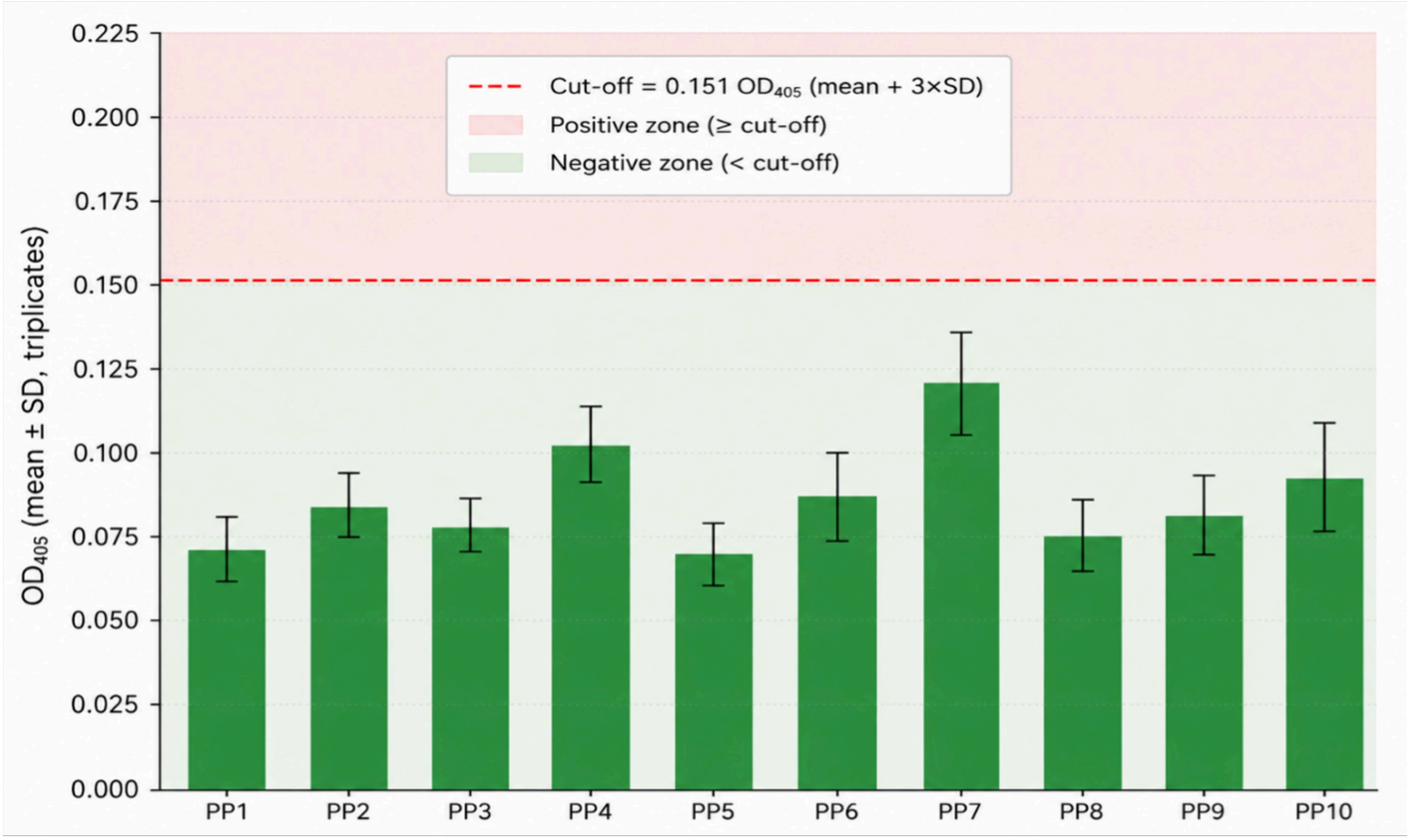


Figure 7

Cut-off determination from pre-pandemic sera. OD_{405} (mean ± SD) of ten pre-pandemic sera (PP1–PP10) analysed in triplicate under optimal assay conditions (2 µg/mL coating; 1:100 serum dilution). The red dashed line indicates the positivity threshold (cut-off = 0.151 OD_{405} = mean + 3 × SD). No pre-pandemic serum exceeded the threshold, yielding zero false positives at the cut-off.

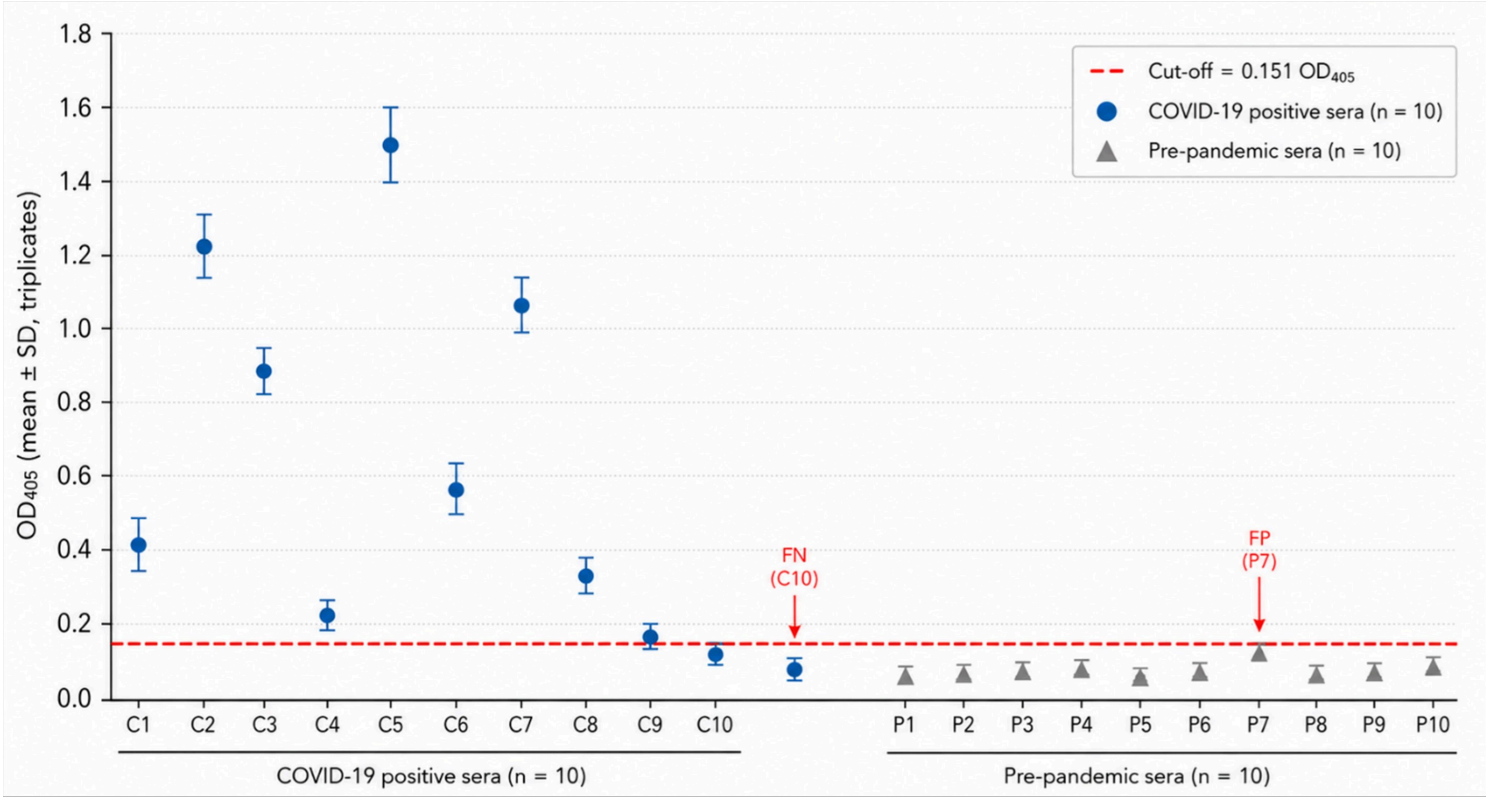


Figure 8

Preliminary diagnostic performance of the in-house anti-Spike SARS-CoV-2 ELISA. OD_{405} (mean ± SD) for RT-PCR-confirmed COVID-19 sera (C1–C10, blue circles) and pre-pandemic sera (P1–P10, grey triangles). The red dashed line indicates the positivity cut-off (0.151 OD_{405}). FN: false negative (COV10, OD_{405} = 0.12); FP: false positive (PP7, OD_{405} = 0.128). Sensitivity = Specificity = Diagnostic accuracy = 90.0%.

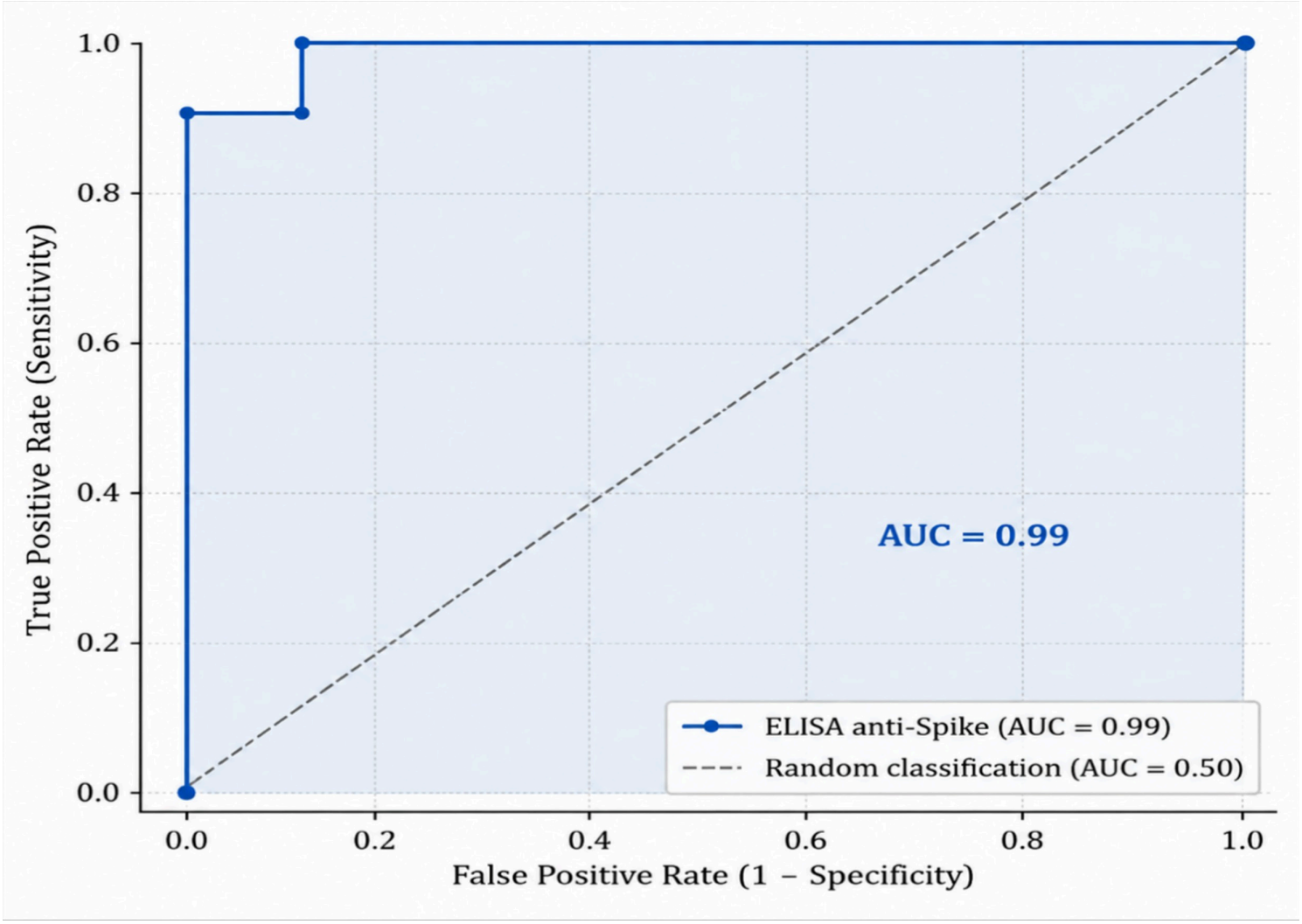


Figure 9

Receiver Operating Characteristic (ROC) curve of the in-house anti-Spike SARS-CoV-2 ELISA (pilot cohort, n = 20). The AUC of 0.92 indicates excellent global discriminant capacity. The diagonal dashed line represents chance-level discrimination (AUC = 0.50). The shaded area represents the area under the ROC curve. Complete ROC curve coordinates (threshold, TPR, FPR) are provided in Supplementary Table S4.

Supplementary Files

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- floatimage1.png