A Multiplex Noninvasive Salivary Antibody Assay for SARS-CoV-2 Infection and Its Application in a Population-Based Survey by Mail

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ABSTRACT
Noninvasive salivary antibody immunoassays can enable low-cost epidemiological surveillance of infections. This study involved developing and validating a multiplex suspension immunoassay on the Luminex platform to measure immunoglobulin G (IgG) responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleocapsid and spike (S) proteins, and the spike protein’s S1 and S2 subunits and receptor binding domain. Multiple versions of these recombinant proteins acquired from commercial and noncommercial sources were evaluated. Assay development and validation utilized saliva and serum samples from coronavirus disease 2019 (COVID-19) cases procured from commercial sources and negative controls from a prepandemic survey. Saliva was also collected in a demonstration survey by mail involving adult individuals in the United States who were diagnosed with SARS-CoV-2 infection 15 to 80 days prior to sample collection. The survey had an 83% valid sample return rate (192 samples from 38 states). Most COVID-19 cases (93%) reported mildly symptomatic or asymptomatic infections. The final salivary assay based on the best-performing spike and nucleocapsid proteins had a sensitivity of 87.1% (95% bootstrap confidence interval, 82.1 to 91.7%) and specificity of 98.5% (95.0 to 100%) using 227 and 285 saliva samples, respectively. The same assay had 95.9% (92.8 to 98.9%) sensitivity and 100% (98.4 to 100%) specificity in serum (174 and 285 serum samples, respectively). Salivary and serum antibody responses to spike and nucleocapsid proteins were strongly correlated in 22 paired samples ($r = 0.88$ and $r = 0.80$, respectively). Antibody responses peaked at approximately 50 days postonset; greater illness severity was associated with stronger responses. This study demonstrated that a salivary antibody assay can be used in large-scale population surveys by mail to better characterize public health impacts of COVID-19.

IMPORTANCE
Given the enormous impacts of the COVID-19 pandemic, developing tools for population surveillance of infection is of paramount importance. This article describes the development of a multiplex immunoassay on a Luminex platform to measure salivary immunoglobulin G responses to the spike protein, its two subunits and receptor binding domain, and the nucleocapsid protein of SARS-CoV-2. The assay validation utilized serum and saliva samples from prepandemic controls and recent COVID-19 cases. A survey by mail targeting recent COVID-19 cases across the United States also demonstrated the utility of safe, at-home self-collection of saliva. By incorporating multiple SARS-CoV-2 proteins, this assay may differentiate responses to natural SARS-CoV-2 infections from responses to most vaccines. Application of this noninvasive immunoassay in COVID-19 surveillance can help provide estimates of...
The coronavirus disease 2019 (COVID-19) pandemic reduced life expectancy in the United States in 2020 by over a year compared to 2019 and disproportionally impacted minority communities, with estimated life expectancy declines of approximately 2 years and 3 years in Black and Hispanic populations, respectively (1, 2). Given the immense public health and economic impacts of COVID-19, developing tools for population surveillance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is of paramount importance. Reverse transcription (RT)-PCR tests that detect the presence of viral genetic material in nasopharyngeal swabs or saliva are used for diagnosing an active infection. In contrast, tests of serum antibodies to SARS-CoV-2 enable identification of convalescent (previously infected) individuals but not necessarily those with active infection. Given the invasive nature of venous blood sampling, large-scale population serological surveys are likely to be costly and logistically challenging. Noninvasive salivary antibody tests for other viruses and parasites have been developed and applied in low-cost population surveys involving self-collection at home (3–8). Experimental salivary antibody tests have also been developed to measure immunoglobulin G (IgG), IgA, and IgM responses to SARS-CoV-2 (9–11). IgG responses remain elevated for several months after SARS-CoV-2 infection, longer than other antibody isotypes (12). Therefore, IgG-based serum and saliva tests are more suitable than IgA and IgM tests for assessing cumulative incidence rates in population surveys.

To support potential efforts aimed at understanding the broad impacts of the COVID-19 pandemic, this study sought to develop a multiplex salivary IgG assay on a microsphere-based Luminex platform to detect SARS-CoV-2 infection. In addition, a survey by mail drawing a convenience sample of the general U.S. population of convalescent individuals was conducted to validate this assay and demonstrate its utility.

RESULTS
Saliva and serum samples. Assay development and validation tests included the following: (i) saliva samples from 35 adult individuals and serum samples from 174 individuals who were previously diagnosed with COVID-19, purchased from commercial entities and a biobank, including paired serum samples obtained from 22 of these individuals; (ii) a nationwide saliva survey by mail involving adults who were diagnosed with COVID-19; and (iii) leftover prepandemic paired saliva and serum samples from 285 participants of a previous EPA population study in North Carolina (Table 1).

The saliva survey by mail involved shipping sampling kits to 231 individuals; of these, 207 (90%) returned their samples to EPA. After excluding four samples with insufficient volume, three samples exposed to ambient temperature for more than 3 days due to shipping delays, and eight samples collected outside the 15-to-80-day postonset window, samples from 192 (83%) individuals from 38 states were included in data analysis (Fig. S1). North Carolina, where the core recruitment team was located, had the most participants, at 46 (24%).

Overall, most COVID-19 cases whose saliva samples were used in this project were female (64.3%) and White (83.3%); the average age was 42.8 years (range, 18 to 77 years), the most common symptom severity levels were mild and moderate (43.2% and 42.7%, respectively), and the average interval from the onset to sampling was 36.4 days (Table 1). COVID-19 cases who donated serum samples (not shown in Table 1) were generally similar to saliva donors: the average age was 43.7 years (range 18 to 77 years), 59.2% were female, 78.2% were White, 53.4% had mild and 27.6% moderate symptoms, and the average interval from symptom onset was 39.3 days. Prepandemic samples were obtained from slightly older (average age of 51.8 years)
and more racially diverse donors (Table 1). In total, 512 saliva samples and 456 serum samples were used for assay validation.

**Immunos assay development.** The development of this multiplex assay involved testing more than 30 recombinant protein candidates, including several versions of spike protein (S), spike subunit 1 (S1), spike subunit 2 (S2), receptor binding domain (RBD), and nucleocapsid (N) proteins of SARS-CoV-2 and recombinant S and S1 proteins of four endemic human coronaviruses, 229E, NL63, OC43, and HKU1, acquired from 11 commercial and noncommercial sources (Table 2). Following the initial assessment of recombinant S and RBD proteins of SARS-CoV-2 from BEI Resources (Manassas, VA), in-house versions of these proteins were produced using plasmids acquired from the same source. Initially, each protein was covalently coupled to

| TABLE 1 Descriptive statistics of saliva donors |
|-------------------------------------------------|
| Parameter                                      | No. of cases (column %) or mean value (range) |
| COVID-19 cases (44.3% of total [n = 512])      | 227 (100)                                    |
| Source                                         |                                               |
| EPA survey                                     | 192 (84.6)                                   |
| Purchased samples                              | 35 (15.4)                                    |
| Age (yr)                                       | 42.8 (18–77)                                 |
| Sex                                            |                                               |
| Male                                           | 75 (33.0)                                    |
| Female                                         | 146 (64.3)                                   |
| Not reported                                   | 6 (2.6)                                      |
| Race                                           |                                               |
| African American                               | 9 (4.0)                                      |
| White                                          | 189 (83.3)                                   |
| American Indian or Pacific Islander            | 1 (0.4)                                      |
| Asian                                          | 5 (2.2)                                      |
| Mixed race                                     | 6 (2.6)                                      |
| Not reported                                   | 17 (7.5)                                     |
| Ethnicity                                      |                                               |
| Hispanic                                       | 14 (6.2)                                     |
| Not Hispanic                                   | 192 (84.6)                                   |
| Not reported                                   | 21 (9.3)                                     |
| Disease severity                               |                                               |
| No symptoms                                    | 16 (7.0)                                     |
| Mild                                           | 98 (43.2)                                    |
| Moderate                                       | 97 (42.7)                                    |
| Severe                                         | 6 (2.6)                                      |
| No data                                        | 10 (4.4)                                     |
| Interval from onset or diagnosis (days)        | 36.4 (16–80)                                 |
| Prepandemic controls (55.7% of total [n = 512])| 285 (100)                                    |
| Age (yr)                                       | 51.8 (20–88)                                 |
| Sex                                            |                                               |
| Male                                           | 118 (41.4)                                   |
| Female                                         | 165 (57.9)                                   |
| Not reported                                   | 1 (0.4)                                      |
| Race                                           |                                               |
| African American                               | 109 (38.2)                                   |
| White                                          | 153 (53.7)                                   |
| American Indian or Pacific Islander            | 0 (0.0)                                      |
| Asian                                          | 4 (1.4)                                      |
| Mixed race                                     | 11 (3.9)                                     |
| Not reported                                   | 8 (2.8)                                      |
| Ethnicity                                      |                                               |
| Hispanic                                       | 14 (4.9)                                     |
| Not Hispanic                                   | 263 (92.3)                                   |
| Not reported                                   | 8 (2.8)                                      |
| Coronavirus species | Protein | Source | Catalog no. | Protein ID | Expression system | Purification tag | Coupling concn (µg per 500 µL) | Note |
|---------------------|---------|--------|-------------|------------|-------------------|-----------------|-------------------------------|------|
| SARS-CoV-2          | N       | AcroBiosystems | NUN-C5227 | AB_N       | HEK293 cells     | His             | 5                             | Final assay |
|                     | N       | RayBiotech | 230-30164-100 | RB_N       | HEK293 cells     | His             | 5                             | |
|                     | N       | R&D Systems | 10474-CV | RD_N       | Baculovirus-insect cells | His             | 5                             | Added to final assay subsequent to initial screening step |
|                     | N       | Vioregen | 00221-V | VR_N       | E. coli         | His             | 25                            | |
|                     | N       | RayBiotech | 230-30164-100 | RB_P       | HEK293 cells     | His             | 5                             | |
|                     | N       | R&D Systems | 10474-CV | RD_P       | Baculovirus-insect cells | His             | 5                             | |
|                     | N       | AcroBiosystems | SPD-C52H2 | AB_RBD    | HEK293 cells     | His             | 5                             | Final assay |
| S                   | N       | BEI Resources | NR-52306 | BEI_RBD   | HEK293 cells     | Hexa-his        | 15                            | EPA_RBD version in final assay |
|                     | N       | Creative Diagnostics | DAGC089 | CD_RBD   | HEK293 cells     | Mouse IgG1 Fc   | 5                             | |
|                     | N       | ProSci | 97-093 | PS_RBD    | HEK293 cells     | Mouse IgG Fc    | 5                             | |
|                     | N       | R&D Systems | 10500-CV | RD_RBD    | HEK293 cells     | His             | 15                            | |
|                     | N       | Sino Biological | 40592-V02H | SB_RBD    | HEK293 cells     | His             | 5                             | |
|                     | N       | Sino Biological | 40591-V08H | SB_S      | Baculovirus-insect cells | His             | 15                            | |
|                     | N       | BioVendor | RN0005100 | BV_S1     | HEK293 cells     | Fc              | 15                            | |
|                     | N       | Creative Diagnostics | DAGC091 | CD_S1     | HEK293 cells     | His             | 5                             | Final assay |
|                     | N       | GenScript | Z03501 | GS_S1     | Human cells      | Tag-free        | 5                             | |
|                     | N       | NativeAntigen | REC31806 | NA_S1    | HEK293 cells     | Sheep IgG Fc    | 15                            | |
|                     | N       | Sino Biological | 40591-V08H | SB_S1     | HEK293 cells     | His             | 5                             | Selected for final assay, but later removed |
| S                   | N       | Vioregen | 00220-V | CR_S1     | E. coli         | His             | 15                            | |
|                     | N       | BioVendor | RN0004100 | BV_S2     | HEK293 cells     | IgG Fc          | 15                            | |
|                     | N       | NativeAntigen | REC31807 | NA_S2    | HEK293 cells     | Sheep IgG Fc    | 15                            | |
|                     | N       | ProSci | 97-079 | PS_S2     | HEK293 cells     | His             | 5                             | Replacement with PS_S2i version |
|                     | N       | ProSci | 19-115 | PS_S2i    | Baculovirus-insect cells | His             | 5                             | Final assay |
| α-CoV 229E          | S       | Sino Biological | 40605-V08B | SB_229E_S | Baculovirus-insect cells | His             | 15                            | Final assay |
|                     | S       | AcroBiosystems | SIN-V52H4 | AB_229E_S1 | HEK293 cells     | His             | 15                            | |
|                     | S       | Sino Biological | 40601-V08H | SB_229E_S1 | HEK293 cells     | His             | 15                            | |
| α-CoV NL63          | S       | Sino Biological | 40604-V08B | SB_NL63_S | Baculovirus-insect cells | His             | 15                            | Final assay |
|                     | S       | AcroBiosystems | SIN-V52H3 | AB_NL63_S1 | HEK293 cells     | His             | 15                            | |
|                     | S       | Sino Biological | 40600-V08H | SB_NL63_S1 | HEK293 cells     | His             | 15                            | |
| β-CoV HKU1          | S       | Sino Biological | 40606-V08B | SB_HKU1_S | Baculovirus-insect cells | His             | 15                            | Final assay |
|                     | S       | AcroBiosystems | SIN-V52H6 | AB_HKU1_S1 | HEK293 cells     | His             | 15                            | |
|                     | S       | Sino Biological | 40602-V08H | SB_HKU1_S1 | HEK293 cells     | His             | 15                            | |
| β-CoV OC43          | S       | Sino Biological | 40607-V08B | SB_OC43_S | Baculovirus-insect cells | His             | 15                            | Final assay |
|                     | S       | AcroBiosystems | SIN-V52H5 | AB_OC43_S1 | HEK293 cells     | His             | 15                            | |
Luminex microspheres at a range of concentrations. Coupling confirmation tests were conducted using 11 primary antibodies specific to SARS-CoV-2 antigens and corresponding secondary detection antibodies (Table S1). The optimal coupling concentration was identified for each protein (Table 2) as described in Materials and Methods.

To reduce nonspecific reactivity and maximize the signal-to-noise ratio, a total of 10 anti-human IgG detection antibodies were evaluated (Table S1). Preliminary selection of detection antibodies was conducted using serum samples from COVID-19 cases. Four polyclonal detection antibodies conjugated with biotin (these antibodies were assayed with streptavidin R-phycoerythrin [SAPE], one of them was excluded from further evaluation due to a strong cross-reactivity) and two antibodies conjugated with phycoerythrin were evaluated. The phycoerythrin-conjugated goat anti-human IgG F(ab)₂-specific polyclonal detection antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was initially determined to have the best average signal-to-noise ratio among these polyclonal antibodies (Table S2 and Fig. S2). This detection antibody was then used to analyze a subset of 401 saliva samples from commercial sources, EPA’s survey by mail, and prepandemic controls, resulting in a sensitivity of 79% and specificity of 96%. Further assay optimization was conducted to improve the sensitivity and specificity. Second-stage optimization tests involved comparison of the preliminary assay version with four alternative versions utilizing monoclonal detection antibodies using positive- and negative-control saliva samples. The results showed that the anti-human IgG Fc-specific monoclonal antibody from Leinco Technologies (St. Louis, MO) was the overall best-performing antibody with the greatest average ratio of responses in positive- and negative-control saliva samples (Table S3 and Fig. S3).

**Selection of proteins.** Initial screening of 19 SARS-CoV-2 proteins was conducted using the preliminary version of the assay. It involved testing serum samples from COVID-19 cases and prepandemic controls. Based on receiver operating characteristic (ROC) area under the curve (AUC) analysis, the nine best-performing proteins were selected for further assay development and testing (Fig. S4). Subsequently, minor revisions were made to the panel of proteins. Specifically, S and RBD proteins from BEI Resources were replaced with similar proteins expressed and purified at EPA, and the assay was further modified to incorporate improved versions of three proteins for a total of 10 SARS-CoV-2 proteins. In addition, S and S1 proteins of four endemic coronaviruses were evaluated. In preliminary analysis of saliva samples, antibody responses to S and S1 proteins of homologous viruses were found to be strongly correlated. In order to reduce the cost and complexity of the assay, the final assay included only four S proteins of four endemic coronaviruses that were acquired from Sino Biological USA (Wayne, PA), for a total of 14 proteins (Table 2).

**Immunosassay validation.** Correlations between log-transformed antibody responses to homologous SARS-CoV-2 proteins in 22 paired serum and saliva samples from COVID-19 cases ranged from 0.73 to 0.88 (Table S4), with the strongest being 0.88 for the S protein produced at EPA (protein ID [identifier], EPA_S) (Fig. 1).

Adjusting the results for control serum samples that were assayed on each plate did not improve assay sensitivity or specificity (data not shown); therefore, all results presented below are for unadjusted data. Random forest analysis of all salivary assay data with the full set of 10 SARS-CoV-2 proteins produced 87.5% (95% confidence interval 81.6% to 93.6%) sensitivity (n = 227) and 97.6% (92.2% to 100%) specificity (n = 285). A serological assay with the full set of 10 proteins had 7.2 percentage points higher specificity (n = 174), at 94.7% (89.6% to 98.8%), and 1.7 percentage points higher specificity (n = 282), at 99.3% (96.1% to 100%) (Table 3). The results also demonstrated that the S protein produced at EPA utilizing a plasmid obtained from BEI Resources (EPA_S) was the best-performing protein (ranking top in all 500 runs) while Acro Biosystems (Newark, DE, USA) S and N proteins (AB_S and AB_N, respectively), RBD protein expressed at EPA using another plasmid from BEI Resources (EPA_RBD), and ProSci (Fort Collins, CO, USA) S2 protein (PS_S2i) were among the top five best-performing SARS-CoV-2 proteins (Table 4). The parsimonious set of SARS-CoV-2
proteins for more in-depth analysis included four of these five proteins, while the AB_S protein, which ranked second after the EPA_S protein, was excluded to avoid redundancy. Salivary antibody responses to the four selected proteins by COVID-19 status and symptom severity categories are presented in Fig. 2. Distributions of responses to the best-performing EPA_S protein were bimodal in asymptomatic, mildly, and moderately symptomatic individuals (Fig. 2A), with the proportion of nonresponders diminishing with increasing symptom severity. Scatterplots of associations between the best-performing EPA_S protein and the other three SARS-CoV-2 proteins in the parsimonious set are shown in Fig. 3. Intraplate coefficients of variation (CV) for salivary antibody responses to EPA_S, EPA_RBD, AB_N, and PS_S2i antigens were 2.4%, 2.6%, 2.4%, and 2.8%, respectively, based on 19 samples assayed in duplicate. Intraplate CVs for serum antibody responses were similar, with an average of 2.7% for all four proteins based on 31 samples. Interplate variability was estimated from four control serum samples analyzed on 14 plates and an additional nine serum and saliva samples assayed on two plates; the CVs were 8.1% for IgG responses to EPA_S antigen, 10.6% for EPA_RBD, 16.3% for AB_N, and 22.3% for PS_S2i.

In further random forest analysis using data on the four top-ranking heterologous proteins, the sensitivity and specificity of the salivary antibody assay was reduced slightly (by 1.4 and 0.9 percentage points, respectively) compared to the sensitivity and specificity of the full set of 10 SARS-CoV-2 proteins. Logistic regression analysis showed that a model with only two of these four proteins, EPA_S and AB_N, produced

**Table 3** Sensitivity and specificity estimates of salivary and serum assays with 95% confidence intervals

| Model            | Protein(s)          | Index value or % (95% CI) for: | | |
|------------------|---------------------|---------------------------------|---|---|
|                  |                     | Saliva assay                    | Serum assay                  |
|                  |                     | Youden’s J index | Sensitivity | Specificity | Youden’s J index | Sensitivity | Specificity |
| 1. Random Forest | Set of 10 SARS-CoV-2 proteins | 0.851 | 87.5 (81.6–93.6) | 97.6 (92.2–100) | 0.941 | 94.7 (89.6–98.8) | 99.3 (96.1–100) |
| 2. Random Forest | EPA_S, EPA_RBD, AB_N, PS_S2i | 0.828 | 86.1 (80.2–91.6) | 96.7 (91.9–99.3) | 0.935 | 94.1 (89.5–97.9) | 99.4 (96.6–100) |
| 3. Logistic      | EPA_S, AB_N         | 0.856<sup>a</sup> | 87.1 (82.1–91.7) | 98.5 (95.0–100) | 0.959<sup>c</sup> | 95.9 (92.8–98.9) | 100 (98.4–100) |
| 4. Logistic      | EPA_S               | 0.835 | 85.7 (80.7–90.4) | 97.8 (94.6–100) | 0.940 | 94.7 (90.8–98.7) | 99.3 (97.1–100) |
| 5. Logistic      | EPA_RBD             | 0.804 | 87.9 (79.6–93.7) | 92.5 (87.2–97.6) | 0.916 | 93.0 (88.5–97.3) | 98.6 (95.2–100) |
| 6. Logistic      | AB_N                | 0.815 | 83.7 (78.9–89.2) | 97.8 (94.2–99.3) | 0.929 | 94.4 (90.5–97.8) | 98.4 (96.3–100) |
| 7. Logistic      | PS_S2i              | 0.785 | 82.5 (76.6–88.0) | 96.1 (91.0–99.2) | 0.940 | 95.5 (91.5–98.8) | 98.5 (94.9–100) |

<sup>a</sup>Protein IDs are defined in Results and Table 2.
<sup>b</sup>Best value for saliva.
<sup>c</sup>Best value for serum.
the best fit to the data. In the analysis of saliva data, this model produced 0.4 percentage point lower sensitivity and 0.9 percentage point higher specificity than the full random forest model for 10 proteins, and it produced the top sensitivity and specificity values in the analysis of serum data (Table 3). This bivariate model also had superior

### TABLE 4 Random forest ranking of proteins included in the final salivary IgG assay

| SARS-CoV-2 protein       | Protein ID | Gini coefficient | Gini_OOB | Parsimonious model |
|--------------------------|------------|------------------|----------|--------------------|
| Spike                    | EPA_S      | 0.352            | 0.312    | Yes                |
| Spike                    | AB_S       | 0.037            | 0.024    | No                 |
| Nucleocapsid             | AB_N       | 0.012            | 0.002    | Yes                |
| Spike stalk subunit      | PS_S2i     | 0.012            | 0.001    | Yes                |
| Receptor binding domain  | EPA_RBD    | 0.007            | 0.000    | Yes                |
| Spike                    | RD_S       | 0.009            | -0.005   | No                 |
| Receptor binding domain  | AB_RBD     | 0.018            | -0.006   | No                 |
| Spike globular subunit   | CD_S1      | 0.009            | -0.007   | No                 |
| Nucleocapsid             | RD_N       | 0.011            | -0.009   | No                 |
| Receptor binding domain  | SB_RBDn    | 0.025            | -0.016   | No                 |

*Protein IDs are defined in Results and Table 2.

*OOB, out-of-box.

![FIG 2](image-url) Antibody responses to a parsimonious set of SARS-CoV-2 proteins by COVID-19 status and symptom severity. (A) Spike protein (EPA_S); (B) receptor binding domain (EPA_RBD); (C) nucleocapsid protein (AB_N); (D) stalk subunit S2 of S protein (PS_S2i). Protein IDs are defined in Results and Table 2. MFI, mean fluorescence intensity.
performance compared to univariate logistic regression models for each of the four selected proteins. The sensitivity of the salivary assay was 8.8 percentage points lower and its specificity was 1.5 percentage points lower than the values for the serological assay. A cutoff line separating negative and positive test results based on this model is shown for the EPA$_S$-versus-AB$_N$ plot in panel A.
shown on the scatterplot of salivary IgG responses to EPA_S and AB_N proteins (Fig. 3A). Overall, this logistic model produced the highest Youden’s J index value for both salivary and serological assays.

**Associations of antibody response with symptom severity and interval from onset.** Symptom severity was a strong predictor of antibody responses to the four selected SARS-CoV-2 proteins after adjusting for age and a spline function of time interval from the date of symptom onset or positive diagnostic test (Table 5). For example, adjusted responses to the EPA_S protein were 3.28 (1.43 to 7.53), 5.32 (2.31 to 12.3), and 8.03 (1.86 to 34.7) times stronger in COVID-19 cases with mild, moderate, and severe symptoms, respectively, than in asymptomatic COVID-19 cases. Antibody responses to each protein declined after approximately 50 to 60 days following the onset of symptoms (Fig. 4), consistent with previously published results (9, 12).

**Evaluation of cross-reactivity to endemic coronaviruses.** Associations between salivary antibody responses to S proteins of endemic coronaviruses and a homologous EPA_S protein of SARS-CoV-2 were significantly stronger ($P < 0.001$ for an interaction effect in all regression models, data not shown) in COVID-19 cases than in prepandemic controls (Fig. 5). Moreover, among COVID-19 cases, there were stronger associations between responses to SARS-CoV-2 and $\beta$-coronaviruses HKU1 and OC43 (Fig. 5A and B) than between responses to SARS-CoV-2 and $\alpha$-coronaviruses NL63 and 229E (Fig. 5C and D). At the same time, associations between responses to SARS-CoV-2 and all four endemic coronaviruses were not statistically significant in prepandemic samples.

**DISCUSSION**

This study involved the development and validation of a salivary antibody assay for population surveillance of SARS-CoV-2 infection. It demonstrated that this noninvasive method enables large-scale population surveys of SARS-CoV-2 infections by mail in a clinically, geographically, and demographically diverse population and that salivary antibodies were strongly correlated with serum antibodies in paired samples. This method can be applied to identify disproportionately affected socioeconomic or demographic groups and to conduct surveys in disadvantaged communities to assess risk factors for infection. Its application can provide critically important information for managing and mitigating impacts of COVID-19, such as SARS-CoV-2 seroprevalence estimates, spatial and temporal patterns of population immunity, and behavioral or sociodemographic risk factors of infection. Noninvasive saliva sampling can also support prospective surveys to detect incident infections.

The survey by mail was built upon EPA’s previous experience conducting similar salivary antibody surveys of environmentally transmitted viral, bacterial, and parasitic infections by mail (4, 7, 8). In the present nationwide survey, most saliva samples were delivered to an EPA laboratory overnight and remained refrigerated upon delivery. Salivary antibody responses are not significantly affected by storage at room temperature for up to several days (13), and population-based surveys by mail in the United Kingdom and Belgium involved mailing saliva samples at ambient temperature (5, 6).

Previous studies demonstrated that serological enzyme-linked immunosorbent assays (ELISAs) based on S or N protein had similar rates of false-negative results but misclassified different subsets of positive samples (14), while a serological assay based on combined S and N proteins had higher sensitivity than single protein tests (15). The present study demonstrated that an assay using the best-performing versions of S and N proteins can produce results similar to those of a multiplex assay involving a total of 10 versions of S, S1, S2, RBD, and N proteins. Although the S protein produced at EPA using a plasmid provided by BEI Resources was the best-performing protein overall, the N protein (AcroBiosystems) helped to identify several COVID-19 cases where responses to the S protein were not detectable (Fig. 3A). In future surveys, an assay involving S and N proteins is also expected to be able to differentiate antibody responses to natural infections that target both S and N proteins from responses to most vaccines that target S protein only.

This project has some limitations as well as important strengths. The salivary antibody assay had an appreciably lower sensitivity than the serum antibody assay (Table 3), likely...
TABLE 5: Multiplicative effects with 95% confidence intervals of disease severity categories and age on median salivary antibody responses to SARS-CoV-2 proteins from generalized additive models, adjusted for a spline function of interval from the onset.

| Parameter | Spike protein EPA_S | Nucleocapsid protein AB_N | Spike stalk subunit PS_S2i | Receptor binding domain EPA_RBD |
|-----------|---------------------|---------------------------|---------------------------|--------------------------------|
| Severity of symptoms | Multiplicative effect | P value | Multiplicative effect | P value | Multiplicative effect | P value | Multiplicative effect | P value |
| Asymptomatic | Reference | | Reference | | Reference | | Reference | |
| Mild | 3.28 (1.43–7.53) | 0.005 | 2.79 (1.10–7.10) | 0.03 | 3.69 (1.58–8.61) | 0.003 | 2.35 (1.01–5.47) | 0.05 |
| Moderate | 5.32 (2.31–12.3) | <0.001 | 4.68 (1.83–12.0) | 0.001 | 5.94 (2.53–13.9) | <0.0001 | 4.09 (1.75–9.55) | 0.001 |
| Severe | 8.03 (1.86–34.7) | 0.01 | 8.28 (1.59–43.0) | 0.01 | 11.6 (2.59–51.5) | 0.001 | 5.66 (1.28–25.1) | 0.02 |
| Age, per 10-yr increase | 1.05 (0.92–1.19) | 0.5 | 1.30 (1.12–1.51) | 0.0004 | 1.12 (0.98–1.28) | 0.10 | 1.14 (1.00–1.31) | 0.05 |
| Spline of interval from onset | NA* | 0.0004 | NA | 0.0005 | NA | <0.0001 | NA | 0.15 |

*NA, not applicable.
due to lower levels of IgG in saliva. However, the ease of saliva sampling and the ability to conduct nationwide surveys by mail justify this trade-off. This project utilized samples from adults; therefore, its results are applicable to adults only, as children’s antibody responses to SARS-CoV-2 may differ (16). Our mail survey also relied on self-reported medical diagnosis of COVID-19; if some survey participants were, in fact, not infected with SARS-CoV-2, the results might underestimate the sensitivity of this assay.

The sensitivities and specificities of previously developed SARS-CoV-2 antibody tests varied widely and depended on immunoassay characteristics, time interval from symptom onset to sample collection, participant age, and the presence and severity of COVID-19 symptoms (15). As all antibody assays have imperfect sensitivity and specificity, crude population seroprevalence estimates need to be adjusted for these parameters using one of several available statistical techniques (17). In this study, individuals with more severe COVID-19 symptoms tended to have stronger antibody responses than mildly symptomatic and asymptomatic individuals, which is consistent with previous research (18, 19). Asymptomatic and mildly symptomatic individuals may have much more robust immune responses (non-antibody dependent, such as CD8+ T cell responses) that clear the infection faster; consequently, these individuals often do not produce detectable antibody responses (20, 21). As a result, the sensitivity of antibody assays in individuals who had mild COVID-19 symptoms tends to be substantially lower than in individuals who had severe illness (22). Therefore, the results of assay validation surveys that oversampled severely ill or hospitalized patients by design (11, 23) were
likely affected by the upward sensitivity spectrum bias (24). The present population survey involved a convenience sample of COVID-19 cases; the resulting sample consisted of mainly mildly and moderately symptomatic individuals with smaller proportions of asymptomatic (7.5%) and severely symptomatic (2.4%) persons. The sensitivity estimates are likely to be unbiased in individuals who experienced at least mild COVID-19 symptoms, which is an important strength of this project. At the same time, the survey undersampled asymptomatic individuals, who may represent about one-third of SARS-CoV-2 infections in the general population (25). Therefore, this assay’s sensitivity may still be overestimated for a general population.

Previously published sensitivity and specificity estimates of SARS-CoV-2 antibody tests were based on sample-derived cut-points for dichotomizing antibody responses (10, 11, 23). It has been shown that sensitivity and specificity values based on optimal sample-derived cut-points tend to be substantially inflated, especially in small studies using fewer than 200 samples in each analysis (26), such as the three studies cited above. To address this problem, the present study employed a bootstrap method to produce unbiased estimates of sensitivity and specificity with 95% confidence intervals.

This study involved testing and evaluating multiple versions of S, S1, S2, RBD, and N proteins from different sources. The random forest machine learning procedure that was applied in the statistical analysis can handle a large set of interrelated variables, such as highly correlated antibody responses to homologous SARS-CoV-2 proteins. It

![FIG 5 Associations between salivary IgG responses to SARS-CoV-2 spike protein (EPA_S) and to spike proteins of endemic coronaviruses procured from Sino Biological (SB). Lines show 95% confidence intervals (dashed lines) and mean values (solid lines). (A) HKU1 (SB_HKU1_S); (B) OC43 (SB_OC43_S); (C) NL63 (SB_NL63_S); (D) 229E (SB_229E_S).](image-url)
has been shown to be superior to other statistical methods for selecting the most informative biomarkers and building prediction models (27).

The multiplex assay employed in this project enabled comparative analysis of antibody responses to SARS-CoV-2 and four endemic coronaviruses. There were much stronger correlations between responses to S proteins of SARS-CoV-2 and endemic coronavirus among COVID-19 cases than among prepandemic controls. Antibodies specific to SARS-CoV-2 could cross-react with endemic human coronaviruses, especially with more closely related $\beta$-coronaviruses. Alternatively, SARS-CoV-2 infections could boost antibody responses to endemic human coronaviruses, as demonstrated previously (28).

This was the first demonstration of a nationwide application of a noninvasive salivary antibody assay for SARS-CoV-2 surveillance. Information generated by salivary antibody surveys can help to inform public health policies and risk mitigation strategies and ultimately contribute to limiting the impacts of this and future pandemics.

MATERIALS AND METHODS

Study design. This study aimed to develop and validate a multiplex immunoassay to measure antibody responses to SARS-CoV-2 for potential application in population-based epidemiological studies of COVID-19 transmission patterns and risk factors for infection. Human serum and saliva samples were obtained from SARS-CoV-2 cases and from control individuals whose samples were collected prior to the pandemic. Samples were evaluated for IgG responses to recombinant antigens from two of the major SARS-CoV-2 structural proteins, the nucleocapsid and spike proteins, along with the spike protein’s globular and stalk subunits and receptor binding domain. In addition to the immunoassay development objective, we set out to determine whether saliva can be utilized as a noninvasive alternative to serum for monitoring antibody responses to SARS-CoV-2 and demonstrate the feasibility of population-level surveillance of SARS-CoV-2 antibodies using a nationwide saliva survey by mail.

Selection and expression of proteins. Assay development involved evaluating more than 30 recombinant protein candidates acquired from 10 commercial sources and a noncommercial entity affiliated with the U.S. National Institute of Allergy and Infectious Diseases, BEI Resources (Manassas, VA). These proteins included multiple versions of spike (S), globular spike subunit 1 (S1), stalk spike subunit 2 (S2), receptor binding domain (RBD), and nucleocapsid (N) proteins of SARS-CoV-2, as well as recombinant S and S1 proteins of endemic human coronaviruses 229E, NL63, OC43, and HKU1 (Table 2).

Following the initial assessment of S and RBD proteins from BEI Resources, recombinant S and RBD proteins were expressed and purified in-house using puH-Spike and pCAGGS-RBD plasmids (catalog numbers NR-52563 and NR-52309; BEI Resources) in accordance with a previously published protocol (29), with substantial modifications involving the use of lentiviral transfer vector to transduce HEK293T human kidney cells, as described below.

The cytomegalovirus (CMV) expression cassettes for both S and RBD proteins were excised from the plasmids by SpeI/HindIII and subcloned into the lentiviral transfer vector pTRED (EPA). Both expression cassettes encode an amino-terminal signal peptide that facilitates secretion of the expressed proteins into the extracellular medium and a hexahistidine tag for protein purification purposes (29). The resulting plasmids were purified by cesium chloride-ethidium bromide gradient centrifugation, and their sequences verified by fluorescent DNA capillary sequencing. Human HEK293T cells (ATCC, Manassas, VA) were cotransfected with purified pTRED-CMV/Spike or pTRED-CMV/RBD transfer vector plasmids and a lentiviral packing mix (Open Biosystems, Huntsville, AL). The resulting lentiviral stock was harvested and the titer determined as previously described (30). HEK293T cells were subsequently transduced with CMV-Spike or CMV-RBD lentivirus at a multiplicity of infection of 20. These stable transgenic cell lines, designated 293T-Spike and 293T-RBD, respectively, were seeded into collagen-coated T-175 flasks overnight at a density of $2 \times 10^5$ cells in 25 ml of complete growth medium consisting of high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Waltham, MA) supplemented with 10% fetal calf serum (FCS; Gibco, Waltham, MA), 5 mM HEPES, and penicillin-streptomycin (Sigma, St. Louis, MO) at 37°C in a humidified 5% CO$_2$ atmosphere. Cells were then exchanged to complete DMEM with 5% FBS and incubated for 72 h. Medium was collected and stored at $–20°C$ until processed.

Spike and RBD proteins were purified using a previously published protocol (29) with modifications. Briefly, collected medium was thawed overnight at 4°C and then filtered through a 0.22 μM vacuum filter unit (Corning, Corning, NY). The filtrate was transferred to Amicon ultra centrifuge filters (100 kDa for spike and 10 kDa for RBD) and concentrated by centrifugation at 3,000 × g at 4°C for 20 min. Retentate medium (45 ml) was preequilibrated to 5 mM imidazole (final) by adding 918 μl of elution buffer (56 mM Na$_2$HPO$_4$, 300 mM NaCl, 250 mM imidazole). A 2-ml volume of Ni-nitrilotriacetic acid (NTA) bead slurry (Qiagen; Germantown, MD) was washed with 45 ml of cold 1× phosphate-buffered saline (PBS) and aspirated. The retentate was added to the washed Ni-NTA beads and mixed by rotation at ambient temperature for 2 h. Ni-NTA beads were pelleted by centrifugation at 3,500 × g for 5 min at 4°C, all but 2 ml of the medium was aspirated, and the remaining volume was used to resuspend the beads into a slurry that was transferred to a 2-ml Pierce centrifuge column (Thermo Fisher Scientific). After residual medium flowed through the column by gravity, the beads were washed four times by gravity flow, each with 14 ml of wash buffer (56 mM Na$_2$HPO$_4$, 300 mM NaCl, 20 mM imidazole). Protein was collected by eluting once by gravity flow with 3.5 ml of elution buffer. Eluted protein was transferred to an Amicon
ultra centrifuge filter, raised in volume to 15 ml with cold 1 × PBS, and then centrifuged at 3,000 × g at 4°C for 30 min or until the retentate volume was <1 ml. The retentate volume was again raised to 15 ml with cold 1 × PBS and reprocessed twice for a total of three filtration steps. The concentration of the final retentate was assessed using the Bradford assay (Thermo Fisher Scientific) with bovine serum albumin (BSA) standards. Protein integrity was confirmed by SDS-PAGE analysis and verification of bands with apparent molecular weights of approximately 180 kDa and 35 kDa, for the spike and RBD proteins, respectively.

**Luminex multiplex immunoassay technology.** This project used Luminex xMAP technology (Luminex Corp., Austin, TX), a suspension sandwich immunoassay involving spectrally distinct sets of 6.5-μm MagPlex microspheres covalently coupled to target proteins (in the present study, recombinant proteins of coronaviruses). Different dye ratios in MagPlex microspheres allow simultaneous detection of multiple analytes in a single sample. In the indirect immunoassay format employed in this project, saliva or serum samples were incubated with a mixture of coupled microspheres to allow antiviral and antimitochondrial antibodies to react with their target antigens. This was followed by incubation with a biotinylated secondary detection antibody targeting the primary antibody and a streptavidin R-phycocerythrin (SAPE) fluorescent reporter. A dual laser Luminex 200 analyzer classified each microsphere by dye ratio and quantified the reporter signal of each captured analyte, which corresponds to the number of primary antibodies attached to a given microsphere. Results are expressed as median fluorescence intensity (MFI) values for each microsphere set based on data for at least 50 individual microspheres.

**Coupling proteins to Luminex MagPlex microspheres.** Each candidate protein was covalently coupled to a distinct set of carboxylated MagPlex microspheres following the standard Luminex carbodi-imide coupling protocol (Luminex xMAP Cookbook at https://info.luminexcorp.com/en-us/research/immunoassays/luminext-multiplex-booklet.pdf, accessed on 20 July 2022). Briefly, 5 × 10⁴ MagPlex microspheres were washed in reagent-grade water and resuspended in 80 μl of 1 M monobasic sodium phosphate, pH 7.4, with subsequent addition of 10 μl of 50 mg/ml N-hydroxysulfo succinimide (sulfo-NHS) (catalog number A39269; Thermo Fisher Scientific) and 10 μl of 50 mg/ml EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] (catalog number 22980; Thermo Fisher Scientific), followed by 20 min of incubation at room temperature for surface activation. Activated beads were washed twice in 250 μl of 50 mM MES (4-morpholineethanesulfonic acid) (pH 5.0) coupling buffer (catalog number M2933; Millipore Sigma, Burlington, MA), and resuspended in 100 μl of coupling buffer. Each protein was added to the coupling solution to a specific concentration. The total volume was brought to 500 μl with MES coupling buffer, followed by rotational mixing for 2 h at room temperature. Beads were then pelleted and resuspended in pH 7.4 blocking and storage buffer comprised of PBS (catalog number P3813; Millipore Sigma), 0.1% BSA (catalog number A2934; Millipore Sigma), 0.05% sodium azide, and 0.02% Tween 20, washed twice and resuspended in 1 ml of the same buffer, and stored at 4°C. For coupling optimization, each protein was initially coupled at a range of concentrations, followed by coupling confirmation tests with various antigen-specific primary antibodies (Table S1) assayed at serial dilutions as described previously (31). The optimal protein coupling concentration was the lowest concentration that saturated the highest primary antibody dilution. Samples were analyzed using a pH 7.4 assay buffer comprised of PBS, 1% BSA (catalog number A2934; Millipore Sigma) in accordance with a standard indirect immunoassay protocol described in the Luminex cookbook.

**Serum and saliva samples.** Leftover paired saliva and serum samples collected from adult residents of North Carolina in 2017 to 2018, prior to the SARS-CoV-2 pandemic, were used as negative controls. The protocol of that prepandemic study was approved by the Institutional Review Board of the University of North Carolina in 2017 to 2018, prior to the SARS-CoV-2 pandemic, were used as negative controls. Enrollees were sent a prepaid insulated return shipping box containing an Oracol sampler, cooler, ice pack, detailed sampling instructions, consent form, and short questionnaire to ascertain basic demographic information, characterize COVID-19 symptoms (if any) and their severity, and provide dates of symptom onset and positive diagnostic test result (Text S1). Symptom severity was defined based on

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**Coupling proteins to Luminex MagPlex microspheres.** Each candidate protein was covalently coupled to a distinct set of carboxylated MagPlex microspheres following the standard Luminex carbodi-imide coupling protocol (Luminex xMAP Cookbook at https://info.luminexcorp.com/en-us/research/immunoassays/luminext-multiplex-booklet.pdf, accessed on 20 July 2022). Briefly, 5 × 10⁴ MagPlex microspheres were washed in reagent-grade water and resuspended in 80 μl of 1 M monobasic sodium phosphate, pH 7.4, with subsequent addition of 10 μl of 50 mg/ml N-hydroxysulfo succinimide (sulfo-NHS) (catalog number A39269; Thermo Fisher Scientific) and 10 μl of 50 mg/ml EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] (catalog number 22980; Thermo Fisher Scientific), followed by 20 min of incubation at room temperature for surface activation. Activated beads were washed twice in 250 μl of 50 mM MES (4-morpholineethanesulfonic acid) (pH 5.0) coupling buffer (catalog number M2933; Millipore Sigma, Burlington, MA), and resuspended in 100 μl of coupling buffer. Each protein was added to the coupling solution to a specific concentration. The total volume was brought to 500 μl with MES coupling buffer, followed by rotational mixing for 2 h at room temperature. Beads were then pelleted and resuspended in pH 7.4 blocking and storage buffer comprised of PBS (catalog number P3813; Millipore Sigma), 0.1% BSA (catalog number A2934; Millipore Sigma), 0.05% sodium azide, and 0.02% Tween 20, washed twice and resuspended in 1 ml of the same buffer, and stored at 4°C. For coupling optimization, each protein was initially coupled at a range of concentrations, followed by coupling confirmation tests with various antigen-specific primary antibodies (Table S1) assayed at serial dilutions as described previously (31). The optimal protein coupling concentration was the lowest concentration that saturated the highest primary antibody dilution. Samples were analyzed using a pH 7.4 assay buffer comprised of PBS, 1% BSA (catalog number A2934; Millipore Sigma) in accordance with a standard indirect immunoassay protocol described in the Luminex cookbook.

**Serum and saliva samples.** Leftover paired saliva and serum samples collected from adult residents of North Carolina in 2017 to 2018, prior to the SARS-CoV-2 pandemic, were used as negative controls. The protocol of that prepandemic study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill and EPA’s human subjects research review official. All samples utilized in the present study were collected from individuals who consented to long-term storage and reuse of their leftover samples. Saliva sampling was conducted using Oracol sampling devices designed to collect gingival crevicular fluid enriched with serum IgG (Malvern Medical Developments, UK). Sampling involved rubbing the gums with the sampler sponge for 1 min or until the sponge was fully saturated.

Convalescent serum and saliva samples from recovered COVID-19 cases collected in the summer and fall of 2020, before the introduction of vaccines, were purchased from commercial vendors and a university biobank for initial assay validation. Information regarding subject age, symptom severity, and interval between diagnosis and sample collection was provided. Saliva samples were collected using Oracol samplers or a comparable method aimed at collecting gingival crevicular fluid.

To further validate the assay and demonstrate the feasibility of saliva self-collection by mail, a popula-tion-based survey of individuals who recovered from COVID-19 was conducted in June to December 2020. Adults who had recent SARS-CoV-2 infection were recruited by distributing flyers and placing advertisements in newspapers, community centers, testing and medical facilities, social media, electronic mailing lists, and through word-of-mouth. The study was also advertised at the EPA clinical studies website (https://epastudies.org/). Interested individuals were directed to call a study recruiter to be screened and enrolled. Eligible individuals were those at least 18 years of age, residing in the United States, and capable of communicating in English who self-reported being medically diagnosed with SARS-CoV-2 infection within the previous 60 days. Samples were collected from 15 to 80 days following symptom onset or diagnosis of COVID-19, whichever happened earlier. This time interval was selected to maximize the proportion of IgG-seropositive individuals, based on previous research (12, 32, 33). Enrollees were sent a prepaid insulated return shipping box containing an Oracol sampler, cooler, ice pack, detailed sampling instructions, consent form, and short questionnaire to ascertain basic demographic information, characterize COVID-19 symptoms (if any) and their severity, and provide dates of symptom onset and positive diagnostic test result (Text S1). Symptom severity was defined based on
the degree of incapacitation as asymptomatic, mild (could perform usual activities), moderate (unable to perform usual activities), severe (hospitalized), or life-threatening. Samples and ice packs were frozen in a household freezer prior to overnight shipping to an EPA laboratory in Chapel Hill, North Carolina. The study protocol was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (protocol number 20-1206) and EPA’s human subjects research review official.

**Saliva and serum processing.** Oracol samples were processed upon delivery to the laboratory by inverting the sampler in the collection tube and centrifuging at 700 × g for 5 min at 4°C to separate saliva from the sponge, followed by centrifugation at 1,900 × g for an additional 5 min to pellet debris. Samples were transferred to 1.5-ml microcentrifuge tubes and centrifuged at 1,500 × g for 3 min at 4°C to further separate sample debris; the supernatant was transferred to new microcentrifuge tubes and stored at −80°C.

Prior to analysis, saliva samples were thawed and diluted 1:2 in PBS–1% BSA assay buffer, heat treated at 56°C for 30 min in a dry bath, and centrifuged at 10,000 × g for 5 min to separate mucin precipitate that can form as a result of freezing saliva.

Serum samples were diluted 1:400 in PBS–1% BSA assay buffer and centrifuged at 10,000 × g for 5 min prior to analysis to separate cryoprecipitate. To adjust for potential plate-to-plate variability, the final version of the assay included four positive-control serum samples that were assayed on each microplate.

**Assay optimization.** Assay optimization involved comparing saliva sample pretreatment and processing options (e.g., heat treatment, centrifugation, dilution buffer, etc.), secondary detection antibodies (Table S1), and candidate SARS-CoV-2 proteins (Table 2). Optimization experiments to evaluate secondary antibodies and processing options involved retesting the same set of selected human saliva and serum samples from COVID-19 cases and prepandemic controls. Candidate SARS-CoV-2 proteins were evaluated as described below.

**Statistical analysis.** Data were analyzed using SAS version 9.4 (SAS Institute, Cary, NC). The initial screening and selection of SARS-CoV-2 proteins were conducted by comparing areas under ROC curves (logistic procedure). Further analyses of multiplex assay data were performed to estimate assay sensitivity and specificity with 95% confidence intervals and identify a parsimonious set of proteins. These analyses were conducted using a random forest procedure ($hpforest$ and $hp4score$ procedures). Log-transformed data on selected proteins were also analyzed using logistic regression; due to collinearity, multivariate logistic regression models were limited to a small set of heterologous proteins. In both methods, a set of saliva or serum samples was randomly divided into similarly sized training and testing subsets. The analysis was repeated 500 times; bootstrap point estimates of sensitivity and specificity and 95% confidence intervals were determined using the 50th, 2.5th, and 97.5th percentiles of the empirical distribution, respectively. To rank proteins in the final assay, the Gini coefficient and out-of-bag (OOB) Gini values from each random forest run were averaged over 500 runs.

The $hp4score$ and logistic procedures produce a probability value for each observation with a default dichotomization cut point at $P = 0.5$. By shifting the cut point, it is possible to produce a set of sensitivity and specificity estimates that are optimized for different scenarios, e.g., with increased specificity for low prevalence and increased sensitivity for high prevalence. In this analysis, we reported results corresponding to the maximum Youden’s J statistics (sensitivity + specificity − 1), which maximize the assay accuracy at 50% population seroprevalence. Generalized additive models (gampl procedure) were used to assess factors affecting the intensity of log-transformed antibody responses to SARS-CoV-2 and produce plots of predicted responses versus time intervals from onset or diagnosis.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.9 MB.

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All authors reviewed the manuscript. A.I.E., S.M.G., and T.J.W. designed the study. S.S. and D.R.H. expressed and purified recombinant proteins. M.F., J.K., R.G., W.P., and L.W. conducted all other laboratory experiments and contributed to data collection and...
laboratory tests. E.S. and E.H. contributed to project organization and specimen collection. A.I.E. conducted statistical data analysis and wrote the first draft of the article. A.I.E., S.M.G., and T.J.W. edited the article.

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