

Validation and performance of a quantitative IgG assay for the screening of SARS-CoV-2 antibodies

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Running Title: UPR-MS CovIgG-Assay

Abstract

The current COVID-19 epidemic imposed an unprecedented challenge to the scientific community in terms of treatment, epidemiology, diagnosis, social interaction, fiscal policies and many other areas. The development of accurate and reliable diagnostic tools (high specificity and sensitivity) is crucial in the current period, the near future and in the long term. These assays should provide guidance to identify immune presumptive protected persons, potential plasma, and/or B cell donors and vaccine development among others. Also, such assays will be contributory in supporting prospective and retrospective studies to identify the prevalence and incidence of COVID-19 and to characterize the dynamics of the immune response. As of today, only thirteen serological assays have received the Emergency Use Authorization (EUA) by the U.S. Federal Drug Administration (FDA). In this work we describe the development and validation of a quantitative IgG enzyme-linked immunoassay (ELISA) using the recombinant SARS-CoV-2 Spike Protein S1 domain, containing the receptor-binding domain (RBD), showing 98% sensitivity and 98.9% specificity. The assay showed to be useful to test for SARS-CoV-2 IgG antibodies in plasma samples from COVID-19-recovered subjects as potential donors for plasmapheresis. This assay is currently under review by the Federal Drug Administration for an Emergency Use Authorization request (Submission Number EUA201115).

Introduction

The current severe acute respiratory syndrome coronavirus 2 (SARS CoV-2) pandemic and the resulting unprecedented outbreak of coronavirus disease 2019 (COVID-19) have shifted the paradigm for viral research, epidemiology and diagnostic. Both molecular and serological methods have been developed at an extraordinary speed. As of April 2, 2020 only four months after the virus was detected for first time in Wuhan region, 28 companies obtained Emergency Use Authorization (EUA) approvals from US Federal Drug Administration (FDA) for their commercial Reverse Transcription-Polymerase Chain Reaction (RT-PCR) diagnostics. Those assays are intended to detect the virus during the acute phase of the infection, providing no information regarding the immunological status of these patients. By the same time, from the more than 25 rapid serological tests available only one had the EUA granted. These rapid tests are relatively simple to perform and interpret and therefore require limited test operator training. The main drawback of these rapid tests is that the specificity and particularly the sensitivity are lower than the standard Enzyme-linked Immunosorbent Assays (ELISA). As of June 1, 2020 FDA had received more than 198 notifications from manufacturers confirming they have validated and intend to distribute their tests in the market. However only 13 of those tests have indeed the EUA from FDA. Moreover, in May 2020, FDA removed 28 SARS-CoV-2 serological tests from the notification list of tests offered during the COVID-10 emergency for not having an EUA request. Choosing an appropriate test to screen for the presence of humoral immune response to SARS-CoV-2 is critical. Such serologic tests are expected to play a key role in the fight against COVID-19 by helping to identify individuals who had developed an adaptive immune response and may be at lower risk of infection. Also, validated serological tests are needed to confirm which subjects, being confirmed positive for COVID-19, truly developed a substantial humoral immune response and may be considered as plasma donors (1). Different antigens have been used to detect antibodies against other novel coronavirus such as SARS-CoV and MERS-CoV (2-5). From these previous works it can be concluded that spike-derived (S) antigens are more sensitive, specific

and accurate than nucleocapsid protein-derived (NP) antigens. Also results from assays using S antigens correlated much better with the neutralizing titers than those using NP antigens (6). A recent work showed the usefulness for the Receptor Binding Domain (RBD) and the full Spike protein to detect SARS-CoV-2 specific antibodies (7) and their correlation with neutralizing antibodies nAb (8). For these reasons we choose to use a recombinant SARS-CoV-2 Spike Protein, S1 domain containing the RBD.

With this work we described the validation of a quantitative ELISA, CovIgG-Assay (<https://prsciencetrust.org/the-covigg-assay-kit/>), showing a very low background and lack of cross reactivity with other respiratory and non-respiratory pathogens in more than 132 samples collected before June 2019. Also the limited correlation with a rapid test and another serology test available in the market is described. Finally, we confirm the usefulness of the assay detecting anti-SARS-CoV-2 antibodies in plasma samples from potential plasma donors. CovIgG-Assay is a useful tool to characterize, quantify and to study the dynamics of the humoral immune response to SARS-CoV-2.

Materials and Methods

Study Design

The study population included 49 positive samples from individuals with symptomatic infection and positive SARS-CoV-2. Forty-eight (48) were confirmed by RT-PCR tests EUA authorized and one (1) diagnosed by COVID-19 ELISA IgG Antibody Test – Mount Sinai, also EUA authorized. De-identified serum or plasma specimens were obtained from local clinical laboratories and Blood Banks and no personal identifiers were retained. For specificity, we tested a total of 132 samples taken previously to 2019 from the Virology UPR MSC or the Immunology UPR-MSC serum bank. From these samples, 78 had no previous history of viral, allergic or bacterial infections according to our cross-reactivity panel. Nine (9) were previously diagnosed with Zika, three (3) with Dengue, thirteen (13) with history of respiratory allergies and one (1) with Influenza H1N1. We also included a cross reactivity panel with 28 samples kindly donated by the Centers for Disease Control and Prevention (CDC) Dengue Branch, San Juan, PR. These samples included six (6) positives for Respiratory Syncytial Virus (RSV)-IgM, twelve (12) RT-PCR positive for Influenza A or B, five (5) Zika-IgM positive and five (5) positive for Dengue-IgM. In addition, to complete our cross-reactivity panel, we tested nine (9) samples IgM positive for Mycoplasma and three (3) positives for Chikungunya. This cross-reactive panel was selected according to the most common viral and respiratory infections affecting our population. All samples were stored at -80°C until use.

For comparison with two other serological test (CoronaCheck and Abbott Architect) holding an EUA, we used a set of nine (9) samples assumed to be positive for IgG and IgM and eighteen (18) assumed to be IgG positive for SARS-CoV-2 antibodies. Those samples were also received de-identified from local laboratories.

CovIgG-Assay

CovIgG Assay is an indirect ELISA for quantitative determination of human IgG antibody class, which was optimized by checkerboard titration. Disposable high bind flat-bottomed polystyrene 96-wells microtiter plates (Costar, Corning MA No. 3361) were coated overnight at 4°C with 2µg/ml of recombinant SARS-CoV-2 S1-RBD protein (GenScript No. Z03483-1) in carbonate-bicarbonate buffer (Sigma Aldrich No. 08058). Plates were washed 3 times with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST) and blocked for 30 min at 37°C with 250µl/well of 3% non-fat, skim milk in PBST. Samples (serum or plasma) were diluted 1:100 in PBST; 100µL/well was added in duplicates and incubated at 37°C for 30 min. The excess antibody was washed off with PBST. Horseradish peroxidase (HRP) labeled-mouse anti-human IgG-Fc specific (GenScript No. A01854) diluted 1:10,000 in PBST was added (100µl/well) and incubated for 30 min at 37°C. After another washing step, the substrate solution (Sigma Aldrich No. P4809) was added (100µl/well) followed by 15 min incubation in dark. The reaction was stopped by the addition of 50µl/well 10% HCl and the absorbance was measured at 492nm (A_{492}) using a Multiskan FC reader (Thermo Fisher Scientific). In every CovIgG-Assay determination two in-house controls, a high positive control (HPC) and negative control (NC) were included. HPC and NC were prepared by diluting an IgG anti-SARS-CoV-2 at a concentration of 30µg/ml and 0.070µg/ml, respectively in PBST containing 10% glycerol. The IgG anti-SARS-CoV-2 was purified from plasma of a convalescent patient using a 5/5 HiTrap rProtein-A column (GE Healthcare, USA) (see detailed information about this procedure in **Supplementary method No.1**).

Antibody class specificity

To confirm that our assay accurately detects antibody IgG class and excludes the potential for human IgM to cross-react with IgG, five (5) COVID-19 samples (1:100 diluted) were treated with 5mM DTT for 30 min at 37°C prior testing. After treatment, samples were added in duplicate

(100µl/well) followed by the addition of the anti-human IgG-Fc-HRP (GenScript No. A01854) conjugate (diluted 1:10,000) or the addition of an anti-human IgM-HRP conjugate (Abcam No. ab97205) diluted 1:8,000 in PBST and the assay progressed as described above.

Estimation of Antibody Titer

To estimate the IgG antibody titer, 26 COVID-19 RT-PCR-confirmed samples were subjected to serial dilutions from 1:100 to 1:12,800, each dilution was tested in duplicate in the CovIgG-Assay and each experiment was replicated twice. A standard curve was created in which the mean individual absorbance (A_{492}) of each sample at 1:100 dilutions was correlated with its corresponding IgG antibody titer. Antibody titer was defined as the highest serum dilution that renders A_{492} values greater than the cut point estimated by the ROC analysis.

Comparison with two serological assays approved for emergency use

We tested a set of 9 samples reported as IgM/IgG positives and 18 reported as IgG positives for SARS-CoV-2 antibodies by CoronaCheck (20/20 BioResponse, 20/20 Genesystems, Inc, Rockville, MA, USA). The information provided by the manufacturer claims that this assay use Roche's technology (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim, Germany). Same set of 18 samples reported as SARS-CoV-2 IgG positive were also tested by Abbott Architect SARS-CoV-2 IgG (Abbott Laboratories Diagnostics Division Abbott Park, IL 60064 USA). For comparison, both set of samples (n=27) were tested with our CovIgG-Assay.

Data analysis

Each CovIgG-Assay determination was performed in duplicate and the results expressed as the mean absorbance at 492 nm (A_{492}) for each determination. The optimal cut point for the assay was established within a 95% confidence interval (CI) by receiver operating characteristic (ROC) curve analysis using the EpiTools epidemiological calculator (<http://epitools.ausvet.com.au>).

Arbitrary guidelines were followed for analyzing the area under curve (AUC) as follows: non-informative, $AUC=0.5$; low accurate, $0.5 < AUC < 0.7$; moderately accurate, $0.7 < AUC < 1$; perfect, $AUC = 1$ (9). Intra-plate repeatability was evaluated for CovIgG-Assay by measuring the coefficient variation (CV) of 60 repeats of a High Positive Control (HPC) and a Negative Control (NC). For reproducibility evaluation, we completed three independent runs for the CovIgG Assay using HPC, NC, four (4) negative and four (4) COVID-19 positive sera for each of the three runs. Correlation between the A_{492} at 1:100 dilutions and the antibody titer as well as between the results of CovIgG Assay and the RT-PCR test results were evaluated using the Pearson correlation coefficient (with the 95% CI). To evaluate the agreement between the CovIgG-Assay and the RT-PCR, CovIgG-Assay and CoronaCheck or Abbott Architect SARS-CoV-2 IgG, inter-rater agreement (kappa) was calculated according to the method described by Thrusfield (10). The Kappa values (κ) were considered as follows: poor agreement, $\kappa < 0.02$; fair agreement, $\kappa = 0.21$ to 0.4 ; moderate agreement, $\kappa = 0.41$ to 0.6 ; substantial agreement, $\kappa = 0.61$ to 0.8 ; very good agreement, $\kappa = 0.81$ to 1.0 .

Results

Distribution of absorbance values of sera and ROC analysis

We used the RT-PCR for COVID-19 positive samples, as recommended standard reference method, to build ROC curves on the basis of the absorbance values (A_{492}) obtained with specimens from two reference populations: subjects infected with SARS-CoV-2 that were all RT-PCR positive (assumed infected population) and healthy subjects or subjects that had been diagnosed with other respiratory or viral infections prior to the COVID-19 pandemic (uninfected population). The A_{492} values of uninfected population ranged between 0.011 and 0.312 with a mean $\pm$ SD A_{492} value of 0.075 ± 0.052 whereas samples from assumed infected population showed A_{492} values that ranged between 0.045 (one sample) and 3.21 with a mean A_{492} value of 1.99 ± 0.727 . The mean value of the infected population was significantly different from the mean

value of the uninfected population ($p < 0.0001$). The distribution of A_{492} values of these two reference populations was very different. Approximately the 75% of infected population had A_{492} values between 0.828 and 2.5 (median 2.01), whereas that the 95% of uninfected population had A_{492} values between 0.011 and 0.176 (median 0.065) (**Figure-1**). Receiving operating characteristic analysis was used to determine the best cut-points for the CovIgG-Assay. The ROC optimized cut-point was 0.312. The selection of this cut-point derived from three different conditions: (a) maximum specificity at which the sensitivity was still 100%, (b) maximum sensitivity at which the specificity of the assay was also maximized, and (c) maximum value for Youden's J index ($S + Sp - 1$) and test efficiency (**Table-1**).

Table-1. Specificity (Sp) and sensitivity (Se) of the CovIgG-Assay based on cut-points results obtained when compared infected and uninfected populations.

Target Sp	Cut-point	Specificity	Sp lower 95% CL	Sp Upper 95% CL	Sensitivity	Se Lower 95% CL	Se Upper 95% CL
0.999	0.745	1	0.961	1	0.98	0.893	0.996
0.995	0.745	1	0.961	1	0.98	0.893	0.996
0.990	0.745	1	0.961	1	0.98	0.893	0.996
0.98	0.312	0.989	0.943	0.998	0.98	0.893	0.996
0.95	0.202	0.958	0.897	0.984	0.98	0.893	0.996
0.9	0.129	0.905	0.83	0.949	0.98	0.893	0.996
0.8	0.099	0.8	0.709	0.868	0.98	0.893	0.996

The area under curve values (AUC) (accuracy) for the ROC curve was 0.985 (**Figure-2**). Based on the established cut-point only one seronegative was detected in the infected group whereas no seropositive was detected in the uninfected group. A sample from the uninfected group, collected between 1995 and June 2019, had A_{492} values equal to the cut-point and was considered negative (**Figure-3**).

To verify the cross reactivity of the assay we tested 67 samples known to be positive to common respiratory and non-respiratory infections (RSV, Flu A and B, Mycoplasma, Zika, dengue, Chikungunya) or allergies which are very common in the local population. As it is showed in figure

3, all those samples were negative showing no-cross reactivity in CovlgG-Assay. Based on these results the estimated sensitivity is 98% (95% CI 89.35-99.95%) and the specificity is 100% (95% CI 97.24-100%). Also the predictive positive value (PPV) and predictive negative value (NPV) for CovlgG-Assay were estimated to be 100% and 99.25% (95% CI 94.99-99.89%), respectively (**Table-2**). There was substantial agreement ($\kappa=0.71$) between CovlgG-Assay and RT-PCR. Detailed optical densities (ODs) values of the positive and negative samples, including the cross-reactivity panel are provided (**Supplementary tables 1 and 2 respectively**)

Table-2. Agreement between test results of CovlgG-Assay and the PCR-based assay used as reference method for COVID-19 diagnosis.

		PCR-based assay		
		Positive	Negative	Total
CovlgG-Assay	Positive	48	0	48
	Negative	1	132	133
	Total	49	132	181

Positive Predictive Value (PPV)=100% is calculated as the number of individuals with a positive result by CovlgG-Assay / the total of true positive individuals x 100. Negative Predictive Value (NPV)= 99.25% is calculated as the number of individuals reported as negative by CovlgG-Assay / the total of true negative individuals x 100.

We also assessed the reproducibility of the CovlgG-Assay by calculating the CV of data from 3 different assays and 30 repeats of controls and 6 repeats of selected samples. The intra-assay and inter-assay reproducibility values were both lower than 15% (**Supplementary Table 3**).

Class antibody specificity of CovlgG-Assay

To confirm that the positivity showed by CovlgG-Assay with the COVID-19 samples was mostly due to the presence of IgG antibody class and not due to potential cross-reactions with IgM antibody, five samples treated with DTT were tested in parallel on the CovlgG-Assay using as

secondary antibody anti-human IgG- and anti-human IgM-HRP conjugates and the results obtained were compared with those obtained for the same samples previous to the DTT treatment. As expected, the A_{492} values of DTT-treated samples tested with the anti-IgM-HRP conjugate significantly dropped to values similar to the background. In contrast, the A_{492} values for the same DTT-treated samples tested with the anti-IgG-HRP conjugate were similar to those obtained with the untreated samples, confirming that positive results were from IgG antibodies. (Table-3).

Table 3. Samples before and after treatment with DTT (Dithiothreitol) showing IgG class specificity for CovIgG-Assay. Each result represent OD at 492nm absorbance.

Numeric ID	IgG			IgG DTT			IgM			IgM DTT		
	OD1	OD2	Average OD	OD1	OD2	Average OD	OD1	OD2	Average OD	OD1	OD2	Average OD
45	2.7594	2.6820	2.7207	1.7638	1.7889	1.7763	1.6503	1.5807	1.6155	0.0244	0.0235	0.0239
121	3.1175	2.9996	3.0585	2.7779	2.6761	2.7270	3.5162	3.5980	3.5571	0.3303	0.3293	0.3298
122	2.7393	2.3745	2.5569	1.9655	1.9516	1.9585	2.6131	2.5388	2.5759	0.1060	0.1019	0.1039
146	2.7958	2.7973	2.7965	2.3467	2.3361	2.3414	1.0818	1.0508	1.0663	0.0898	0.0876	0.0887
147	2.8958	2.7455	2.8206	2.4553	2.3915	2.4234	3.2084	3.2769	3.2426	0.8243	0.8349	0.8296
183	1.4743	1.4506	1.4624	0.8341	0.8457	0.8399	3.3603	3.3997	3.3800	0.0607	0.0519	0.0563

Samples tested were previously confirmed by PCR and IgG/IgM rapid tests. Sample 183 was previously confirmed by PCR.

Correlation between the A_{492} values and the IgG antibody titer

To determine whether the magnitude of the A_{492} values correlate with the antibody titer we selected 27 samples from infected individuals with different A_{492} values, which were titrated at dilutions from 1:100 to 1:12,800 and each dilution was tested in duplicate in the CovIgG-Assay. The number of individuals with different antibody titers (defined as the maximal dilution that renders a positive result) is shown in Table-4. We found a lineal correlation ($r^2=0.984$) between the antibody titer (maximal dilution that render $A_{492} = 0.312$) and the individual A_{492} value at the working dilution (1:100). Thus, results reported by CovIgG-Assay could be quantitatively reported by estimating the titer, using the lineal equation ($Y= 1.185 \cdot X - 1.773$) derived from the lineal correlation between antibody titer and the magnitude of absorbance values (Figure-4). Titers are reported from 1:100 to 1:12,800 which were the dilutions tested to establish the OD at A_{492} and the IgG titers. Positive samples rendering titers below or above of these values are reported as <1:100 or >1:12,800.

Table 4. Experimental antibody titers of SARS-CoV-2 infected subjects in relation to their absorbance value at 492nm (1:100 dilution).

Antibody Titer*	Number of Individuals	Absorbance range at 492 nm (A_{492})	Mean A_{492} + SD
1:100	0	0	0
1:200	6	0.745-1.03	0.896 + 0.103
1:400	4	1.08-1.55	1.24 + 0.18
1:800	0	0	0
1:1600	7	1.98-2.52	2.2 + 0.203
1:3200	6	2.17-2.57	2.43 + 0.166
1:6400	2	2.37-3.05	2.71 + 0.34
1:12800	2	2.85-3.12	2.985 + 0.135

*Antibody titer is defined as the maximal serum dilution that renders A_{492} greater than the optimized cut-point ($A_{492} = 0.312$) determined by ROC analysis.

Agreement between CovIgG-Assay and other Rapid tests

To evaluate the performance of CovIgG-Assay with other tests in the market, we analyzed a group of samples that have been previously reported as positive for IgG/IgM (n=9) or only positive for IgG (n=18) by CoronaCheck rapid test. CovIgG-Assay had 100% agreement with the CoronaCheck results for the IgG/IgM positive samples. These samples were all reported as positive by CovIgG-Assay with antibody titers that ranged between 1:100 and 1:3,251 (Supplementary table 4). However, the agreement was fair (38.8%) for samples only reported positive for IgG by CoronaCheck since only 7 from 18 samples were reported as positive. From these sera 3 had antibody titers <1:100 and 4 had antibody titers among 1:219 to 1:254 (Table-5). Interestingly, all 18 presumptive IgG positive samples reported by CoronaCheck were found negative by Abbot Architect SARS-CoV-2 IgG method, which might subject that these 18 could be considered as false positive and reveal a better agreement between Abbot Architech SARS-

CoV-2 IgG method and our CovIgG-Assay. The correlation between CovIgG-Assay and Abbot Architect method was of 61% (**Table 5**). Detailed OD values for this experiment are provided in **supplementary table 4**.

Table 5. Comparison of two rapid tests against CoVlgG-Assay for the detection of anti SARS CoV-2 IgG antibodies.

Sample	CoronaCheck ¹ (Rapid COVID-19 IgG)	CoVlgG-Assay ² Estimated titer	CoV-2 IgG ³ (Abbott Architect) Index value
46	+	-	.05
47	+	†	.03
48	+	-	.03
49	+	254	.05
50	+	-	.03
51	+	-	.05
52	+	-	.02
53	+	-	.04
54	+	254	.05
55	+	-	.04
56	+	-	.07
57	+	-	.37
58	+	†	.08
59	+	†	.02
60	+	-	.05
61	+	-	.01
62	+	219	.69
63	+	220	.05

Serum samples obtained during April 2020 were tested for SARS CoV-2 IgG antibodies using two different assays. All samples tested positive with the Coronacheck test, when tested with the CoVlgG-Assay only 7 were positive for a 38.8% concordance. Same set of samples were tested with Abbott Architect CoV-2 (EUA) assay; all samples were negative for a 61% match when compared with CoVlgG-Assay. Positive results for CoVlgG-Assay are expressed as estimated titers from the average of duplicates OD₄₉₂.

¹Lateral flow rapid test intended to qualitatively detect antibodies to SARS-CoV-2. Presence of IgG/IgM antibodies are indicated by a visible line in the specific region on the device. Positive results may be due to cross reactivity with other Coronavirus strains (non CoV-2). Information regarding antigen used is not available. ²Quantitative ELISA for the detection SARS CoV-2 IgG antibodies using Spike S1-RBD (GenScript) as capture antigen. Samples with OD₄₉₂<0.312 are considered negative. [†]IgG titration of positive samples by CovlgG-Assay range from 1:100 to 1:12,800. Values below or above those limits are reported as < or > respectively.

³Chemiluminescent microparticle immunoassay for the qualitative detection of IgG antibodies against SARS CoV-2. Assay is designed to detect IgG antibodies to the nucleocapsid protein of SARS CoV-2. Samples with an index value <1.4 are considered negative.

Discussion

308 Since the circulation of SARS-CoV-2 was spillover outside of China, the global efforts to develop
309 serological assays have been unprecedentedly huge. The precise diagnostic of COVID-19 poses
310 multiples challenges. The proposed method reference is the molecular diagnostic which
311 determine the presence of an active infection. However the timing of viral replication and the
312 development of immune response is quite variable (reviewed in (11)) and the presence of IgM
313 and or IgG at the time of the molecular diagnostic is merely speculative. While the molecular
314 testing results are a guide, they should not be considered gold standards as it has been the
315 practice so far. Other factors as clinical presentation and epidemiological aspects need to be
316 considered at the time of selecting the appropriate samples to validate any assay. Here we
317 selected 48 samples reported positive by authorized molecular methods and 1 sample reported
318 as positive by an authorized serological assay which is not considered a rapid test (7). As negative
319 samples we used a set of 132 sera that were collected in the period of 1995 to June 2019, before
320 COVID-19 period. The CovIgG-Assay data were subjected to ROC curve analysis. During the last
321 two decades this type of analysis has become a popular method for evaluating the accuracy of
322 medical diagnostic systems and has been used not only to evaluate the ability of a test to
323 discriminate between infected and healthy subjects (12) but also to compare the diagnostic
324 performance of a number of tests (13). The ROC curve is obtained by plotting the true-positive
325 rate (sensitivity) as a function of the false-positive rate (100-specificity) that is associated with
326 each cut-point. The AUC is then used as a measure of the accuracy of the test. If the assay can
327 distinguish between infected and normal populations the AUC will be equal to 1 and ROC curve
328 will reach the upper left corner. As our results demonstrate, the AUC value obtained from the
329 ROC curve analysis conducted on the CovIgG-Assay data was very high, indicating the accuracy
330 of this test. The only sample that was not detected by CovIgG-Assay could be a false positive in
331 the RT-PCR. Currently, there are few reports addressing those discordant results. Several
332 molecular assays have been developed with high specificity and low limit of detections (14-17)
333 and are considered the reference method for SARS-CoV-2 diagnostic (18). However everyday

there are more reports addressing problems with the RT-PCR accuracy (14, 19-21). Otherwise that sample may be collected within a window where the immune response was not developed yet. Nevertheless, our results reinforce the complexity of the diagnostic of COVID-19 and the need for prospective studies with more samples and better-characterized cohorts to better understand the dynamics of the immune response to this novel coronavirus.

We also considered fundamental to develop a quantitative test, in addition to suggesting a qualitative result. This would provide a guide about quantity or the dimension of the immune response mounted by an individual. Up to today, few works on COVID-19 addressed the relation between the titer of the IgG and the neutralizing capability of that sample. But all of them coincide that there is a direct correlation (1, 21-23). While the scientific community develops safe (BSL-2) and reproducible neutralization assays to determine nAbs against SARS-CoV-2, quantitative assays like CovIgG-Assay are useful tools for a reliable serological characterization.

When we compared the performance of an available rapid test and a high throughput assay, both with EUA by the FDA. The results are highly worrisome showing 100% and 0% of positive samples reported by CoronaCheck and Abbott Architect SARS-CoV-2 IgG respectively while our assay reported a 38.8% % of the samples as positive. These results reinforce the need for better assays and for better validations in the context of clinical presentations (24).

These results reinforce the concept recently proposed by the CDC to implement an orthogonal testing algorithm (i.e., employing two independent tests in sequence when the first test yields a positive result ([shorturl.at/AHUY2](https://www.cdc.gov/media/releases/2020/s110520-covid-19-testing.html))). However the best characteristics of CovIgG-Assay are its PPV of 100% along a PNV of 99.2% which render highly trustable positive results. These characteristics made this assay suitable to be implemented both in a high or low prevalence settings. A subject confirmed as positive by this assay, with a high degree of confidence can be reincorporating to a normal life in support of the rest of the community with a high degree of confidence of having certain level of immunity. On the other hand the high NPV of the assay increase its value to know which individuals may be still at risk to be infected.

Furthermore, the quantitative aspect of CovIgG-Assay, after a subject being reported as positive, provides a second step of certainty of possible protection against SARS-CoV-2 infection. However such correlation studies using a larger set of samples are under way.

Statistical analysis

The sensitivity of the developed ELISAs was calculated as (the number of true positive samples / the total number of true positive and false negative samples) x 100. The specificity was calculated as (the number of true negative samples / the total number of true negative and false positive samples) x 100. As we do not know the prevalence of the tested population, the PPV (probability that the disease is present when the test is positive) was calculated as the number of individuals with a positive result by CovIgG-Assay / the total of true positive individuals x 100. The NPV was calculated as the number of individuals reported as negative by CovIgG-Assay / the total of true negative individuals x 100. Agreement was calculated as (the total number of true positive and negative samples / the total number of samples) X 100 and evaluated with kappa values. Receiver operating characteristic (ROC) analysis was calculated using GraphPad Prism software.

Authors Contribution

AME and CAS conceived and supervised the studies. PP and AME performed the experiments. CAS and AME drafted the manuscript. CAS, AME and PP reviewed the final version of the manuscript. CAS and AME obtained the funds. CAS prepared and CAS and AME submitted the Emergency Use Authorization request to the US Federal Drug Administration.

Acknowledgement

Authors want to thank Ilia Toledo, MT and Drs. Consuelo Climent and Gerardo Latoni and Ivelisse Martin for their contribution and diligent access to the samples from subject presumptive exposed to SARS-CoV-2 and from plasma donors. Also, thanks to Dr. Jorge L. Muñoz-Jordan for providing the panel to improve the cross-reactivity testing. Particular acknowledgement deserves all administrative and supportive staff at the Medical Sciences Campus, University of Puerto Rico,

386 Laboratorio Clinico Toledo, Laboratorio Clinico Martin, Banco de Sangre Centro Médico and
387 Banco de Sangre Servicios Mutuos for their availability and commitment during the curfew
388 imposed by the quarantine period. This work was supported by the COVID Rapid Grant from the
389 Puerto Rico Science Technology and Research Trust (PRSTRT <https://prsciencetrust.org/>) to
390 AME and CAS. The excellent support of Mss. Andreica Maldonado and Grace Rendon from
391 PRSTRT was instrumental in support of the work described here.

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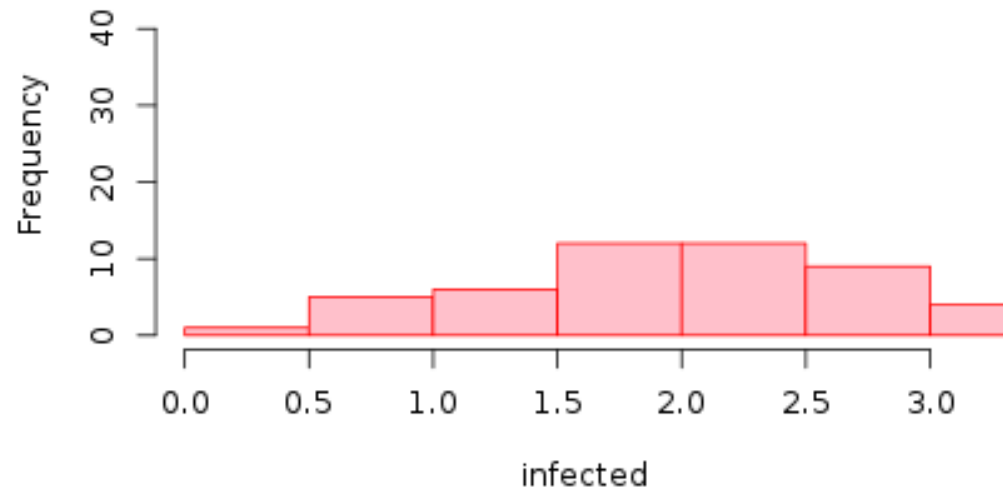
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FIGURES

Distribution of test results for infected



Distribution of test results for uninfected

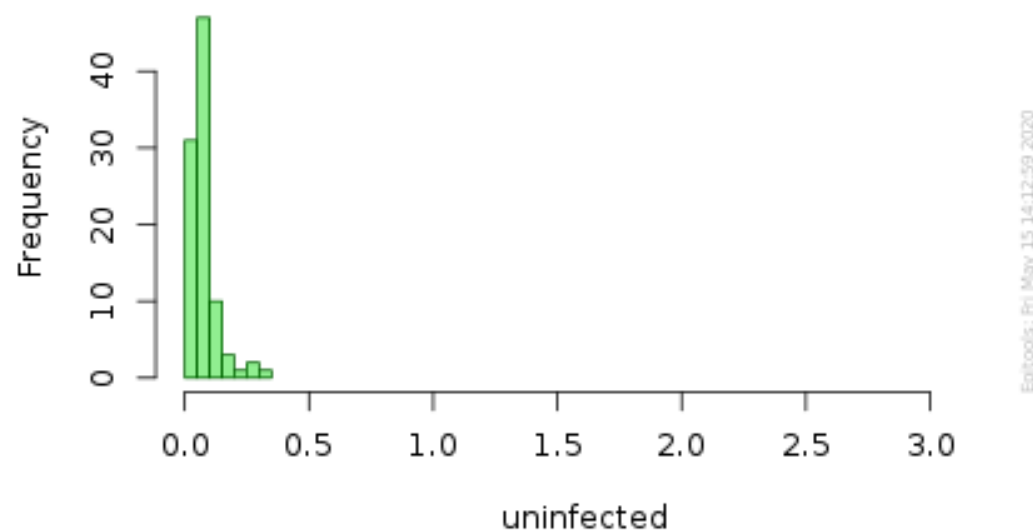


Figure 1: Distribution of absorbance values obtained with CovIgG-Assay. The CovIgG-Assay was optimized using as antigen recombinant Spike-S1-RBD from SARS-CoV-2. Absorbance values were distributed in form of frequency histograms to clearly visualize the separation between true positives (COVID-19 sera) (upper figure) and true negative populations

(sera from healthy subjects and subjects carrying other respiratory and viral infections) (lower figure).

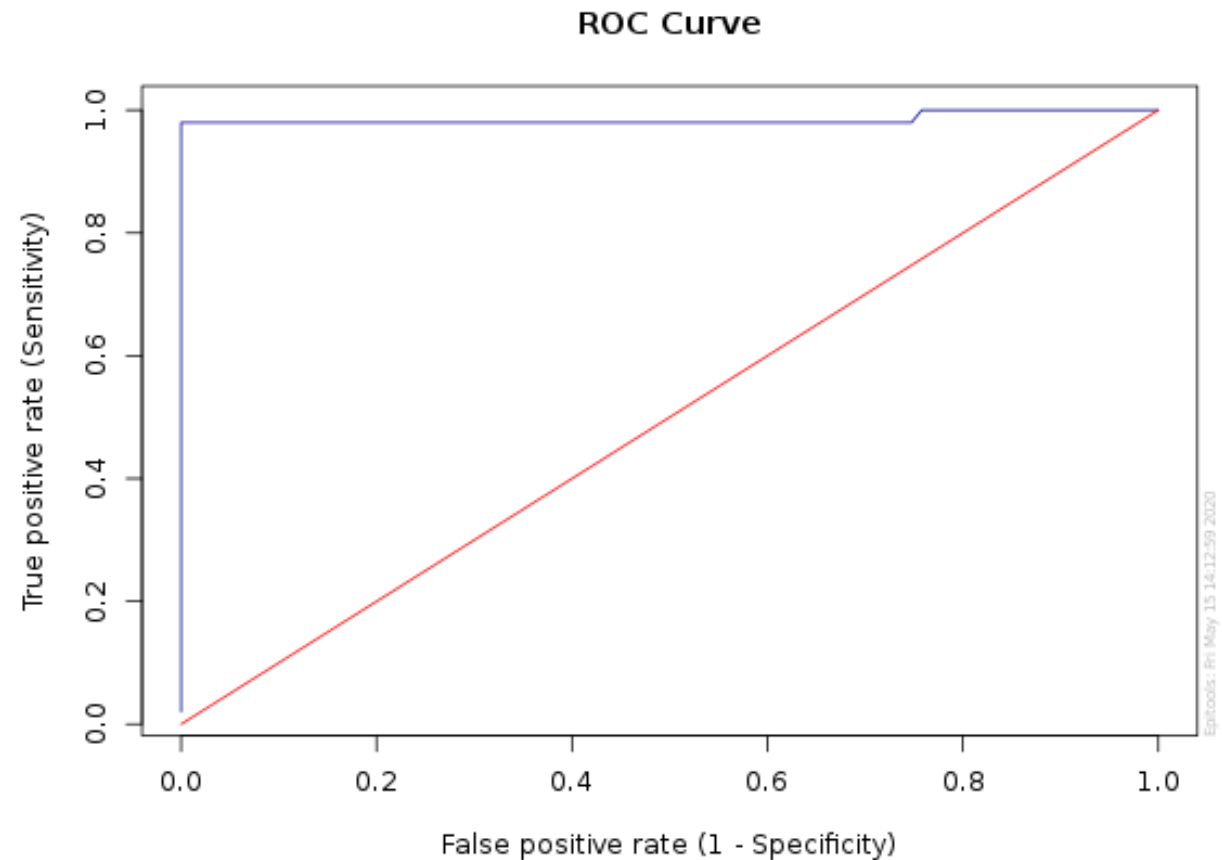


Figure 2: Receiver-operator characteristic (ROC) curve. The ROC curve was built for 132 sera from healthy subjects or subjects carrying other respiratory or other viral infections and 49 COVID-19 confirmed subjects. The area under the ROC curve (accuracy) was 0.985 and 95% for AUC= 0.954-1.

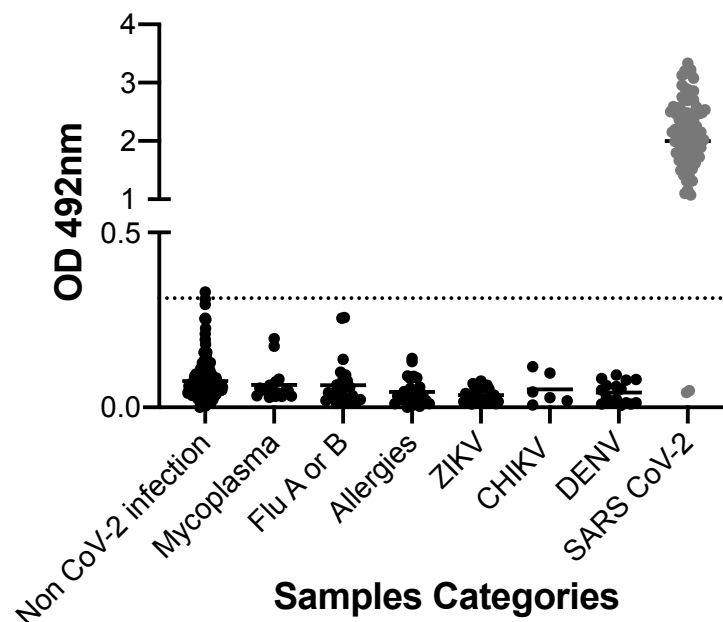
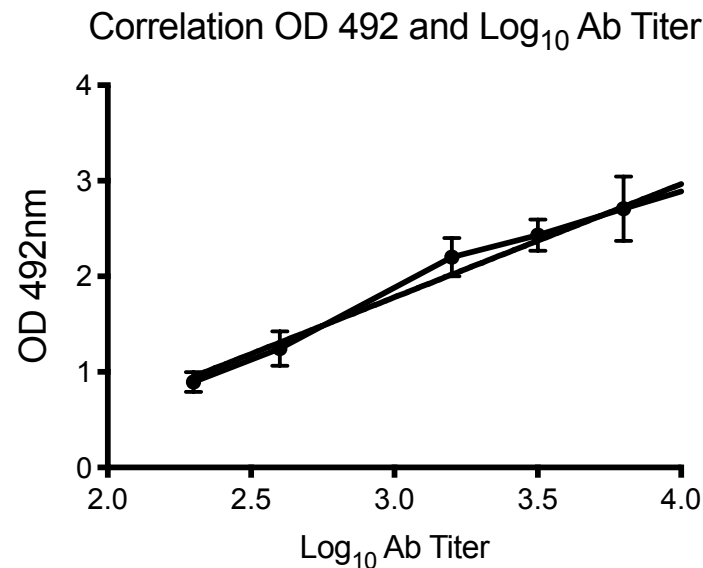


Figure 3: Validation of use of Spike S1-RBD ELISA for detection of SARS-CoV-2 IgG antibodies. Non-CoV-2 infection indicate negative cohorts from samples prior 2019 with no previous history of selected viral infections or respiratory allergies (n= 78). Black dots indicate samples which tested positive for Mycoplasma IgM, (n = 9), Influenza A or B (n=13), respiratory allergies (n=13), ZIKV (n=14), CHIKV (n=3), DENV (n=8). Gray dots indicate SARS CoV-2 infections (n=49). Dotted horizontal line indicate CoVlgG ELISA cutoff value ($OD_{492} = 0.312$). S, spike; RBD, receptor-binding domain; ZIKV, Zika virus; CHIKV, Chikungunya virus; DENV, Dengue virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2. ODs in duplicate for every sample are showed.

493



Best-fit values	
Slope	1.185 ± 0.07537
Y-intercept when X=0.0	-1.773 ± 0.2496
X-intercept when Y=0.0	1.497
1/slope	0.8440
95% Confidence Intervals	
Slope	0.9756 to 1.394
Y-intercept when X=0.0	-2.466 to -1.080
X-intercept when Y=0.0	1.101 to 1.779
Goodness of Fit	
R square	0.9841

Equation: $Y = 1.185 \cdot X - 1.773$

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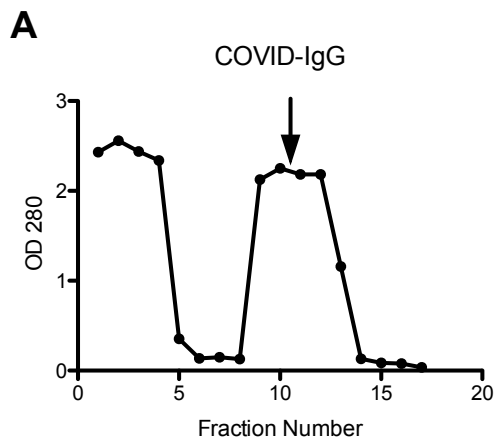
Figure 4: Correlation between absorbance at 492nm (A_{492}) and antibody titer. A total of 27 sera from confirmed COVID-19 subjects that resulted positive by CovlgG-Assay were titrated at dilutions among 1:100 to 1:12,800. A lineal regression analysis was then done in which the mean A_{492} of sera with similar antibody titer were plotted with their corresponding A_{492} values. We found a lineal correlation ($r^2=0.984$) between the antibody titer (maximal dilution that render $A_{492} = 0.312$) and the individual A_{492} value at the working dilution (1:100). From this analysis the following lineal equation ($Y = 1.185 \cdot X - 1.773$) was obtained, which was further used to estimate the antibody titer of all sera reported as seropositive by CovlgG-Assay.

SUPPLEMENTARY DATA

Supplementary Method-1

IgG Purification for positive and negative controls elaboration

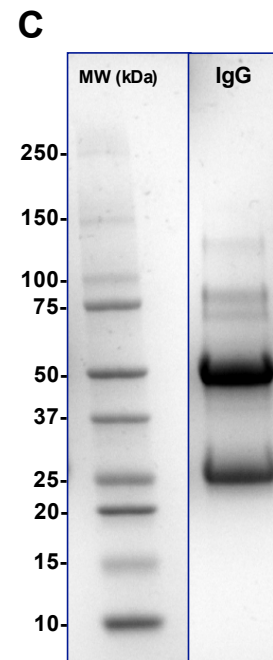
For the assay quality control; positive and negative controls were included for each assay. These controls were prepared in-house from plasma from a convalescent subject with COVID-19. **(A)** 20mL of the plasma sample was mixed 1:1 with phosphate buffer saline (PBS) and loaded onto a 5/5 HiTrap rProtein-A column (GE Healthcare, USA) at a flow rate: of 1.5mL/min. The equilibration buffer used was PBS (pH 7.2), for elution we used 0.1M Glycine-HCl (pH 2.85) and the neutralization buffer used was 1M Tris pH 8.5. **(B)** A total of 17 fractions were collected and optical density (OD) was determined for each fraction at 280nm absorbance (A280). Fractions 9 to 14 were pooled in a total volume of 20ml and OD was determined (A280=2.179). Pooled samples were desalted by PD-10 column and OD was also determined for the desalted fraction (A280= 2.076). The final concentration for this fraction was 1.48mg/mL $(2.076 / 14) \times 10$ in 28mL, for a total of 41.4mg. **(C)** A total of 2µg-purified IgG was suspended in loading SDS-buffer containing 5mM DTT and analyzed for purity by 4-20% sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and stained with Coomassie-blue. A high positive control and a negative control were prepared by diluting the aforementioned IgG anti-SARS-CoV-2 at a concentration of 30µg/mL and 0.078µg/mL respectively in PBST + 10% glycerol.



Column HiTrap rProtein-A FF 5/5
Loading buffer: PBS pH 7.2
Sample: 20-ml Plasma BB#1 + 20-ml PBS
Elution Buffer: 0.1M Glycine-HCL pH 2.85

B

Fraction	OD 280nm
1	2.438
2	2.563
3	2.438
4	2.341
5	0.353
6	0.135
7	0.149
8	0.13
9	2.126
10	2.251
11	2.184
12	2.184
13	1.16
14	0.132
15	0.087
16	0.078
17	0.036



Supplementary Table 1: Positive samples used for CovIgG-Assay validation.
Each result represent OD at 492nm absorbance.

Immunological status/Source	Numeric ID	Confirmatory Method	Sample Date	OD1	OD2	Average OD	Estimated Titer
PCR+/ IgG/IgM Positive Local Laboratory	45	LabCorp	4/12/20	2.034	2.251	2.291	1:2691
Blood Bank 1 PCR+	102	Roche or LabCorp or CDC	4/27/20	2.533	2.591	2.555	1:4487
	103	Roche or LabCorp or CDC	4/27/20	2.584	2.463	2.539	1:4355
	104	Roche or LabCorp or CDC	4/27/20	0.972	0.975	0.973	1:207
	105	Roche or LabCorp or CDC	4/27/20	2.422	2.487	2.447	1:3639
	106	Roche or LabCorp or CDC	4/27/20	0.797	0.876	0.826	1:156
Local Major Hospital PCR+	120	Cepheid	4/27/20	2.022	2.005	2.014	1:1570
Local Major Hospital PCR+ / IgG/IgM Positive	121	Cepheid	4/24/20	2.390	2.355	2.372	1:3148
Local Major Hospital PCR+/ IgG+/IgM-	143	Cepheid	4/28/20	2.885	2.818	2.851	1:7980
PCR+ / IgG/IgM Positive Local Laboratory	122	LabCorp	4/16/20	1.991	1.970	1.981	1:1472
Blood Bank 1 PCR+	132	Roche or LabCorp or CDC	4/30/20	2.153	2.198	2.176	1:2148
	133	Roche or LabCorp or CDC	4/30/20	1.163	0.998	1.081	1:256
	134	Roche or LabCorp or CDC	4/30/20	0.828	0.816	0.822	1:155
	135	Roche or LabCorp or CDC	4/30/20	1.953	2.009	1.981	1:1472
	136	Roche or LabCorp or CDC	4/30/20	0.048	0.043	0.045	N/A
	137	Roche or LabCorp or CDC	4/30/20	0.973	1.100	1.037	1:235
Blood Bank 2 PCR+	155	Quest PCR	5/11/20	1.429	1.314	1.371	1:450
	156	Quest PCR	5/11/20	1.793	1.724	1.759	1:955
	157	Quest PCR	5/11/20	2.464	2.501	2.483	1:3899
	158	Roche	5/11/20	3.080	3.334	3.207	>12800
	159	PR ASEM PCR	5/11/20	2.906	3.206	3.056	1:11,885
	160	Quest PCR	5/11/20	1.916	1.879	1.897	1:1,250
	161	Quest PCR	5/11/20	2.746	3.170	2.958	1:9,817
	162	Roche	5/11/20	2.264	1.981	2.122	1:1936
	163	Roche	5/11/20	1.415	1.460	1.437	1:512
	164	Roche	5/11/20	2.694	2.513	2.604	1:4932
	165	Roche	5/11/20	2.199	2.194	2.196	1:2239
	166	Roche	5/11/20	1.715	1.851	1.783	1:1002

	167	Roche	5/11/20	1.796	1.800	1.798	1:1032
	170	VA Orlando, FL PCR	5/11/20	3.128	3.152	3.140	>12800
	171	Roche	5/11/20	2.468	2.487	2.477	1:3864
	173	PR Dept. of Health PCR	5/11/20	3.185	3.223	3.204	>12800
	176	VA San Juan PCR	5/11/20	2.484	2.541	2.512	1:4130
	177	PR Dept. of Health PCR	5/11/20	2.103	2.296	2.200	1:2254
	178	Quest PCR	5/11/20	1.621	1.521	1.571	1:664
	181	Roche	5/11/20	1.748	1.682	1.715	1:879
	182	Quest PCR	5/11/20	2.857	2.955	2.906	1:8872
	183	COVID-19 Ab Assay - Titer 2880 Mount Sinai	5/11/20	1.758	1.663	1.711	1:871
	184	Quest PCR	5/11/20	1.889	1.616	1.752	1:944
	185	Roche	5/11/20	1.362	1.497	1.430	1:505
	186						
PCR+ Local Laboratory	146	LabCorp	5/11/20	2.707	2.756	2.732	1:6324
	147	LabCorp	5/11/20	2.573	2.700	2.636	1:5260
Blood Bank 1 PCR+	148	Roche or LabCorp or CDC	5/11/20	2.480	2.019	2.250	1:2483
	149	Roche or LabCorp or CDC	5/11/20	2.052	2.131	2.092	1:1824
	150	Roche or LabCorp or CDC	5/11/20	0.925	0.848	0.886	1:175
	151	Roche or LabCorp or CDC	5/11/20	2.153	2.220	2.187	1:2193
	152	Roche or LabCorp or CDC	5/11/20	1.316	1.073	1.195	1:319
	153	Roche or LabCorp or CDC	5/11/20	2.365	2.155	2.260	1:2529
	154	Roche or LabCorp or CDC	5/11/20	0.984	0.990	0.987	1:213

Estimated titer was calculated , using the lineal equation ($Y = 1.185 \cdot X - 1.773$) derived from the lineal correlation between antibody titer and the magnitude of absorbance values.

Supplementary Table 2

Sample Source	Numeric ID	Immunological status	Sample Date	OD1	OD2	Average OD
Virology Serum Bank	VB1		5/23/95	0.028	0.027	0.027
	VB2		12/7/95	0.211	0.194	0.202
	VB3		2/10/97	0.082	0.087	0.085
	VB4		5/25/97	0.054	0.053	0.054
	VB5		2/17/06	0.041	0.054	0.047
	VB6		6/28/16	0.054	0.059	0.056
	VB7		4/10/17	0.092	0.106	0.099
	VB8		8/15/16	0.067	0.102	0.084
	VB9		8/15/16	0.310	0.225	0.268
	VB10		6/27/17	0.075	0.058	0.067
	VB11		1/10/17	0.023	0.000	0.011
	VB12		4/28/17	0.076	0.061	0.068
	VB13		6/26/18	0.330	0.294	0.312
	VB14		6/26/18	0.025	0.025	0.025
	VB15		6/28/18	0.036	0.030	0.033
	VB16		9/8/00	0.040	0.050	0.045
	VB17		5/8/19	0.026	0.033	0.029
	VB18		6/6/19	0.069	0.077	0.073
Immunology Bank	IB95		2012	0.087	0.094	0.090
	IB96		2012	0.042	0.050	0.046
	IB97		2012	0.081	0.076	0.078
	IB100		2012	0.032	0.031	0.032
	IB144		2012	0.100	0.100	0.100
	IB145		2012	0.048	0.036	0.042
	IB146		2012	0.072	0.067	0.069
	IB147		2012	0.035	0.038	0.036
	IB148		2012	0.028	0.031	0.029
	IB149		2012	0.043	0.049	0.046
	IB135		2012	0.044	0.044	0.044
	IB136		2012	0.112	0.095	0.103
	IB137		2012	0.024	0.027	0.025
	IB138		2012	0.023	0.034	0.029
	IB139		2012	0.028	0.027	0.027
	IB140		2012	0.107	0.106	0.106
	IB141		2012	0.033	0.025	0.029
	IB142		2012	0.049	0.040	0.044

	IB143		2012	0.041	0.040	0.040
	IM		2012	0.032	0.029	0.030
	AO		2012	0.096	0.090	0.093
	OF		2012	0.075	0.069	0.072
	VB83		2012	0.064	0.060	0.062
	IB37		2012	0.069	0.078	0.074
	IB58		2012	0.055	0.059	0.057
	IB86		2012	0.016	0.015	0.015
	IB84		2012	0.014	0.016	0.015
Virology Serum Bank	RB	ZIKV +	2016	0.084	0.082	0.083
	FM		2016	0.019	0.024	0.021
	VB82		2016	0.083	0.088	0.085
	JN		8/11/16	0.076	0.054	0.065
	JR		8/15/16	0.157	0.157	0.157
	EXP		4/1/17	0.040	0.015	0.028
Immunology Bank	IB133		2012	0.029	0.004	0.017
	IB130		2012	0.073	0.061	0.067
	IB131		2012	0.024	0.025	0.024
	IB132		2012	0.253	0.251	0.252
Immunology Bank	IB1	Resp. Allergies	2012	0.081	0.084	0.083
	IB2		2012	0.044	0.048	0.046
	IB3		2012	0.048	0.049	0.049
	IB4		2012	0.055	0.123	0.089
	IB5		2012	0.127	0.061	0.094
	IB6		2012	0.050	0.050	0.050
	IB7		2012	0.103	0.107	0.105
	IB8		2012	0.081	0.080	0.081
	IB9		2012	0.090	0.095	0.093
	IB10		2012	0.003	0.045	0.024
	IB11		2012	0.255	0.128	0.192
	IB12		2012	0.065	0.064	0.065
	IB13		2012	0.058	0.317	0.188
Immunology Bank	BVF		2012	0.052	0.059	0.056
	VA		2012	0.058	0.064	0.061
	JJF		2012	0.070	0.075	0.073
	IB53		2012	0.067	0.062	0.065
	IB54		2012	0.058	0.059	0.059
	IB55		2012	0.049	0.050	0.050
	IB56		2012	0.180	0.158	0.169

	IB64		2012	0.095	0.082	0.089
	IB65		2012	0.051	0.062	0.057
	IB66		2012	0.065	0.066	0.066
	IB74		2012	0.062	0.063	0.063
	IB75		2012	0.398	0.327	0.363
	IB77		2012	0.063	0.062	0.063
	IB78		2012	0.360	0.287	0.324
	IB79		2012	0.137	0.121	0.129
	IB80		2012	0.108	0.103	0.106
	IB81		2012	0.089	0.093	0.091
	IB83		2012	0.121	0.118	0.120
	IB84		2012	0.106	0.102	0.104
	IB85		2012	0.059	0.055	0.057
	IB86		2012	0.066	0.067	0.067
	IB89		2012	0.064	0.062	0.063
	IB90		2012	0.100	0.139	0.120
	IB22		2012	0.066	0.063	0.065
	IB23		2012	0.068	0.068	0.068
	IB35		2012	0.063	0.061	0.062
	IB69		2012	0.069	0.068	0.069
	IB82		2012	0.059	0.060	0.060
CDC*	CDC233	DENV+	4/28/20	0.013	0.013	0.013
	CDC255	DENV+	4/28/20	0.010	0.009	0.010
	CDC269	DENV+	4/28/20	0.014	0.006	0.010
	CDC713	DENV+	4/28/20	0.052	0.050	0.051
	CDC736	DENV+	4/28/20	0.082	0.080	0.081
	CDC315	ZIKV +	4/28/20	0.058	0.052	0.055
	CDC324	ZIKV +	4/28/20	0.017	0.024	0.021
	CDC432	ZIKV +	4/28/20	0.011	0.012	0.011
	CDC493	ZIKV +	4/28/20	0.075	0.064	0.069
	CDC518	ZIKV +	4/28/20	0.055	0.062	0.059
	CDC101	Influenza A+	4/28/20	0.011	0.018	0.014
	CDC102	Influenza A+	4/28/20	0.023	0.023	0.023
	CDC103	Influenza A+	4/28/20	0.042	0.045	0.044
	CDC104	Influenza A+	4/28/20	0.015	0.020	0.017
	CDC105	Influenza B+	4/28/20	0.064	0.064	0.064
	CDC106	Influenza B+	4/28/20	0.257	0.255	0.256
	CDC107	Influenza B+	4/28/20	0.077	0.137	0.107
	CDC108	Influenza B+	4/28/20	0.035	0.036	0.035

	CDC109	Influenza B+	4/28/20	0.014	0.017	0.015
	CDC110	Influenza B+	4/28/20	0.080	0.022	0.051
	CDC111	Influenza B+	4/28/20	0.093	0.101	0.097
	CDC112	Influenza B+	4/28/20	0.058	0.063	0.060
	CDC113	RSV	4/28/20	0.058	0.070	0.064
	CDC114	RSV	4/28/20	0.036	0.034	0.035
	CDC115	RSV	4/28/20	0.050	0.050	0.050
	CDC116	RSV	4/28/20	0.022	0.025	0.024
	CDC117	RSV	4/28/20	0.098	0.101	0.100
	CDC118	RSV	4/28/20	0.036	0.037	0.037
Virology Serum Bank	1		4/26/17	0.061	0.069	0.065
	3	FluA H1N1+	6/26/18	0.039	0.041	0.040
	5	DENV +	10/11/17	0.092	0.078	0.085
	7	DENV +	8/17/14	0.024	0.029	0.026
	9	DENV +	6/24/16	0.060	0.062	0.061
	29	ZIKV +	6/29/16	0.027	0.022	0.024
	VB83	ZIKV +	2016	0.014	0.009	0.012
	VB84	ZIKV +	2016	0.012	0.016	0.014
Mycoplasma IgGM+	107	Mycoplasma IgGM+	4/28/20	0.0289	0.0309	0.0299
	123		4/28/20	0.0472	0.0456	0.0464
	124		4/28/20	0.0313	0.0331	0.0322
	125		4/28/20	0.1966	0.1743	0.1854
	126		4/28/20	0.0499	0.0554	0.0526
	127		4/28/20	0.0641	0.0804	0.0722
	128		4/28/20	0.0516	0.0536	0.0526
	129		4/28/20	0.0286	0.0402	0.0344
	130		4/28/20	0.0323	0.0315	0.0319
	131		4/28/20	0.0631	0.0738	0.0684
Virology Serum Bank	119	Chikungunya +	5/4/20	0.0067	0.0445	0.0256
	20		4/15/20	0.1159	0.0982	0.1070
	118		5/4/20	0.0184	0.0281	0.0232

OD values of all negative and cross reactive samples. Each result represent OD at 492nm absorbance.

*Samples dates refer to the date of panel assemble no to the sample collection which was prior 2020

Supplementary Table 3.

Sample	Replicates	OD range	Mean $\pm$SD
HPC	30	2.29-3.33	2.476 $\pm$ 0.211
NC	30	0.0003-0.0164	0.022 $\pm$ 0.021
1	20	0.0197-0.0649	0.046 $\pm$ 0.0185
2	20	2.29-3.60	2.914 $\pm$ 0.462
3	6	2.01-2.72	2.35 $\pm$ 0.33
4	6	0.79-1.27	0.96 $\pm$ 0.21
5	6	1.48-2.25	1.87 $\pm$ 0.36
6	6	1.48-2.45	1.95 $\pm$ 0.4
7	6	1.81-2.36	2.08 $\pm$ 0.36
8	6	2.0-2.17	2.08 $\pm$ 0.075

HPC-high positive control; NC-negative control

Supplementary Table 4: Comparison of CovIgG assay with two alternative rapid tests.

Previous Testing	Numeric ID	Date	OD1	OD2	Average OD	Estimated Titer
IgG Positive by CoronaCheck kit IgG Negative by Abbott Architect	46	4/7/20	0.0462	0.0401	0.043	N/A
	47	4/8/20	0.4019	0.3484	0.375	<100
	48	4/8/20	0.0132	0.0136	0.013	N/A
	49	4/8/20	1.1209	1.0302	1.076	254
	50	3/31/20	0.0376	0.0400	0.039	N/A
	51	4/16/20	0.0211	0.0220	0.022	N/A
	52	4/22/20	0.0264	0.0322	0.029	N/A
	53	4/7/20	0.2408	0.2675	0.254	N/A
	54	4/22/20	1.0786	1.0731	1.076	254
	55	4/8/20	0.0804	0.0864	0.083	N/A
	56	4/20/20	0.1052	0.1287	0.117	N/A
	57	4/22/20	0.0187	0.0175	0.018	N/A
	58	4/14/20	0.4744	0.4319	0.453	<100
	59	4/20/20	0.5221	0.6148	0.568	<100
	60	4/22/20	0.1433	0.1669	0.155	N/A
	61	4/22/20	0.1230	0.1197	0.121	N/A
	62	4/9/20	1.0005	1.0023	1.001	219
	63	4/22/20	0.9949	1.0091	1.002	220
IgG/IgM Positive by Corona Check kit	34	4/16/20	0.2901	0.3395	0.315	<100
	35	4/16/20	2.0120	2.0430	2.027	1611
	36	4/16/20	0.7907	0.7226	0.757	136
	37	4/16/20	1.5401	1.4808	1.510	590
	38	4/16/20	1.4867	1.5621	1.524	607
	39	4/16/20	1.8183	1.8326	1.825	1089
	40	4/16/20	2.2866	2.4907	2.389	3251

Each result represent OD at 492nm absorbance.

Estimated titer was calculated , using the lineal equation ($Y = 1.185 * X - 1.773$) derived from the lineal correlation between antibody titer and the magnitude of absorbance values.