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RESEARCH ARTICLE

COVID-19 Antibody Detection and Assay Performance Using Red Cell Agglutination

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ABSTRACT Red cells can be labeled with peptides from the SARS-CoV-2 spike protein (C-19 kodecytes) and used as reagent cells for serologic screening of SARS-CoV-2 antibodies. We evaluated 140 convalescent COVID-19 donors and 275 healthy controls using C19-kodecytes. The analytical performance of the C19-kodecyte assay was compared with a virus neutralizing assay and two commercial chemiluminescent antibody tests (Total assay and IgG assay, Ortho). The C19-kodecyte assay detected SARS-CoV-2 antibodies with a sensitivity of 92.8% and specificity of 96.3%, well within the minimum performance range required by FDA for EUA authorization of serologic tests. The Cohen's kappa coefficient was 0.90 indicating an almost perfect agreement with the Total assay. The Spearman's correlation coefficient was 0.20 with the neutralizing assay (0.49 with IgG, and 0.41 with Total assays). The limited correlation in assay reaction strengths suggested that the assays may be influenced by different antibody specificities. The C19-kodecyte assay is easily scalable and may vastly improve test capacity in any blood typing laboratory using its routine column agglutination platforms.

IMPORTANCE We recently developed a red cell based assay to detect SARS-CoV-2 antibodies in human plasma. In the current study, we show the hands-on application of this assay in a group of COVID-19 convalescent plasma donors and healthy individuals. We compared our assay against three published assays, including two that are widely used for patient care in the United States. Our assay compared well with all three assays. Our easily scalable assay can be used for population-wide screening of SARS-CoV-2 antibody status. It can be readily established in any hospital blood bank worldwide using its routine equipment.

KEYWORDS COVID-19, SARS-CoV-2, C19-kodecyte, column agglutination technique, assay evaluation, red cell agglutination

The coronavirus disease 2019 (COVID-19) pandemic, caused by the novel betacoronavirus SARS-CoV-2, has resulted in a global public health crisis. As of July 7, 2021, there have been more than 184 million confirmed cases of COVID-19, including more than 3.9 million deaths reported globally (1). Vaccines against SARS-CoV-2 were first approved in China on June 25, 2020 for limited use in the military (2), followed by Russia on August 11, 2020 for emergency use (3). In the United States, the United States Food and Drug Administration (FDA) issued the first emergency use authorization (EUA) for Pfizer-BioNTech COVID-19 vaccine on December 11, 2020 (4). Worldwide, 18 COVID-19 vaccines have been authorized or approved, as of July 7, 2021 (5).

On August 23, 2020, the FDA issued an EUA for COVID-19 convalescent plasma (CCP) for the treatment of hospitalized COVID-19 patients (6). While some patients benefitted

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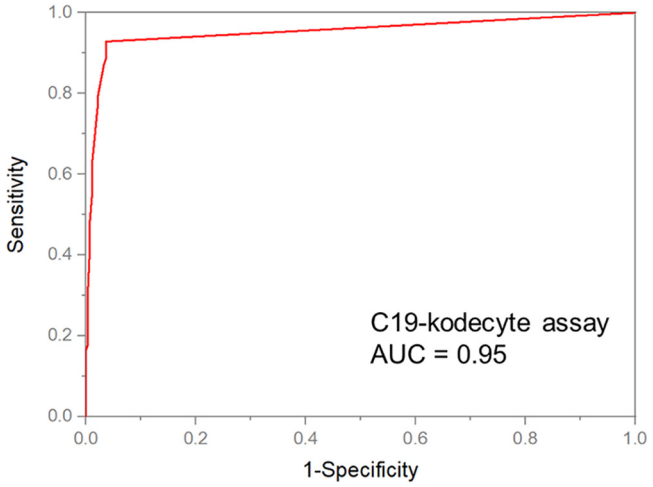

FIG 1 ROC-AUC analysis of C19-kodecyte assay. Receiver operating characteristic curves were obtained based on the agglutination scores for the donors in the COVID-19 convalescent plasma group and the control group consisting of healthy donors.

from the passive immunity through neutralizing antibodies (6–8), more recent studies have concluded no clear benefit of the CCP therapy (9) with many blood centers suspending a collection of convalescent plasma.

Serologic assays detecting antibodies against the SARS-CoV-2 spike or nucleocapsid proteins have been developed to advance our understanding of the prognosis and clinical course of the COVID-19 disease. These serologic assays are also used to identify asymptomatic individuals, evaluate immune response in patients, identify high-titer CCP units, determine duration and magnitude of immunity conferred by SARS-CoV-2 vaccine, and assist in the prediction of disease progression and epidemiology (10, 11). As of July 7, 2021, the FDA has authorized 84 SARS-CoV-2 antibody assays under EUA (12). These antibody assays are based on chemiluminescence, fluorescence, or colorimetric chemistry. Only 12 have been accepted by the FDA for CCP manufacture (6).

In Kode technology, function-spacer-lipid (FSL) constructs are attached to the surface of cells (13, 14). Each cell is thus loaded with structurally defined antigenic epitopes at known and controllable concentrations. Such cells, called kodecytes, have been successfully used for the qualitative and quantitative detection of antibodies for diagnostic purposes (15–19).

Grounded on the principles of the Kode technology (13–19), we recently developed a red cell agglutination-based assay to detect SARS-CoV-2 antibodies (20, 21). This assay uses peptide fragments of the SARS-CoV-2 spike protein to label red cells (C19-kodecytes). These C19-kodecytes are agglutinated in the presence of SARS-CoV-2 antibodies using routine serology techniques available in most hospitals worldwide. We performed a clinical evaluation of this C19-kodecyte assay in CCP donors previously assessed with two commercial immunoassays and a virus neutralizing assay (22).

RESULTS

A total of 140 CCP donors and 275 healthy controls (125 from year 2020 and 150 from year 2008) were analyzed in the C19-kodecyte assay (Table S1). We compared the C19-kodecyte assay results with those from the two Ortho chemiluminescent immunoassays and with a virus neutralizing assay.

C19-kodecyte assay. The area under the receiver operating characteristic (ROC) curve (AUC) for the C19-kodecyte assay reached 0.95 (95% CI 0.93 to 0.97) with sensitivity of 92.8% (95% CI 86.9% to 96.3%) and specificity of 96.3% (95% CI 93.2% to 98.1%) (Fig. 1). The median log anti-SARS-CoV-2 score was 1.41 (interquartile range: 1.20–1.60).

Ortho SARS-CoV-2 assays. The Ortho Total assay was positive in 97.8% of CCP samples (137/140) and the Ortho IgG assay in 92.1% (129/140). The median log anti-

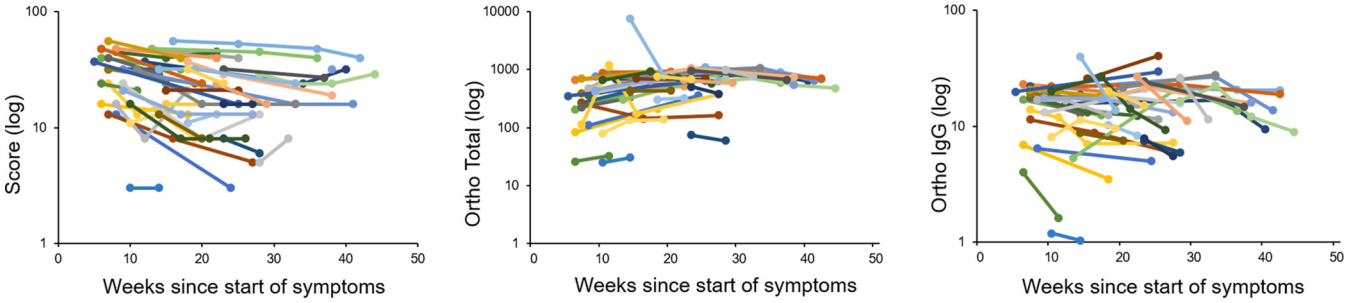

FIG 2 Change in Strength (score), Ortho Total, and Ortho IgG S/CO ratio over time for each individual donor. Each data point represents the antibody levels calculated from an individual convalescent plasma donation, and each color represents an individual donor followed longitudinally. Each time point was calculated using the day of donation and the donor's self-reported first day of symptoms. The median time interval from the onset of symptoms and plasma donation was 78 days (Quartile 1–Quartile 3, 53–111 days; range, 33–331 days). Total score was calculated by adding the individual scores at 2-fold serial dilutions.

SARS-CoV-2 S/CO ratio and interquartile range for each assay were similar to a previous report (22) (Fig. S2).

SARS-CoV-2 virus neutralizing assay. The NT50 titers of the neutralization assay varied widely among the 140 CCP samples (Fig. S3). The highest NT50 titer of 640 was observed in two samples (1.4%). In 45 samples (32.1%), the NT50 titers were below the detection threshold of 40.

Longitudinal follow-up. In almost all of the 40 CCP donors with longitudinal data, the antibody concentration decreased during the follow-up, which ranged between 7 to 44 weeks (Fig. 2). The neutralizing assay was not tested with the follow-up samples.

Assay correlation. In the 140 CCP donors, we compared the C19-kodecyte score to the antibody concentrations from the two FDA authorized assays (Ortho Total and Ortho IgG) and the titer in the neutralizing assay. There was a positive relationship between the results of all four assays (Fig. 3). The Spearman's correlation *r* ranged from 0.20 to 0.49 and the R^2 from 0.0796 to 0.2959, which is considered a weak linear relationship (23, 24).

Cohen's kappa. The assay agreement was calculated using the results of the 140 CCP donors and the 125 healthy controls from 2020, known to be Ortho Total negative. The Cohen's kappa, as an indicator of agreement between assays, was 0.90 (95% CI 0.85 to 0.95) (25) which is considered almost perfect (26) (Table 1).

False positive and false negative rate. We found 17 CCP donors who tested negative in one or several of the five assays, three of them without detectable antibodies in the two kodecyte and two Ortho assays (Table 2). We found six Ortho Total negative donors from 2020, not tested with the Ortho IgG assay, who were positive with the C19-kodecyte assay (Table 1). Additionally, the C19-kodecyte assay gave positive result in four of the 150 donors collected in 2008 (false positive rate: 2.7%; data not shown) and gave negative results in 10 of the 140 rRT-PCR SARS-CoV-2 positive donors (false negative rate: 7.1%; Table 2).

Antibody detection after vaccination. The Ortho Total assay turned positive in a vaccinated individual on day 14 after the vaccination and the C19-kodecyte assay on day 28 when the second dose was scheduled (data not shown). Additional results were reported separately (27).

DISCUSSION

Red cells coated with peptides can be used for the screening of SARS-CoV-2 antibodies in undiluted human plasma (20). Unlike the other 84 serologic tests for SARS-CoV-2 (12), this C19-kodecyte assay is a simple, rapid test that can be established in any hospital blood bank using the already available routine blood typing equipment. Our assay can be automated and is well-suited for large-scale surveillance studies. We compared the C19-kodecyte assay with two FDA authorized assays (Ortho Total and Ortho IgG) and an NIH developed virus neutralizing assay. All four assays detect antibodies directed against the SARS-CoV-2 spike glycoprotein (20, 28, 29).

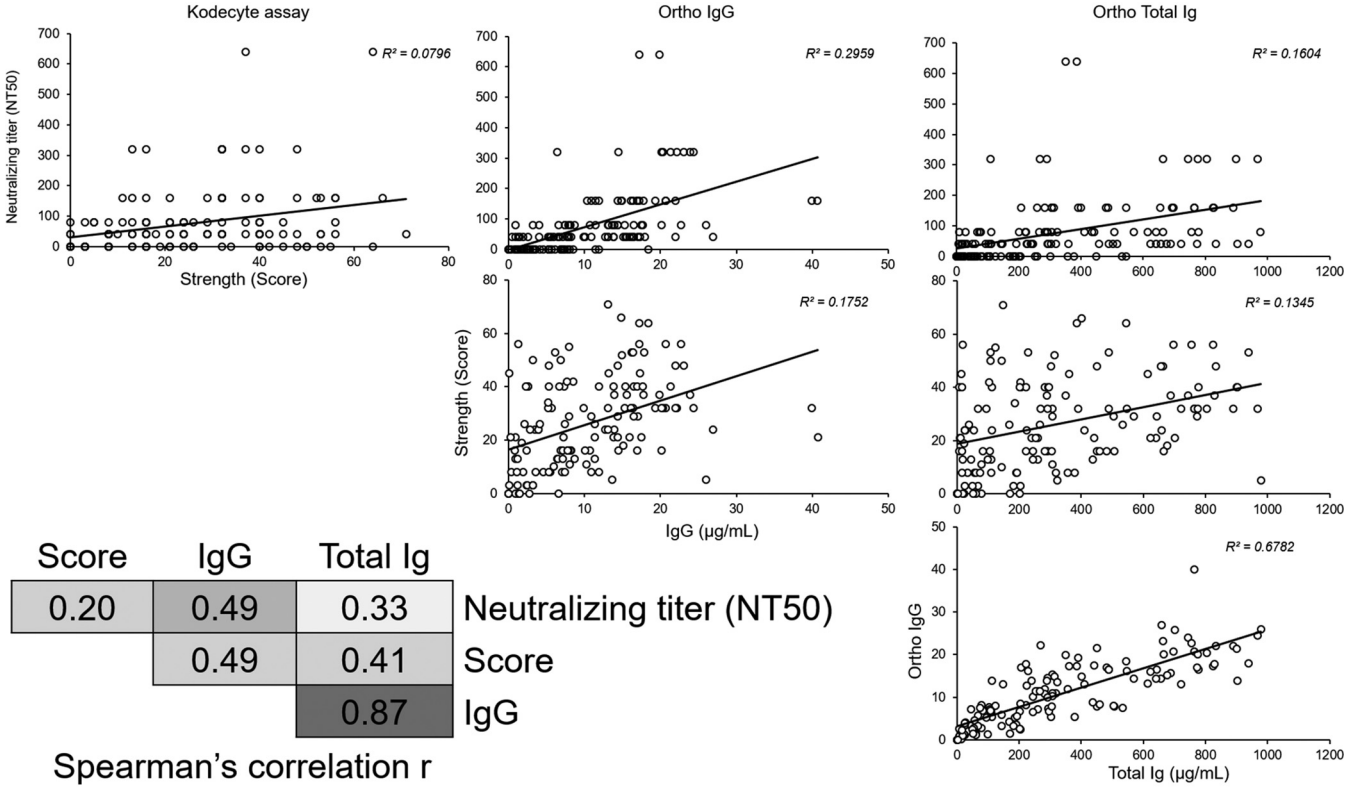

FIG 3 Correlation plots comparing all assays tested in 140 CCP donors, with linear curve fitting R^2 value shown. Spearman's correlation (*r*) for pairwise comparisons of each assay tested is also shown. Each circle represents one donor. Total score was calculated by adding the individual scores at 2-fold serial dilutions.

The C19-kodecyte assay demonstrated an almost perfect agreement with the Ortho Total assay (Cohen's kappa = 0.90). The C19-kodecyte assay sensitivity (92.1%) was higher than that published for the two Ortho assays (IgG, 80.8% and Total, 87.7%) (28). The C19-kodecyte assay also demonstrated a high specificity (95.2%) comparable with the Ortho assays (IgG, 98.1% and Total, 95.2%). The sensitivity and specificity of the C19-kodecyte assay are well within the minimum performance range of serologic tests with FDA issued EUA (90% sensitivity and 95% specificity) (30).

Despite an excellent analytical performance, there was a weak correlation between the C19-kodecyte assay and the other three assays (Fig. 3). A small proportion of CCP donors (10%; 14/140) and healthy controls (3.6%; 10/275) gave discrepant results. This inconsistency may be explained by subtle differences in sensitivity or specificity or both among the four assays. The Ortho Total assay is known to detect IgG, IgM, and IgA antibodies. In contrast, the C19-kodecyte assay primarily detects the IgG antibody, because the peptide antigens attached to the kodecytes react poorly with IgM anti-

TABLE 1 C19-kodecyte and ortho total assay results: Agreement and Cohen's kappa[a]

	C19-kodecyte assay		
Ortho Total assay	**Negative**	**Positive**	**Total (%)**
Negative	122	7	129 (48.7%)
Positive	6	130	136 (51.3%)
Total (%)	128 (48.3%)	137 (51.7%)	265 (100%)*
Agreement[b]		95%	

[a]Cohen's kappa coefficient (95% CI; standard error) = 0.90 (0.85, 0.95; 0.027) kappa coefficient <0.20 = poor agreement, 0.21–0.40 = fair agreement, 0.41–0.60 = moderate agreement, 0.61–0.80 = substantial agreement, and 0.81–1.00 = almost perfect agreement (26).
[b]Agreement: (122 + 130)/265 = 0.95.
*rRT-PCR positive samples (140) + Ortho Total negative samples (125) = 265.

TABLE 2 CCP donors with inconsistent results among the 5 COVID-19 antibody assays

Sample ID[a]	COVID-19 antibody assays					CCP donation after start of symptoms (days)
	Ortho Total assay	Ortho IgG assay	Virus neutralizing assay[b]	C19-kodecyte assay	Peptide 808-kodecyte assay	
1	+	+	−	−	−	65
2	+	+	+	−	+	89
3	+	+	−	−	−	85
4	+	+	+	−	+	77
5	+	+	+	−	+	88
6	+	+	−	−	−	273
7	+	−	+	+	−	Unknown[c]
8	+	−	−	+	−	52
9	+	−	+	+	+	65
10	+	−	+	+	−	47
11	+	−	+	+	+	97
12	+	−	+	+	−	110
13	+	−	−	+	−	141
14	+	−	−	−	−	33
15	−	−	−	−	−	180
16	−	−	−	−	−	90
17	−	−	−	−	−	47

[a]Distinct donors (single donations).
[b]The NT50 detection threshold is titer 40.
[c]CCP donor never experienced symptoms (asymptomatic infection).

bodies (17). Moreover, the peptides on the kodecyte surface are located in the glycocalyx of the red cell membrane and are thus embedded in carbohydrates and proteins (20, 31). The glycocalyx may act like a natural buffer against low-affinity IgG antibodies and the IgM antibodies of even lower affinity. We speculate that this possible buffer may allow for the use of undiluted plasma samples.

Another possible reason for the weak correlation among the four assays may be their different chemistries. The two recombinant protein assays and the virus neutralizing assay present multiple epitopes to interact with antibodies of many specificities. In contrast, the peptides on the kodecyte surface present one or few epitopes that react with a single antigen binding (Fab) region of an antibody. Hence, our C19-kodecytes with two epitopes (1147 and 1255), will likely only react with two specific antibodies of the broad antibody repertoire.

A gradual decrease of the SARS-CoV-2 antibody concentrations was observed in all CCP donors (Fig. 2) and the single vaccinated individual, which had been reported previously in multiple studies (22, 32–35). The concentrations are known to decrease quicker in individuals with mild infection or asymptomatic disease (36–38) until the antibodies become undetectable (39, 40).

Although the C19-kodecyte assay was already comparable with FDA authorized tests (Table 1), the assay, as a beta version, can still be refined by adjusting the sequence or the concentration of the peptides. Slightly changing the current peptide sequences by shifting or elongating them either left or right into the flanking sequences has the potential to improve both sensitivity and specificity (17). Different peptides generated from other regions of the spike protein could also be evaluated, which are the subject of ongoing studies. For example, three samples that tested false negative in the C19-kodecyte assay were positive in the peptide 808-kodecyte assay (Table 2) representing a different segment of the SARS-CoV-2 spike protein (20). Likewise, the peptide concentrations may be optimized to improve the assay specificity.

COVID-19 patients develop a broad antibody repertoire against multiple epitopes of the SARS-CoV-2 spike protein and nucleocapsid protein (41). The unusual flexibility of the C19-kodecyte assay will allow easy application of different peptides from those proteins and generate distinct kodecytes. Testing with a set of distinct kodecytes may enable us to distinguish multiple antibody specificities and their titers within the broad antibody repertoire.

In summary, we performed a clinical validation of the C19-kodecyte assay in a cohort of CCP donors and healthy controls. Our scalable assay can be easily established in any blood typing laboratory worldwide using its routine setup for column agglutination or tube technique (20) to evaluate COVID-19 patients, convalescent donors, and likely also vaccinated individuals. The technique could vastly improve assay capacity, particularly in resource limited hospital blood banks.

MATERIALS AND METHODS

Study population. COVID-19 convalescent plasma or EDTA anticoagulated whole blood was prospectively collected between April 2020 and January 2021 from 140 donors (two asymptomatic and 138 with mild to severe disease) (22), who had previously tested positive for SARS-CoV-2 infection by real-time reverse transcription PCR (rRT-PCR). Plasma samples were also collected from 125 consecutive healthy volunteer donors, who tested negative for the Ortho Total assay, during the same time period. An additional 150 plasma samples, collected in 2008, more than a decade before the COVID-19 outbreak, were also included as negative controls (Table S1). An individual vaccinated with Moderna COVID-19 vaccine was prospectively analyzed for antibody response using the C19-kodecyte assay. Plasma, erythrocytes, and buffy coat aliquots were separated and stored at −80°C (plasma) or in the vapor phase of liquid nitrogen at −150°C (erythrocytes and buffy coat).

Institutional Review Board (IRB) approved protocols were NCT04360278, NCT00001846, and NCT00067054 which entailed written informed consent. The plasma controls from 2008 were collected under the Vaccine Research Center's (VRC), National Institutes of Allergy and Infectious Diseases (NIAID), National Institutes of Health sample collection protocol VRC 200 (NCT00067054) in compliance with the NIH IRB approved procedures. All subjects met protocol eligibility criteria and agreed to participate in the study by signing the NIH IRB approved informed consent. Research studies with these samples were conducted by protecting the rights and privacy of the study participants.

C19-kodecytes. Red cells were coated with peptide fragments of the SARS-CoV-2 spike protein (C19-kodecytes) using a blend of FSL constructs 1147 and 1255 at concentrations 1.5 μM and 2.5 μM, respectively (20). FSL construct 808, with lesser sensitivity compared with constructs 1147 and 1255 but having diagnostic potential, was also prepared at a concentration of 5 μM (20). All samples reactive to C19-kodecytes were also tested against negative control cells, either unmodified red cells or kodecytes labeled with an unrelated FSL construct (20).

Gel card column agglutination technique. We mixed 50 μl of C19-kodecytes at 1% concentration with 25 μl of plasma in the gel card wells (ID Cards LISS/Coombs, no. 50531; Bio-Rad Laboratories, Hercules, CA, USA), incubated at 37°C for 15 min, and centrifuged at 1,032 rpm at room temperature for 10 min (Ortho Workstation; Ortho Clinical Diagnostics, Raritan, NJ, USA). All reactions were read visually (Fig. S1). A titer was determined as the highest dilution showing a 1+* (weak positive) agglutination. The strength of the agglutination reaction was 4+, 3+, 2+, 1+, 1+*, and 0 (Fig. S1), which equated with grades 12, 10, 8, 5, 3, and 0, respectively (20, 42). For the purpose of determining the score, we calculated the sum of the individual grades at 2-fold serial dilutions. A higher score against kodecytes with defined antigen levels indicates the presence of higher antibody concentration or a stronger antigen-antibody affinity or both.

Anti-SARS-CoV-2 assays. Two chemiluminescent immunoassays directed against the SARS-CoV-2 spike glycoprotein, Ortho IgG (80.8% overall sensitivity and 98.1% overall specificity) (28), and Ortho Total (IgG, IgA and IgM; 87.7% overall sensitivity and 95.2% overall specificity) (28) (Ortho Clinical Diagnostics, Raritan, NJ, USA), authorized by the FDA under an EUA for use by laboratories certified under Clinical Laboratory Improvement Amendments of 1988 (CLIA), were used to detect total binding antibodies against the SARS-CoV-2 virus in all 140 CCP donors (22, 28). The sample signal was divided by the calibrator signal, with the resulting signal-to-cutoff (S/CO) ratio of $<$1.00 and $\geq$1.00 corresponding to nonreactive and reactive results, respectively (22). The 125 healthy volunteer donors from 2020 were tested with the Ortho Total assay. The 150 plasma samples from 2008 were presumed negative for SARS-CoV-2 and not tested with any of the two immunoassays, unless positive in our C19-kodecyte assay.

SARS-CoV-2 virus neutralizing assay. The surface-exposed location of the spike glycoprotein makes it a major target of neutralizing antibodies (29). An NIH developed fluorescence reduction neutralizing assay (FRNA; not authorized by FDA; sensitivity and specificity data not available) (43) was performed to detect SARS-CoV-2 neutralizing antibody titer in all 140 CCP donors (22). A 12-step 2-fold serial dilution was used for the calculation of the 50% neutralizing titer (NT50) (43).

Sensitivity and specificity. The C19-kodecyte assay was evaluated on consecutive CCP samples collected from participants at various time points after rRT-PCR confirmation.

Assay sensitivity was calculated as the percentage of samples that tested positive with the C19-kodecyte assay relative to the total number of PCR-confirmed COVID-19 positive samples [Sensitivity = True Positive/(True Positive + False Negative)].

Assay specificity was calculated as the percentage of samples that tested negative with the C19-kodecyte assay relative to the total number of COVID-19 negative samples [Specificity = True Negative/(True Negative + False Positive)].

Statistical analyses. Unless stated otherwise, continuous data are given as median (Quartile 1–Quartile 3). Confidence intervals (95% CI) were calculated according to the efficient-score method (corrected for continuity) (44; http://vassarstats.net/clin1.html#note). Assay results were compared using linear

regression and Spearman correlation (23, 24). Cohen's kappa was used to evaluate between methods agreements (25, 26; https://www.graphpad.com/quickcalcs/kappa2/). ROC curves were generated based on positive (140 SARS-CoV-2 rRT-PCR-positive) and negative data (125 Ortho Total negative samples from 2020 and 150 samples from 2008) and used to evaluate overall performance of the C19-kodecyte assay.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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