

1 **Methodological approaches to optimize multiplex oral fluid SARS-CoV-2 IgG assay**  
2 **performance and correlation with serologic and neutralizing antibody responses**

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28 assay

29

## 30 **ABSTRACT**

31 **Background.** Oral fluid (hereafter, saliva) is a non-invasive and attractive alternative to blood for SARS-  
32 CoV-2 IgG testing; however, the heterogeneity of saliva as a matrix poses challenges for immunoassay  
33 performance.

34 **Objectives.** To optimize performance of a magnetic microparticle-based multiplex immunoassay (MIA)  
35 for SARS-CoV-2 IgG measurement in saliva, with consideration of: i) threshold setting and validation  
36 across different MIA bead batches; ii) sample qualification based on salivary total IgG concentration; iii)  
37 calibration to U.S. SARS-CoV-2 serological standard binding antibody units (BAU); and iv) correlations  
38 with blood-based SARS-CoV-2 serological and neutralizing antibody (nAb) assays.

39 **Methods.** The salivary SARS-CoV-2 IgG MIA included 2 nucleocapsid (N), 3 receptor-binding domain  
40 (RBD), and 2 spike protein (S) antigens. Gingival crevicular fluid (GCF) swab saliva samples were collected  
41 before December, 2019 (n=555) and after molecular test-confirmed SARS-CoV-2 infection from 113  
42 individuals (providing up to 5 repeated-measures; n=398) and used to optimize and validate MIA  
43 performance (total n=953). Combinations of IgG responses to N, RBD and S and total salivary IgG  
44 concentration ( $\mu\text{g/mL}$ ) as a qualifier of nonreactive samples were optimized and validated, calibrated to  
45 the U.S. SARS-CoV-2 serological standard, and correlated with blood-based SARS-CoV-2 IgG ELISA and  
46 nAb assays.

47 **Results.** The sum of signal to cutoff (S/Co) to all seven MIA SARS-CoV-2 antigens and disqualification of  
48 nonreactive saliva samples with  $\leq 15 \mu\text{g/mL}$  total IgG led to correct classification of 62/62 positives  
49 (sensitivity [Se]=100.0%; 95% confidence interval [CI]=94.8%, 100.0%) and 108/109 negatives (specificity  
50 [Sp]=99.1%; 95% CI=97.3%, 100.0%) at 8-million beads coupling scale and 80/81 positives (Se=98.8%;  
51 95% CI=93.3%, 100.0%) and 127/127 negatives (Sp=100%; 95% CI=97.1%, 100.0%) at 20-million beads  
52 coupling scale. Salivary SARS-CoV-2 IgG crossed the MIA cutoff of 0.1 BAU/mL on average 9 days post-  
53 COVID-19 symptom onset and peaked around day 30. Among n=30 matched saliva and plasma samples,  
54 salivary SARS-CoV-2 MIA IgG levels correlated with corresponding-antigen plasma ELISA IgG (N:  $\rho=0.67$ ,  
55 RBD:  $\rho=0.76$ , S:  $\rho=0.82$ ; all  $p<0.0001$ ). Correlations of plasma SARS-CoV-2 nAb assay area under the  
56 curve (AUC) with salivary MIA IgG (N:  $\rho=0.68$ , RBD:  $\rho=0.78$ , S:  $\rho=0.79$ ; all  $p<0.0001$ ) and with plasma  
57 ELISA IgG (N:  $\rho=0.76$ , RBD:  $\rho=0.79$ , S:  $\rho=0.76$ ;  $p<0.0001$ ) were similar.

58 **Conclusions.** A salivary SARS-CoV-2 IgG MIA produced consistently high Se (>98.8%) and Sp (>99.1%)  
59 across two bead coupling scales and correlations with nAb responses that were similar to blood-based  
60 SARS-CoV-2 IgG ELISA data. This non-invasive salivary SARS-CoV-2 IgG MIA could increase engagement  
61 of vulnerable populations and improve broad understanding of humoral immunity (kinetics and gaps)  
62 within the evolving context of booster vaccination, viral variants and waning immunity.

## 63 1. INTRODUCTION

64 Monitoring SARS-CoV-2 antibody responses<sup>1,2</sup> induced by natural infection and/or vaccination is  
65 important to estimate seroprevalence and to understand transmission patterns that can be driven by  
66 asymptomatic exposure or infection, waning immunity, and gaps in humoral immunity<sup>3-5</sup>. Most SARS-  
67 CoV-2 seroprevalence studies utilize peripheral blood-based serologic assays, which require phlebotomy  
68 or needle stick (e.g., dried blood spots). Peripheral blood collection is typically justified by the high  
69 performance of blood-based SARS-CoV-2 serologic assays and their correlation with SARS-CoV-2  
70 neutralizing antibody (nAb) activity.<sup>6-8</sup> However, participant burdens associated with phlebotomy makes  
71 it unsuitable for repeated, routine antibody testing. Further, seroprevalence studies requiring  
72 participants to complete in-person visits at a clinical site for study procedures and phlebotomy tend to  
73 miss under-represented groups, such as under-resourced communities, populations with limited access  
74 to healthcare, populations who mistrust the medical establishment, and people who fear needle  
75 sticks.<sup>9,10</sup> To overcome these challenges and increase opportunity for broad population-scale estimates  
76 of SARS-CoV-2 seroprevalence, several groups<sup>11-13</sup> have developed SARS-CoV-2 antibody tests using  
77 saliva and have shown that saliva-based antibody tests can be reasonably accurate; however, none  
78 approach the highly accurate test performance of the best blood-based SARS-CoV-2 antibody tests.  
79 Most achieve high specificity (e.g., >99%), but fail to achieve sensitivity >90-95%.<sup>14,15</sup>

80 We recently developed a multiplex SARS-CoV-2 antibody assay for use with self-collected  
81 saliva.<sup>16</sup> Saliva for this assay is collected using a lollipop-like sponge on a stick, with which participants  
82 were instructed to brush the line between their teeth and gums for 1-2 minutes, in a motion similar to  
83 brushing their teeth. This collection method stimulates leakage of antibody-enriched serum-derived  
84 gingival crevicular fluid (GCF)<sup>17</sup> from between the gums and teeth that is then absorbed by the swab.  
85 Saliva can be collected at home and mailed to a centralized lab for testing of antibodies to SARS-CoV-2  
86 nucleocapsid (N), receptor-binding domain (RBD) and spike (S) proteins with an in-house microparticle  
87 bead-based multiplex immunoassay (MIA) based on Luminex xMAP technology.<sup>4,18,19</sup>

88 Here we aimed to optimize the SARS-CoV-2 IgG multiplex assay for high accuracy, to  
89 characterize and validate the assay using pre-COVID-19 era saliva samples and prospectively collected  
90 saliva samples from study participants who tested positive for SARS-CoV-2 by RT-PCR. We coupled two  
91 MIA bead batches at different scales and determined assay sensitivity and specificity across bead  
92 batches using close to 1,000 saliva samples. We investigated how sample qualification based on the  
93 total salivary IgG concentration affects SARS-CoV-2 IgG assay performance and kinetic trajectories post  
94 COVID-19 symptoms onset. We estimated time to SARS-CoV-2 IgG immune-conversion and time to  
95 establish a robust IgG level and investigated differences in anti-N, -RBD and -S IgG kinetics post-COVID-  
96 19. We correlated salivary IgG levels with blood-based IgG levels (anti-N, -RBD and -S IgG ELISA) and with  
97 plasma SARS-CoV-2 nAb assay area under the curve (AUC). And lastly, motivated by the goal to achieve  
98 comparability with other quantitative serological assays, we calibrated our quantitative assay standards  
99 to the U.S. SARS-CoV-2 serological standard to estimate the concentration of salivary SARS-CoV-2 IgG in  
100 samples from the pre-COVID-19 era and post SARS-CoV-2 infection.

## 101 2. METHODS

### 102 2.1. Study population

103 Samples were collected longitudinally from consented research participants who had tested  
104 positive for SARS-CoV-2 by RT-PCR in the 1-3 days prior to enrollment and before vaccines were  
105 available (April – September 2020).<sup>18,20</sup> Archived saliva samples that had been self-collected with Oracol  
106 swabs as part of different research studies before December 2019 were used as presumed negative  
107 samples.<sup>21-23</sup>

### 108 2.2. Saliva self-collection and sample processing

109 Upon enrollment, each participant received a self-testing kit containing saliva collection swabs  
110 (Oracol S14, Malvern Medical Developments, Worcester, UK) and other study supplies by courier  
111 delivery. After receipt of the testing supplies a study coordinator scheduled a video or phone visit (day  
112 0) to instruct the participant on saliva self-collection procedures, collect basic demographics, and note  
113 the date of COVID-19 symptom onset. Follow-up phone or video visits, during which participants self-  
114 collected additional saliva samples, were scheduled on study days 3, 7, and 14. On study day 28,  
115 participants were invited for an in-person visit during which blood and saliva were collected. Study staff  
116 collected blood by venipuncture into EDTA tubes and the resulting plasma was separated from solid  
117 blood components through centrifugation. Self-collected saliva from study days 0, 3, and 7 were stored  
118 in a freezer (-20°C) by the participant after collection until the last sample was collected at home on day  
119 14. Swabs were then returned to the study by courier in a package with ice packs. Swabs collected  
120 during the day 28 in-person clinical follow-up visit were transferred directly to the processing lab. Upon  
121 receipt at the lab, swabs were centrifuged at 1,500 g for 10 minutes to separate saliva from the sponge  
122 and then heat-inactivated at 56°C for 1 hour in a water bath to inactivate SARS-CoV-2 virus if present.  
123 Samples were stored at ≤-20°C prior to testing. Presumed SARS-CoV-2 IgG negative archived saliva  
124 samples were also heat-inactivated at 56°C for 1 hour prior to testing.

### 125 2.3. Coupling of antigens and antibodies to magnetic beads

126 Saliva samples were tested using a modified version of a previously described multiplex SARS-  
127 CoV-2 immunoassay<sup>16</sup> based on Luminex xMAP technology. The modified multiplex assay version was  
128 composed of 20 unique magnetic bead sets (MagPlex microspheres), instead of 12 sets. Each set was  
129 coupled covalently with antigen, antibody or BSA (controls), as described previously.<sup>16</sup> The assay  
130 included SARS-CoV-2 nucleocapsid (N), receptor binding domain (RBD), and spike (S) antigens (all Wuhan  
131 2019 strain), SARS, MERS, RSV, human coronavirus 229E, NL63, HKU1, and OC43 antigens in addition to  
132 control antibodies and proteins (BSA, anti-human IgG, IgM, IgA antibody; see **Table S1**). Two different  
133 multiplex assay lots, one at 8 million beads coupling scale (sufficient for approximately 80 assay plates)  
134 and one at 20 million beads coupling scale (for ~200 assay plates), were produced and their  
135 performance compared.

### 136 2.4. Salivary SARS-CoV-2 IgG multiplex immunoassay (MIA)

137 Saliva was tested for IgG binding to SARS-CoV-2 and additional virus antigens in the MIA as  
138 described previously.<sup>16</sup> Briefly, saliva was thawed and centrifuged for 5 minutes at 20,000 g. Then 10 µL  
139 saliva supernatant was added to a 96-well microtiter plate containing 40 µL PBST with 1% BSA (assay

140 buffer) and 1,000 coupled beads per bead set in each well. Each plate contained 1-2 blank wells with  
141 assay buffer instead of sample that were used for background fluorescence subtraction. Positive  
142 controls were created by spiking SARS-CoV-2 IgG positive saliva with high IgG levels to SARS-CoV-2  
143 antigens into pre-pandemic negative saliva. Pre-pandemic saliva was used as negative control.  
144 Phycoerythrin-labeled anti-human IgG diluted 1:100 in assay buffer was used to detect the IgG signal in  
145 saliva (see **Table S1**). Assay plates were read on a Luminex MAGPIX instrument.

146 After assay optimization, an 8-point standard curve of 3-fold serial dilutions was included on  
147 each plate in duplicate. The standard consisted of plasma from a PCR-confirmed COVID-19 study  
148 participant with high SARS-CoV-2 IgG levels diluted in assay buffer. Arbitrary units (AU) ranging from  
149 10,000 AU for the highest standard to ~5 AU for the lowest standard were assigned to each standard.  
150 The IgG concentration to SARS-CoV-2 antigens in AU was calculated with xPONENT Software (Luminex)  
151 using a weighted 5-parameter logistic curve fit. The assay precision within and between assay plates was  
152 determined by assessing replicate measurements of contrived saliva high and low positive controls and  
153 a lower limit of quantification control; ten random saliva samples collected prior to the COVID-19  
154 pandemic were tested, percent recovery of a spiked and serially diluted saliva sample was assessed, and  
155 precision around the assay cutoff was assessed with contrived saliva samples near (+/- 25% and +/-50%)  
156 and at the cutoff measured in replicate. Last, the assay performance was validated using pre-COVID-19  
157 samples (presumed negatives) and samples collected from COVID-19 study participants (confirmed by  
158 FDA-EUA RT-PCR test).

## 159 **2.5. Salivary SARS-CoV-2 MIA calibration to the U.S. national SARS-CoV-2 serology** 160 **standard**

161 A dilution series of the U.S. national SARS-CoV-2 serology standard (8 points in duplicate) was  
162 included as unknowns to measure its concentration in AUs. Likewise, a dilution series of our in-house  
163 standard was included in the SARS-CoV-2 IgG multiplex assay as unknowns using dilutions of the  
164 national serology standard as the assay standard to estimate the in-house standard's concentration in  
165 U.S. serology standard units (BAU/mL) to determine the concentration equivalent of the assay cutoff in  
166 BAU/mL.

## 167 **2.6. Salivary total IgG ELISA**

168 The total IgG concentration in saliva was determined using Salimetrics Salivary Human Total  
169 IgG ELISA Kits according to the manufacturer's instructions with two modifications. The incubation times  
170 with diluted saliva sample and with detect antibody were reduced to 1 hour each instead of 2 hours.  
171 This modification has been discussed with and approved by the manufacturer. ELISA standards and high  
172 and low total salivary IgG assay controls provided with the kit with defined total IgG concentration  
173 ranges were assessed on each plate.

## 174 **2.7. Statistical analysis**

175 The blank-subtracted (net) median fluorescence intensity (MFI) was used for data analysis and  
176 for standard curve fitting. IgG binding expressed in AU and in MFI were compared. Cutoffs to  
177 discriminate SARS-CoV-2 IgG positive from negative samples for each individual SARS-CoV-2 antigen  
178 were defined as the average net MFI (or AU) plus three standard deviations (SD) of samples collected

179 prior to December 2019 (pre-COVID-19 negatives). The resulting sensitivity and specificity of individual  
180 SARS-CoV-2 antigens were calculated using saliva from COVID-19 study participants (positives) and pre-  
181 COVID-19 saliva samples (negatives).

182 In addition to relying on individual antigens to determine presence of SARS-CoV-2 IgG, several  
183 algorithms involving IgG binding to multiple antigens were explored. Algorithms tested included positive  
184 classification if the IgG signal is above the cutoff (signal to cutoff [S/Co]>1) for 1) at least one  
185 nucleocapsid and one RBD or S antigen; 2) two or more RBD or spike (S) antigens; or 3) positive for  
186 either algorithm (1) or (2). Additional algorithms included the sum of IgG S/Co to multiple antigens with  
187 the cutoffs for such combinations calculated correspondingly, i.e., the mean plus 3 SDs of each  
188 algorithm tested in pre-pandemic negatives. We determined assay performance based on 4) anti-N IgG;  
189 5) anti-RBD IgG; 6) anti-S IgG binding; and 7) IgG binding to all seven N, RBD and S antigens combined.  
190 Recognizing that samples collected post COVID-19 vaccination will contain anti-RBD and anti-S IgG, we  
191 also determined the sensitivity to classify prior SARS-CoV-2 infection correctly by combining algorithms  
192 (7) and (4). Algorithm 7 is based on IgG binding to any combination of antigens and would thus classify  
193 post-vaccine samples as positive, whereas algorithm 4 tests for presence of anti-N IgG, which should  
194 only be present post natural infection but not post vaccination. Lastly, we also explored whether  
195 normalizing the SARS-CoV-2 IgG signal with the total salivary IgG (tIgG) concentration in  $\mu\text{g/mL}$  would  
196 improve the assay performance; 8) sum S/Co to N/RBD/S divided tIgG.

197 Next, we evaluated the influence of total salivary IgG on assay performance. Three minimum  
198 total IgG concentrations, 5  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$  and 15  $\mu\text{g/mL}$ , to “qualify” algorithm negative samples  
199 were explored. The minimum total IgG requirement quality control (QC) measure was only applied when  
200 samples tested negative using the applied algorithm, i.e., samples testing positive for SARS-CoV-2 IgG  
201 were not subject to the minimum total IgG concentration requirement, whereas samples that tested  
202 negative failed QC (insufficient total IgG for correct classification) if the total IgG concentration was  
203 below the minimum concentration defined. The number of samples that failed QC was also calculated.

204 Spearman correlation coefficients were calculated to estimate the correlation between plasma  
205 neutralizing SARS-CoV-2 antibody area under the curve (AUC)<sup>8</sup>, plasma anti-N, RBD and S IgG AUC  
206 measured by ELISA<sup>8</sup> and corresponding salivary SARS-CoV-2 IgG. Loess regression was used to visualize  
207 IgG antibody kinetics to SARS-CoV-2 in the multiplex test after COVID-19 symptoms onset. Mononuclear  
208 growth models were used to calculate time from COVID-19 symptoms onset to seroconversion and to  
209 reach maximum IgG levels. Means, standard deviations and %CV were calculated to characterize within  
210 and between assay plate precision and precision near the assay cutoff. To determine assay performance,  
211 samples were classified as positive, negative, or indeterminate and sensitivity, specificity and overall  
212 concordance were calculated. Statistical analyses were performed in SAS University Edition Release 3.8,  
213 SAS Institute Inc., Cary, NC, USA, and with RStudio Version 1.4.

## 214 3. RESULTS

### 215 3.1. Saliva samples used for SARS-CoV-2 IgG MIA characterization and 216 optimization

217 Between April and September 2020, 113 ambulatory COVID-19 study participants provided 398  
218 saliva samples. Most samples (66%) were collected more than two weeks after COVID-19 symptoms  
219 started. About two thirds of participants (n=75; 67%) provided at least 2 samples and 50 (45%) provided  
220 saliva samples for all five study events, i.e., one enrollment sample and follow-up samples on study days  
221 3, 7, 14 and 28. Approximately three-quarters of these samples (n=293; 74%) were used to characterize  
222 and optimize the multiplex assay and the remaining quarter (n=105; 26%) were used for assay  
223 validation. Archived saliva samples collected before December 2019 (n=555) were used as negative  
224 controls. Correspondingly, a larger set of pre-COVID-19-era samples was used for assay optimization  
225 (n=362; 65%) and a smaller set for validation (n=193; 35%; **Table 1**).

### 226 3.2. Threshold setting

#### 227 3.2.1. Performance of individual antigens in SARS-CoV-2 IgG MIA

228 The sensitivity and specificity for each individual SARS-CoV-2 antigen were calculated as a first  
229 step to characterize each antigen's performance classifying presumed SARS-CoV-IgG positive samples  
230 from COVID-19 study participants and presumed negative saliva samples collected prior to the COVID-19  
231 pandemic correctly. In accordance with FDA-guidelines for antibody testing, only samples collected at  
232 least two weeks after COVID-19 symptoms onset were used to determine sensitivity.<sup>24</sup> In general, higher  
233 sensitivity was achieved at the cost of lower specificity and vice versa (**Table S2**). For example, the Sino  
234 Biological RBD antigen results in 84% sensitivity but only 98% specificity, whereas GenScript's RBD  
235 antigen results in higher specificity (99%) but lower sensitivity (78%). With the exception of one S  
236 antigen (1% sensitivity) and a SARS N antigen, which showed equivalent performance to GenScript N and  
237 was derived from a SARS 2002, not SARS-CoV-2 strain, we concluded that the remaining antigens could  
238 contribute to a combined algorithm for sample classification.

#### 239 3.2.2. Algorithms to improve salivary SARS-CoV-2 IgG MIA performance

240 The assay performance was calculated using eight algorithms that combined IgG binding to two  
241 or more of the seven SARS-CoV-2 antigens (**Table S3**). Algorithms included positive classification if the  
242 IgG signal is above the cutoff for 1)  $\geq 1$  N and  $\geq 1$  RBD or S antigen; 2)  $\geq 2$  RBD or S antigens; 3) positive for  
243 either algorithm (1) or (2). Additional classifications were based on the sum of IgG S/Co to the SARS-CoV-  
244 2 antigens represented in the assay with the cutoffs for such combinations calculated correspondingly  
245 (mean plus 3 SDs in pre-pandemic saliva samples): 4) N (n=2); 5) RBD (n=3); 6) S (n=2); and 7) IgG binding  
246 to all seven N, RBD and S antigens combined. Recognizing that samples collected post COVID-19  
247 vaccination will contain anti-RBD and anti-S IgG, we also determined the sensitivity to classify prior  
248 SARS-CoV-2 infection correctly by combining algorithms (7) and (4). Algorithm 7 is based on IgG binding  
249 to any combination of antigens and would thus classify post-vaccine samples as positive, whereas  
250 algorithm 4 tests for presence of anti-N IgG, which should only be present post natural infection, not  
251 post vaccination. Lastly, we also explored whether normalizing the SARS-CoV-2 IgG signal with the total  
252 salivary IgG (tIgG) concentration in  $\mu\text{g/mL}$  would improve the assay performance; 8) sum S/Co to  
253 N/RBD/S divided tIgG. Most combinations improved assay performance compared to relying on any

254 single antigen for sample classification. The assay sensitivity increased to 84.5% with algorithm 7 that  
255 combines the IgG response to seven antigens while maintaining high specificity (98.6%). Algorithm 3  
256 (**Table S3**, positive if IgG signal above cutoff for at least one N and one RBD/S antigen or two or more  
257 RBD/S antigens) also improved overall test accuracy. Normalizing the SARS-CoV-2 IgG response with the  
258 total IgG concentration (Algorithm 8, **Table S3**) yielded poor sensitivity (<50%, **Table S3**).

### 259 **3.2.3. Effect of total salivary IgG concentration on SARS-CoV-2 IgG MIA performance**

260 Large differences in total antibody concentration between saliva samples compared to the  
261 relatively small concentration difference between blood samples need to be overcome for salivary SARS-  
262 CoV-2 IgG tests to perform equivalently to a typical blood-based SARS-CoV-2 antibody test. We  
263 therefore investigated differences in total IgG concentration between presumably false-negative saliva  
264 samples and saliva samples that were classified correctly as SARS-CoV-2 IgG positive using the best  
265 performing algorithm, algorithm 7, the sum of SARS-CoV-2 IgG S/Co to N, RBD and S antigens.

266 Total salivary IgG in samples from COVID-19 study participants that tested false negative for  
267 SARS-CoV-2 IgG was significantly lower than in samples classified correctly as positive ( $p < 0.001$ ; **Figure**  
268 **1A** Error! Reference source not found.). However, many samples with low total IgG (e.g.,  $< 10 \mu\text{g/mL}$ )  
269 nonetheless contained enough SARS-CoV-2 IgG to cross the cutoff, likely because the proportion of  
270 SARS-CoV-2-specific IgG to total IgG was particularly high in such samples. We next examined the effect  
271 of total salivary IgG on the SARS-CoV-2 IgG signal in the first week, second week and more than two  
272 weeks post symptoms onset and in pre-COVID-19 samples. For this, saliva samples were divided into  
273 four categories of total IgG concentration in  $5 \mu\text{g/mL}$  increments. SARS-CoV-2 IgG levels increased in  
274 each group with increasing total salivary IgG (**Figure 1B**). The difference in SARS-CoV-2 signal between  
275 archived pre-COVID-19 era samples and COVID-19 study samples collected  $> 14$  days post symptom  
276 onset was highest in the  $> 10 \mu\text{g/mL}$  and  $15 \mu\text{g/mL}$  total IgG categories. However, note that only pre-  
277 COVID-19 era samples in the highest category ( $> 15 \mu\text{g/mL}$ ) crossed the cutoff (false-positives).

### 278 **3.2.4. Establishment of a minimum total salivary IgG concentration as sample** 279 **qualifier**

280 The large proportion of samples with very low total IgG that tested false-negative for SARS-CoV-  
281 2 IgG led us to examine whether the disqualification of samples with insufficient total IgG could improve  
282 test accuracy. We tested this by defining a minimum total IgG concentration for samples to qualify as  
283 true negatives. This minimum total IgG qualification was only applied to samples that tested SARS-CoV-2  
284 IgG negative using one of the algorithms. Samples with a positive classification were retained regardless  
285 of their total IgG concentration. We assessed the assay performance after defining minimum total IgG  
286 concentrations as sample qualifier in  $5 \mu\text{g/mL}$  increments ( $5 \mu\text{g/mL}$ ,  $10 \mu\text{g/mL}$  and  $15 \mu\text{g/mL}$  total  
287 salivary IgG). Pre-COVID-19 samples did not contribute to the cutoffs (mean plus 3 SDs of pre-COVID-19  
288 samples) if the total IgG concentration was below the qualifying concentration. **Table 2** summarizes the  
289 multiplex assay performance for each minimum total IgG concentration chosen using the algorithms  
290 described above.

291 The MIA performance improved with every  $5 \mu\text{g/mL}$  increment in the minimum “required” total  
292 salivary IgG concentration to qualify negative samples (see **Table 2**). Note that the sum of S/Co to all



293 seven SARS-CoV-2 IgG antigens (N & RBD/S, algorithm 7) resulted almost always in the highest test  
294 accuracy regardless of the minimum total salivary IgG concentration set forward to disqualify potentially  
295 false-negatives. We chose to move forward requiring SARS-CoV-2 IgG negative samples to contain a  
296 minimum IgG concentration of 15 µg/mL. This requirement did not apply to samples that were classified  
297 as positive. However, this minimum total IgG requirement also led to a loss or exclusion of samples for  
298 which no result could be determined (indeterminates). The sensitivity of the algorithm that “normalizes”  
299 the SARS-CoV-2 IgG signal with total IgG (algorithm 8) also improved significantly from <50% to >98%  
300 but at the cost of lower specificity (88%). This algorithm results, however, in the lowest proportion of  
301 samples excluded.

302 Using the sum of S/Co to all seven SARS-CoV-2 antigens (algorithm 7), **Error! Reference source**  
303 **not found.**A demonstrates how the minimum total IgG concentration applied to qualify samples as true  
304 SARS-CoV-2 IgG negatives improves the assay sensitivity from 84.5% without total IgG qualification to  
305 98.6% with a minimum concentration of 15 µg/mL total IgG required. The proportion of indeterminate  
306 samples was lowest in the >14 days post symptom onset category (28/174 indeterminate samples; 16%  
307 at 15 µg/mL) and 25% of samples resulted in an indeterminate classification across the four groups.

### 308 **3.2.5. Optimization of salivary SARS-CoV-2 IgG MIA cutoff**

309 To maximize assay accuracy, we defined 15 µg/mL as the minimum total IgG concentration  
310 required to qualify samples that tested SARS-CoV-2 negative as true negatives (**Figure 2; Figure 3A**).  
311 Next, we examined how fine-tuning the cutoff, rather than relying on the mean + 3 SD of pre-pandemic  
312 samples, influences assay performance and sample loss due to exclusion (**Error! Reference source not**  
313 **found.B**). Increasing the cutoff results in improved specificity (98.5% at a cutoff of 5 and 99.6% at a  
314 cutoff of 8 to 10), however, sensitivity decreased at a cutoff of 9 and higher (98.6% at cutoff 5 to 8;  
315 97.8% at cutoff 9 and 95.6% at cutoff 10). The proportion of indeterminate samples increased from 22%  
316 at a cutoff of 5 to 24% at a cutoff of 10. The corresponding analysis and resulting assay performance of  
317 the two different bead batch coupling scales was remarkably similar. We also compared the assay  
318 performance using either the net fluorescence signal intensity (MFI) or standardized concentrations (AU)  
319 derived from the SARS-CoV-2 IgG standard curve, which was nearly identical (**Figures S1 and S2**).

### 320 **3.3. Salivary SARS-CoV-2 IgG MIA validation**

321 Both bead batches (8-million and 20-million coupling scale) were used for threshold setting and  
322 were subsequently validated as described below. Using the 20-million coupling scale, we had introduced  
323 an assay standard and were able to validate the MFI-based result and the result based on standardized  
324 arbitrary units (AU).

325 The 8-million bead batch resulted in 98.6% sensitivity (142/144 samples collected >14 days post  
326 COVID-19 symptoms onset classified correctly as positive) and 99.2% specificity (263/265 pre-COVID-19  
327 era classified correctly as negative) at a cutoff of six and passed validation with very similar  
328 performance. The proportion of pre-COVID-19 era and COVID-19 study participant samples combined  
329 that tested negative and did not pass the total IgG concentration requirement was 22.7% (**Error!**  
330 **Reference source not found.**). For the 20-million batch a cutoff of 9 (MFI) and 10 (AU) were chosen to  
331 maximize assay specificity. The resulting sensitivity (96.7%) was marginally lower in the threshold setting

332 sample set but almost all (80/81 [98.8%]; AU-based classification) or all (81/81 [100%]; MFI-based  
333 classification) of the COVID-19 study participant samples collected >14 days post symptoms onset were  
334 classified correctly as positive during assay validation. There was very little difference between relying  
335 on the standardized classification based on AUs compared to using net MFI.

### 336 ***3.4. Algorithms to classify prior SARS-CoV-2 natural infection***

337 We evaluated SARS-CoV-2 anti-N IgG classification accuracy by tabulating the anti-N IgG result  
338 (Algorithm 4) among samples classified as SARS-CoV-2 IgG positive (anti-N/RBD/S IgG positive, algorithm  
339 7). This sequence resulted in a sensitivity of 94.1% (95% confidence interval [CI]: 90.0%, 96.9%) and  
340 specificity of 99.2% (95% CI: 97.7%, 99.8%) for detection of anti-N IgG positives, which indicates prior  
341 exposure to or natural infection with SARS-CoV-2.

### 342 ***3.5. SARS-CoV-2 IgG MIA precision***

343 Contrived high and low positive controls and a negative control near the limit of detection  
344 (LLOQ) were tested in replicate over several days to assess within-plate and between-plate assay  
345 precision. The overall result (sum[S/Co]) and the signal (MFI and AU) to individual antigens were  
346 assessed. The coefficient of variation (CV) of the result between replicates within batch was under 5%.  
347 Replicates of the high and low control also resulted in less than 5% variation from the mean for  
348 individual antigens. The CV of the of the LLOQ control for individual antigens was between 0% and 33%.  
349 The assay precision between plates/days was around 10% for the sum of S/Co to N/RBD/S and ranged  
350 between 7% and 13% for the high and low controls and between 11% to 32% for the LLOQ control when  
351 assessing each antigen signal individually (**Table S4-S7**).

352 The average sum of S/Co of 10 pre-COVID-19 era saliva samples was 1.0 (range: 0.3-1.6); all  
353 tested negative and serial dilutions of a contrived sample resulted in 93% to 110% recovery and 117%  
354 recovery near the lower limit of quantitation (**Tables S8-S9**). The standard error of replicates of  
355 contrived saliva samples near the cutoff was 2%. All samples contrived to result either 25% or 50%  
356 above or below the cutoff were classified correctly (**Table S10**). The assay precision using MFI for  
357 analysis was nearly identical (not shown).

### 358 ***3.6. Calibration of salivary SARS-CoV-2 IgG MIA to U.S. SARS-CoV-2 serology*** 359 ***standard***

360 Using the U.S. national serology standard as calibrator, we estimated the SARS-CoV-2 IgG  
361 concentration equivalent of our assay cutoff as ~0.1 SARS-CoV-2 IgG BAU/mL (see **Figure 4**). The range  
362 for detection of SARS-CoV-2 binding IgG in oral fluid was 0.002 to ~4.0 BAU/mL using our MIA. The anti-S  
363 and anti-N IgG concentration in BAU/mL relative to the WHO SARS-CoV-2 serological standard (1,000  
364 BAU/mL) is 764 BAU/mL and 681 BAU/mL, respectively, for the US SARS-CoV-2 serological standard and  
365 371 BAU/mL and 330 BAU/mL, respectively, for our undiluted in-house serological standard. The cutoffs  
366 for individual N, RBD and S antigens in the assay range between 0.04 and 0.13 BAU/mL (see Figure 4).  
367 Note that the final cutoff values (sum of S/Co to N, RBD and S) is higher than the average of the  
368 individual cutoffs to maximize assay specificity.

### 369 **3.7. Correlation of neutralizing antibody with blood- and oral fluid-based SARS-CoV-** 370 **2 IgG response**

371 Plasma samples with neutralizing antibody and IgG binding data to RBD, spike and N antigens  
372 were available for 30 of the study day 28 samples. Matching saliva samples were used to estimate  
373 correlations between blood and salivary IgG measurements; indeterminates were excluded. IgG to N,  
374 RBD, and spike in blood correlated with the corresponding measure in saliva (N:  $\rho=0.76$ , RBD,  
375  $\rho=0.83$ , spike:  $\rho=0.82$ ; all  $p<0.001$ ). Salivary IgG levels to RBD ( $\rho=0.78$ ;  $p<0.001$ ) and spike  
376 ( $\rho=0.79$ ;  $p<0.001$ ) correlated equally well or slightly better with plasma neutralizing antibody AUC  
377 than plasma IgG levels (RBD:  $\rho=0.79$ , spike:  $\rho=0.76$ ), whereas IgG to N ( $\rho=0.68$ ) in saliva  
378 correlated less strongly with neutralizing antibody than IgG to N in plasma ( $\rho=0.76$ ) (see **Figure 5**).

### 379 **3.8. Salivary antibody kinetics post SARS-CoV-2 infection**

380 In accordance with the improved assay performance upon removing samples with  
381 indeterminate classification, this concept also refines SARS-CoV-2 IgG kinetics after COVID-19 symptoms  
382 onset. **Figure 6A** shows a Loess regression with all samples, whereas in **Figure 6B** indeterminate samples  
383 were excluded. The exclusion of potentially false-negative samples due to insufficient total salivary IgG  
384 leads to a steeper average rise in SARS-CoV-2 IgG and most individuals reach maximum SARS-CoV-2 IgG  
385 levels earlier, similar to average time to seroconversion (9 days  $\pm$  3) observed in blood samples<sup>25</sup>. Most  
386 individuals “sero”converted for saliva SARS-CoV-2 IgG around 9 days post symptoms onset and had  
387 detectable IgG responses to the three antigen types used in the assay (N, RBD and spike, **Figure 7**). After  
388 14 days post-symptom onset 202/211 samples (96%) tested positive for anti-N IgG, 193/199 (97%)  
389 tested positive for anti-RBD IgG, and 181/193 (94%) tested positive for anti-S IgG. The average time to  
390 seroconversion was slightly faster for nucleocapsid-binding IgG (8.0 days) than for RBD-binding (8.4  
391 days) or spike-binding IgG (10.1 days, **Figure 7**). However, comparing the salivary anti-N, RBD and S  
392 kinetics to each other rather than in reference to when they cross the cutoff we did not see any  
393 difference in the time to reaching a plateau between the antigens (see **Figure S3**). Saliva samples  
394 average a concentration of 0.8 anti-N IgG and 0.5 anti-spike IgG BAU/mL >2 weeks post symptoms onset  
395 with maximum concentrations of 3.4 anti-N IgG and 4.0 anti-S IgG BAU/mL.

396

## 397 **4. DISCUSSION**

398 We established a SARS-CoV-2 antibody assay for saliva with performance characteristics  
399 comparable to the best performing blood-based antibody tests.<sup>26</sup> We accomplished this by  
400 characterizing and addressing the assay limitations systematically. First, we set out to characterize the  
401 assay performance for each component, which led us to conclude that relying on individual antigens of  
402 the assay would at best classify ~80% of positive samples correctly and ~98% of negative samples. Next,  
403 we explored combining IgG signals to multiple antigens, which improved the assay performance  
404 somewhat (85% sensitivity and 99% specificity). We then examined differences between samples that  
405 were classified as false-negatives and those that were classified correctly as positives, which showed  
406 that samples with low total IgG concentration were much more likely to produce false-negative results.  
407 This observation resulted in the establishment of a “quality control step”. The quality control step fails  
408 samples that tested negative for SARS-CoV-2 IgG and contain less than 15  $\mu$ g/mL total IgG. This added

409 QC step greatly improved the assay's performance, which was, with some additional fine-tuning of the  
410 cutoff, comparable to the best blood-based SARS-CoV-2 antibody assays with >98% sensitivity and >99%  
411 specificity. The assay performance was nearly identical at two different bead coupling scales.

412 However, the QC step also leads to sample loss (indeterminate classification), because a  
413 significant proportion of saliva samples cannot be classified with the recommendation that the  
414 participant collects a new sample. If this assay were based on blood, this would be an unacceptable  
415 request, particularly for children, however, we have found that study participants do not mind providing  
416 a second sample if their saliva sample could not be classified as long as they were provided with a  
417 second collection swab. We have also found that the number of samples with indeterminate  
418 classification (i.e., with insufficient total IgG) can be greatly reduced when the sample collection  
419 procedure is explained well and at least for the first time supervised either by video-call or in-person.

420 The salivary total IgG concentration is influenced by the sample collection device, the type of  
421 saliva that is being collected (passive drool, parotid, sublingual, gingival crevicular fluid, etc.), collection  
422 technique, and duration of sample collection. Other factors include time of day, time since last meal and  
423 drink, participant age and gum health. Saliva sample collection devices, clear instructions for sample  
424 collection and sample processing can be standardized and even diurnal effects could be offset by  
425 instructing participants to collect samples, e.g., 30 minutes after their usual breakfast. However, other  
426 factors like age (which affects total salivary antibody concentration ranges) and gum health likely need  
427 to be controlled for through additional measurements, ideally within the same multiplex assay. If most  
428 of these factors were either eliminated or normalizers existed that allow to adjust for the between-  
429 sample differences, then the proportion of the indeterminate class would likely shrink greatly.

430 We showed that salivary and plasma anti-N, RBD and S IgG correlate highly between the two  
431 sample types and found that salivary SARS-CoV-2 anti-RBD and anti-S IgG binding estimates correlate  
432 equally well with neutralizing titers as corresponding blood-based estimates. Other groups have  
433 developed and characterized assays to measure salivary SARS-CoV-2 antibodies primarily using ELISA  
434 with mixed success.<sup>11,12,27</sup> Typically, the sensitivity of oral fluid assays is at least 10% lower than the  
435 sensitivity of serum or plasma assays if cutoffs are optimized such that >99% specificity is maintained  
436 and researchers conclude that testing salivary antibody testing may complement traditional serology  
437 without being a stand-alone alternative to blood-based testing.<sup>28-30</sup> To overcome the challenge  
438 associated with low total IgG concentrations in saliva compared to blood some have resorted to  
439 concentrating saliva, either with or without additional accounting for salivary total IgG, which resulted in  
440 increased assay sensitivity but reduced specificity.<sup>31</sup> Despite the lower assay performance most groups  
441 found that blood SARS-CoV-2 IgG levels correlate well with salivary IgG levels<sup>32</sup>, however, such findings  
442 are often based on very small sample sizes.<sup>31,33,34</sup> Studies with larger sample sizes typically report less  
443 robust correlations between saliva and blood antibody levels.<sup>30,35</sup>

444 In the Ambulatory COVID-19 Study cohort nearly all participants had seroconverted by saliva IgG  
445 to SARS-CoV-2 two weeks post COVID-19 symptoms onset and SARS-CoV-2 IgG levels were stable for the  
446 duration of the study. We did not observe a significant difference in time to seroconversion and time to  
447 plateau between anti-N, anti-RBD and anti-S IgG in saliva. To our knowledge, this is the first study to

448 report salivary anti-N and anti-spike IgG concentrations in oral fluid post infection that were calibrated  
449 to the international SARS-CoV-2 antibody standard in BAU/mL, which seem approximately 200-500-fold  
450 lower than those in serum or plasma. We measured a median anti-S IgG concentration of 0.3 BAU/mL  
451 (mean 0.5 BAU/mL) in saliva collected between 14 days and 90 days post infection, whereas others  
452 reported a median serum anti-spike concentration of 154 anti-spike BAU/mL post infection (using the  
453 Roche Elecsys anti-S assay).<sup>36</sup> Our in-house assay cutoff of 0.09 anti-spike IgG BAU/mL for saliva is ~200-  
454 fold lower than the Ortho Vitros spike IgG cutoff for serum/plasma cutoff that has been established at  
455 17.8 BAU/mL<sup>37</sup>, ~375-fold lower than the Diasorin Trimeric S IgG cutoff of 33.8 BAU/mL but, compared  
456 to the Elecsys anti-SARS-CoV-2 S cutoff of 0.8 anti-S BAU/mL, our in-house cutoff is only ~10-fold lower.  
457 This demonstrates that even though efforts have been made to harmonize quantitative SARS-CoV-2  
458 humoral immune responses between laboratories, cutoffs for binary classification of antibody status  
459 and also quantitative assay ranges between tests that have been calibrated to the WHO international or  
460 the U.S. serological SARS-CoV-2 standard still vary. For example, cutoffs for anti-spike IgG positivity in  
461 serum or plasma range from 0.8 BAU/mL to 33.8 BAU/mL between just five commercial anti-RBD/S  
462 assays used in a comparative “head-to-head” study and means of anti-spike concentrations post  
463 vaccination were reported to vary between ~70 BAU/mL and ~1500 BAU/mL using the same set of  
464 samples.<sup>38,39</sup> Despite absolute concentration discrepancies, most serological SARS-CoV-2 total Ig and IgG  
465 binding assays showed good quantitative correlations ( $\rho > 0.8$ ).<sup>38</sup> The Diasorin Trimeric S IgG assay and  
466 the Elecsys anti-SARS-CoV-2 S assay are among the best performing antibody assays for blood<sup>26</sup>,  
467 however, their respective cutoffs (0.8 anti-spike BAU/mL for Roche Elecsys and 17.8 BAU/mL for Ortho  
468 Vitros) vary by factor of  $> 22$ ; although the absolute difference is small ( $< 20$  BAU/mL). Panels comprising  
469 SARS-CoV-2 antibody negative and low, medium and high titer samples that have been quantified and  
470 characterized with the most commonly used serologic assays would be a useful reference for better  
471 comparison between reported testing data. Continued standardization and calibration of assays for  
472 saliva but also for serum and plasma will be important to confirm absolute concentration differences  
473 between the different specimen types and to harmonize cutoffs for sample classification.

474 Salivary SARS-CoV-2 IgG testing will be useful to determine the robustness of antibody  
475 responses post vaccination<sup>40</sup>, differences in antibody response between the different SARS-CoV-2  
476 vaccine types, and could be a tool to assess whether and when vaccine boosters may be needed due to  
477 waning immunity<sup>41</sup> at the individual level. Several groups have reported on salivary anti-S/RBD  
478 responses post vaccination, which elicits IgG responses that are at least 10-fold higher<sup>42</sup> than post  
479 natural infection and are therefore more easily detected in saliva.<sup>40,43</sup> Additionally, salivary SARS-CoV-2  
480 anti-N IgG testing may continue to be a useful estimate of exposure or infection among both vaccinated  
481 and naïve individuals. Saliva as a non-invasive specimen that can deliver SARS-CoV-2 antibody  
482 prevalence estimates with high accuracy is particularly attractive in studies with repeated sampling time  
483 points and when phlebotomy is either impractical, too costly or not an option, for example in large  
484 serosurveys, remote locations, when young children are participating or in elderly cohorts.<sup>30,35,44</sup> Salivary  
485 secretory IgA responses to SARS-CoV-2 as a surrogate of mucosal immunity<sup>45-47</sup> may predict protection  
486 from (re-) infection and new intra-nasal vaccines that elicit a stronger mucosal antibody response than  
487 intramuscular vaccines might protect better from infection than currently available intra-muscular  
488 vaccines.<sup>48,49</sup> Salivary multiplex assays will be an important tool to assess the role of preexisting and

489 cross-priming mucosal antibodies that may exhibit cross-reactivity with other endemic coronaviruses  
490 (229E, HKU1, NL63 and OC43) and their potential role in COVID-19 prevention and progression.<sup>50</sup>

491

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506

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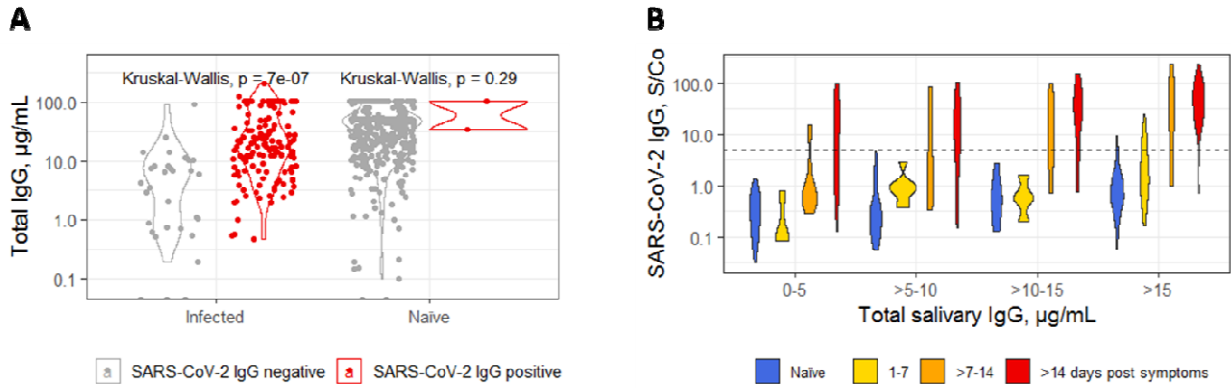
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**Table 1.** Self-collected COVID-19 study participant saliva samples and pre-COVID-19 era archived saliva samples for SARS-CoV-2 IgG multiplex test optimization and validation.

Testing Phase	COVID-19 study participant saliva samples			Pre-COVID-19 saliva samples	Total
	Days after COVID-19 symptoms onset				
	1-7 days	8-14 days	>14 days		
Threshold Setting, n (%)	41 (6%) <sup>#</sup>	78 (12%) <sup>#</sup>	174 (27%) <sup>#</sup>	362 (55%) <sup>#</sup>	655 (69%)*
Validation, n (%)	4 (1%) <sup>#</sup>	14 (5%) <sup>#</sup>	87 (29%) <sup>#</sup>	193 (65%) <sup>#</sup>	298 (31%)*
Total, n (%)	45 (5%) <sup>#</sup>	92 (10%) <sup>#</sup>	261 (27%) <sup>#</sup>	555 (58%) <sup>#</sup>	953 (100%)

<sup>#</sup>Distribution within testing phase/row; \*Distribution between threshold setting and validation phase



**Figure 1. A.** Distribution of salivary total IgG concentration in samples from COVID-19 study participants (Infected) and in archived pre-COVID-19 era samples (Naive) stratified by SARS-CoV-2 IgG test outcome. **B.** SARS-CoV-2 IgG in pre-COVID-19 era and in samples collected 1-7 days, >7-14 days and >14 days post COVID-19 symptoms onset stratified by total IgG concentration. Note: SARS-CoV-2 IgG outcome was determined using the sum of IgG signal to cutoff (S/Co) to 2 N, 3 RBD and 2 S antigens (Table 2). Dotted line: cutoff.

**Table 2. Effect of total IgG qualification of algorithm negative samples on assay performance.**

No exclusion requirement for algorithm negative samples									
Algorithm No.		1	2	3	4	5	6	7	8
		IgG signal above cutoff for multiple antigens				Sum of IgG S/Co of multiple antigens			
Sample Group	n	1 N & 1 RBD/S	2 RBD/S	1 N & 1 RBD/S or 2 RBD/S	N (n=2)	RBD (n=3)	Spike (n=2)	N & RBD/S (n=7)	N & RBD/S divided total IgG
<b>Sensitivity</b>									
1-7 DPSO	41	4.9%	9.8%	9.8%	9.8%	9.8%	14.6%	9.8%	2.6%
>7-14 DPSO	78	43.6%	46.2%	51.3%	47.4%	44.9%	48.7%	53.8%	20.0%
>14 DPSO	174	78.2%	81.0%	83.3%	79.3%	81.0%	75.9%	84.5%	43.8%
<b>Specificity</b>									
pre-COVID	362	99.4%	99.2%	98.6%	97.8%	98.6%	98.9%	98.6%	98.6%
Excluded*		0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.0%
<b>Algorithm negatives must have a minimum of &gt;5 ug/mL total IgG</b>									
<b>Sensitivity</b>									
1-7 DPSO	40	5.4%	10.8%	10.8%	10.8%	10.8%	16.2%	10.8%	20.5%
>7-14 DPSO	77	54.0%	56.3%	61.9%	57.8%	54.7%	58.7%	64.6%	75.0%
>14 DPSO	174	85.5%	89.7%	91.0%	86.2%	89.7%	86.2%	91.8%	94.0%
<b>Specificity</b>									
pre-COVID	355	99.4%	99.1%	98.5%	97.6%	98.5%	98.8%	98.5%	93.6%
Excluded*		9.1%	9.4%	9.6%	9.0%	9.4%	10.1%	8.8%	2.2%
<b>Algorithm negatives must have a minimum of &gt;10 ug/mL total IgG</b>									
<b>Sensitivity</b>									
1-7 DPSO	40	7.1%	14.3%	14.3%	13.8%	14.3%	21.4%	14.3%	31.4%
>7-14 DPSO	77	69.4%	70.0%	74.5%	71.2%	68.0%	68.6%	77.4%	81.7%
>14 DPSO	174	91.9%	94.0%	95.2%	91.3%	94.0%	91.5%	95.4%	95.8%
<b>Specificity</b>									
pre-COVID	355	99.3%	99.0%	98.3%	97.7%	98.3%	98.7%	98.3%	90.1%
Excluded*		18.9%	18.6%	19.7%	18.1%	18.5%	19.4%	17.6%	6.4%
<b>Algorithm negatives must have a minimum of &gt;15 ug/mL total IgG</b>									
<b>Sensitivity</b>									
1-7 DPSO	40	8.3%	16.7%	16.7%	16.0%	8.3%	20.8%	16.7%	44.1%
>7-14 DPSO	77	75.6%	73.9%	76.2%	77.1%	71.7%	72.3%	82.0%	87.3%
>14 DPSO	174	94.4%	97.9%	98.6%	94.4%	97.9%	95.6%	98.6%	98.2%
<b>Specificity</b>									
pre-COVID	355	99.2%	98.9%	98.1%	97.7%	98.5%	98.9%	98.5%	87.9%
Excluded*		26.3%	26.2%	27.4%	25.4%	26.3%	27.0%	24.9%	10.8%

Note: DPSO: days post COVID-19 symptoms onset; N: nucleocapsid (GenScript N and Native Antigen Company N); RBD: receptor binding domain (Sino Biological RBD, Mt. Sinai RBD, GenScript RBD); S: spike (Mt. Sinai S and Sino Biological S1/S2/ectodomain [ECD]). Cutoffs for each algorithm were defined as the mean plus 3 standard deviations of pre-COVID-19 era samples. \*Samples excluded due to total IgG concentration of 0 ug/mL [in denominator] or due to not passing minimum total IgG qualification requirement.

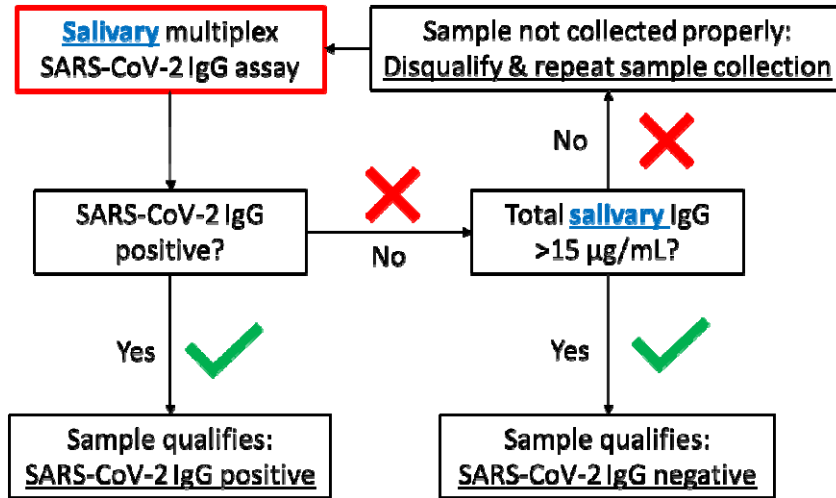
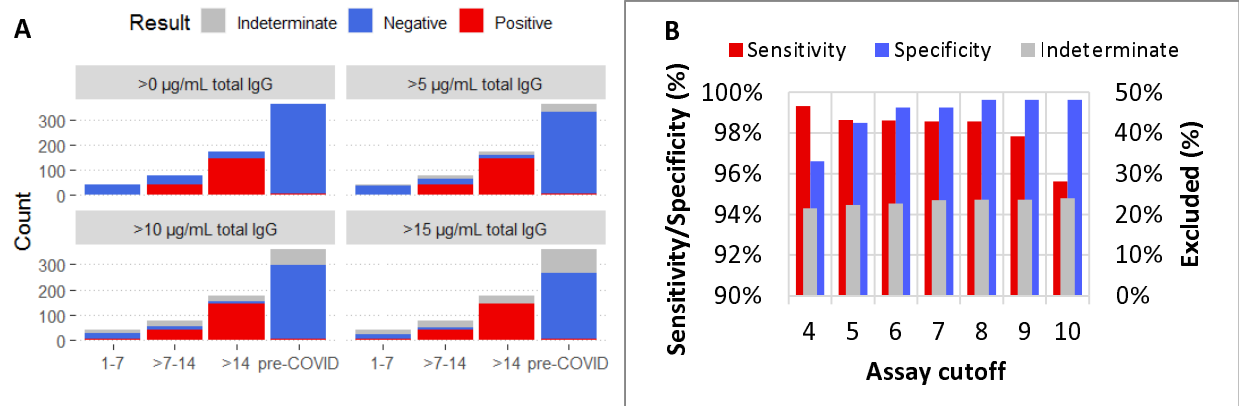


Figure 2: Sequence for salivary SARS-CoV-2 IgG MIA testing and qualification.



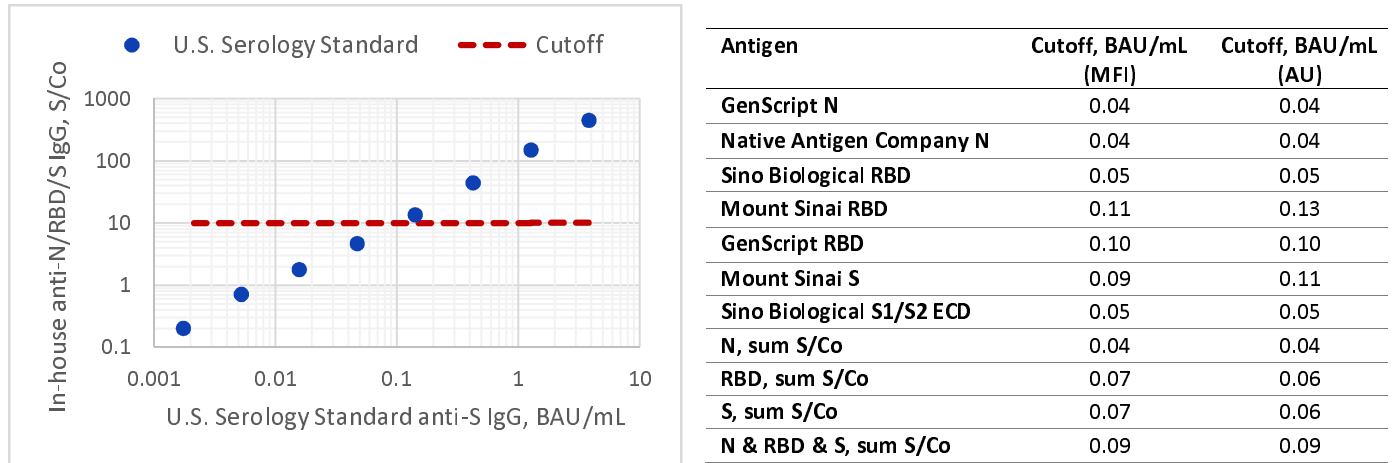
**Figure 3. A)** Effect of establishing a minimum total IgG concentration threshold for saliva qualification on MIA performance; **B)** MIA performance and sample exclusion at various cutoffs (8 million beads coupling scale).

**Table 3.** Salivary SARS-CoV-2 IgG MIA performance during threshold setting and validation phases.

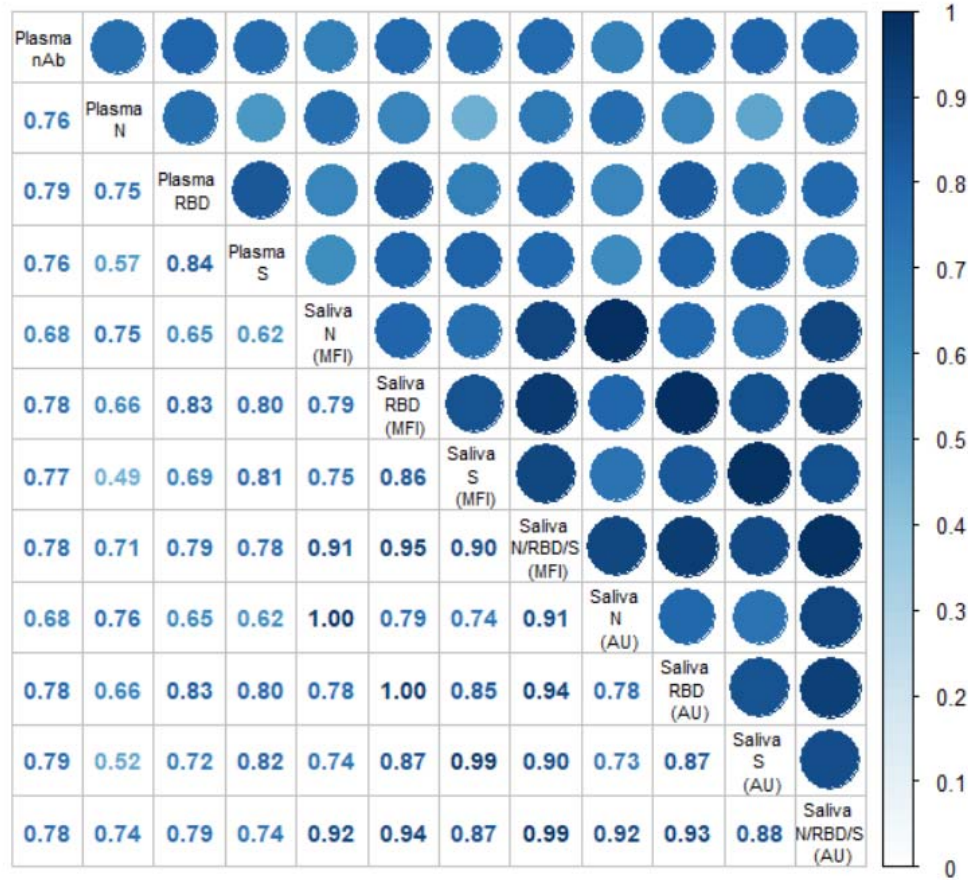
Assay bead scale and phase	Sensitivity			Specificity
	COVID-19 study participant samples			pre-COVID-19 era samples
	Days after COVID-19 symptoms onset			
	1-7 days	8-14 days	>14 days	
<b>8-million coupling scale (MFI)</b>				
Threshold setting	n=40	n=77	n=174	n=355
	3/21 (12.5%)	40/50 (80%)	142/144 (98.6%)	263/265 (99.2%)
n excluded	16/40 (40%)	27/77 (35%)	30/174 (17%)	90/355 (25%)
Validation	n=4	n=11	n=69	n=158
	0/2 (0.0%)	7/8 (87.5%)	62/62 (100.0%)	108/109 (99.1%)
n excluded	2/4 (50%)	3/11 (27%)	7/69 (10%)	49/158 (31%)
<b>20-million coupling scale (MFI)</b>				
Threshold setting	n=37	n=68	n=155	n=287
	2/22 (9.1%)	28/41 (68.3%)	118/122 (96.7%)	218/218 (100.0%)
n excluded	15/37 (41%)	27/68 (40%)	33/155 (21%)	69/287 (24%)
Validation	n=4	n=14	n=87	n=193
	1/3 (33.3%)	9/11 (81.8%)	81/81 (100%)	126/127 (99.2%)
n excluded	1/4 (25%)	3/14 (27%)	6/87 (7%)	66/193 (34%)
<b>20-million coupling scale (AU)</b>				
Threshold setting	n=37	n=68	n=155	n=287
	2/22 (9.1%)	28/40 (70%)	118/122 (96.7%)	218/218 (100.0%)
n excluded	15/37 (41%)	28/68 (41%)	33/155 (21%)	69/287 (24%)
Validation	n=4	n=14	n=87	n=193
	1/3 (33.3%)	8/11 (72.7%)	80/81 (98.8%)	127/127 (100%)
n excluded	1/4 (25%)	3/14 (27%)	6/87 (7%)	66/193 (34%)

Note. Antigens contributing to multiplex assay (algorithm 7; sum[S/Co]): GenScript nucleocapsid (N), Native Antigen Company N, Sino Biological receptor binding domain (RBD), Mt. Sinai RBD, GenScript RBD, Mt. Sinai Spike (S), Sino Biological S1/S2 ectodomain

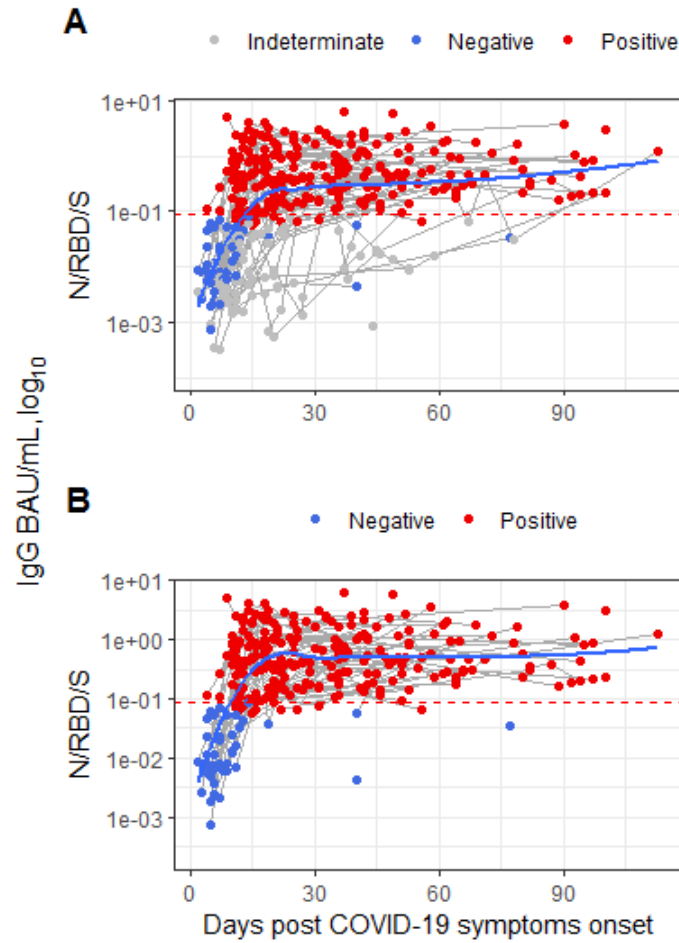




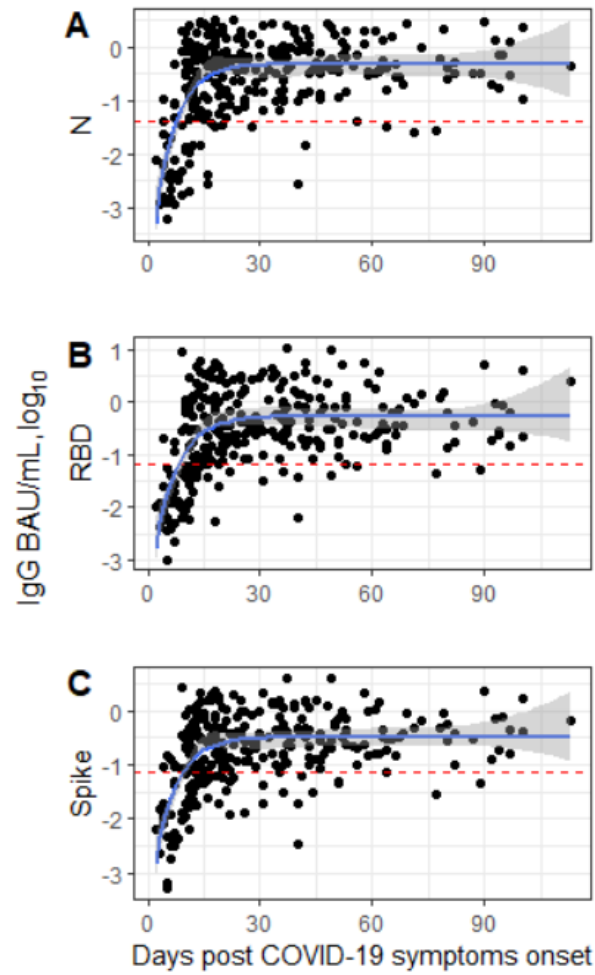
**Figure 4.** Calibration of oral fluid sum of signal to cutoff (S/Co) for N, RBD, and S to the US SARS-CoV-2 serology standard binding anti-S IgG binding antibody units (BAU) per mL. Note. N: nucleocapsid; RBD: receptor binding domain; S: spike; ECD: ectodomain.



**Figure 5.** Correlation between plasma SARS-CoV-2 nAb, plasma SARS-CoV-2 IgG ELISA and salivary SARS-CoV-2 IgG MIA for n=30 matched samples. Note: Plasma anti-N, anti-RBD and anti-S IgG area under the curve (AUC) determined by ELISA, plasma neutralizing antibody AUC (nAb) determined by neutralizing antibody assay. Saliva N, RBD or S AU/MFI: sum of multiplex assay signal to cutoff of anti-N IgG, anti-RBD or anti-S IgG or the sum of S/Co to all antigens (N/RBD/S) measured either in mean fluorescence intensity (MFI) or arbitrary units (AU).



**Figure 6.** SARS-CoV-2 IgG kinetics post infection in saliva. **A.** Samples classified as SARS-CoV-2 positive, negative and indeterminate contributed to Loess regression (n=375). **B.** Samples with insufficient total IgG that tested negative (grey points) were excluded, leading to more naturally plausible IgG signal progression, similar to antibody kinetics observed in blood (n=288). Grey lines connect longitudinal samples from the same participant. **Note.** Y-axis scale reflects a calibration of our in-house sum of anti-N/RBD/S IgG signal to cutoff to the U.S. SARS-CoV-2 serological standard anti-S IgG BAU/mL.



**Figure 7.** Anti-N, RBD and Spike IgG (BAU/mL) kinetics in saliva collected by COVID-19 study participants over time. **A.** Anti-nucleocapsid (N) IgG. **B.** Anti-receptor-binding domain (RBD) IgG. **C.** Anti-spike (S) IgG. Blue line represents a monomolecular model (exponential growth function) fitted to the  $\log_{10}$ -transformed IgG concentration. Red dotted lines represent the cutoff.