Validation of a commercially available SARS-CoV-2 serological Immunoassay

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Abstract:

Objectives: To validate the diagnostic accuracy of a Euroimmun SARS-CoV-2 IgG and IgA immunoassay for COVID-19.

Methods: In this unmatched (1:1) case-control validation study, we used sera of 181 laboratory-confirmed SARS-CoV-2 cases and 176 controls collected before SARS-CoV-2 emergence. Diagnostic accuracy of the immunoassay was assessed against a whole spike protein-based recombinant immunofluorescence assay (rIFA) by receiver operating characteristic (ROC) analyses. Discrepant cases between ELISA and rIFA were further tested by pseudo-neutralization assay.

Results: COVID-19 patients were more likely to be male and older than controls, and 50.3% were hospitalized. ROC curve analyses indicated that IgG and IgA had high diagnostic accuracies with AUCs of 0.992 (95% Confidence Interval [95%CI]: 0.986-0.996) and 0.977 (95%CI: 0.963-0.990), respectively. IgG assays outperformed IgA assays (p=0.008). Taking an assessed 15% inter-assay imprecision into account, an optimized IgG ratio cut-off > 1.5 displayed a 100% specificity (95%CI: 98-100) and a 100% positive predictive value (95%CI: 97-100). A 0.5 cut-off displayed a 97% sensitivity (95%CI: 93-99) and a 97% negative predictive value (95%CI: 93-99). Substituting these thresholds for the manufacturer's, improved assay performance, leaving 12% of IgG ratios indeterminate between 0.5-1.5.

Conclusions: The Euroimmun assay displays a nearly optimal diagnostic accuracy using IgG against SARS-CoV-2 in patient samples, with no obvious gains from IgA serology. The optimized cut-offs are fit for rule-in and rule-out purposes, allowing determination of whether individuals in our study population have been exposed to SARS-CoV-2 or not. IgG serology should however not be considered as a surrogate of protection at this stage.

67 **Introduction:**

68 High throughput and reliable serological assays detecting antibodies against SARS-CoV-2 are
69 essential to determine the proportion of SARS-CoV-2 infected individuals and estimate the
70 current seroprevalence in the general population or in high-risk groups, such as health care
71 workers. Serological assays can complement diagnostic strategies focusing on the identification
72 of the infectious agent during the acute phase of disease. Unlike RT-PCR, they can identify
73 infected individuals that remained asymptomatic or undiagnosed, which are both frequent
74 conditions during SARS-CoV-2 infection, long after the initial infection. Validated serological
75 assays are also key to understanding the (immuno)-pathophysiology of COVID-19 in various
76 patients' groups and will be critical to characterize responses elicited by the numerous vaccine
77 candidates in development (1).

78 Designing serological testing strategies with high sensitivity and specificity and with acceptable
79 positive (PPV) and negative predictive values (NPV) is far from trivial, and requires taking two
80 major analytical aspects into account: analytical specificity and sensitivity (2),(3). The former
81 may largely be determined by the degree of cross-reactivity with other CoVs, which frequently
82 cause common colds in humans (i.e. HCoV-229E, -NL63, -OC43 and -HKU1) (4) resulting in
83 seroprevalence rates usually above 90% in adults (5).

84 This cross-reactivity occurs when virus-specific antigenic epitopes are highly similar and
85 recognized by the same B cells. It is best defined by the proportion of "false-positive" SARS-
86 CoV-2 results in individuals who were never exposed to this pathogen. In contrast to common
87 cold CoVs, the seroprevalence for MERS-CoV is low even in endemic countries (6). Therefore,
88 cross-reactivity between MERS-CoV and SARS-CoV-2 is not a critical factor when assessing
89 population seroprevalence. Previous studies have shown that antibodies against common cold
90 CoVs can cause considerable cross-reactivity in serological assays, depending on the type of
91 assay and antigens used. Particularly, whole virus- or nucleocapsid protein-based assays
92 showed a higher cross-reactivity compared to whole spike or S1 domain-based assays resulting
93 in lower specificity (4). During the early phase of an outbreak, when SARS-CoV-2
94 seroprevalence is low, serological testing strategies must have a very high specificity to reach a
95 high positive predictive value (PPV) and avoid false positive results.

96 On the other hand, analytical sensitivity is strongly influenced by the epidemic course, the
97 disease biology, and numerous analytical factors. All these interrelated items are primarily
98 influenced by the intrinsic immunogenicity of the SARS-CoV-2 antigens and the magnitude and
99 duration of B cell responses elicited by infection, be it asymptomatic, benign, moderate or
100 severe (2),(3).

Last but not least in the context of a pandemic, the availability of high throughput and reliable diagnostic platforms is key for health care systems to effectively handle the testing demand, while respecting clinically compatible diagnostic turnaround times (TAT). In this study, we performed an extensive validation of a high throughput SARS-CoV-2 commercial serological platform quantifying both serum IgG and IgA against the S1 protein. As reference method, we used a whole spike-based recombinant immunofluorescence assay (rIFA) (4),(7),(8). Selected sera from SARS-CoV-2-infected patients were assessed for their neutralization capacity using a pseudovirion assay (see below).

Methods:

Aims

The aim of this study is to validate and define the operational cut-off values of a commercially available ELISA-based SARS-CoV-2 serological assay that could be applied at large scales to reliably determine the presence of specific IgG as a marker of SARS-CoV-2 infection. This study used RT-PCR confirmed cases, but the goal was to be able to identify exposure to SARS-CoV-2 by immunoassay alone. Therefore, the ELISA results were compared against recombinant immunofluorescence assay (rIFA), which was considered as the reference method due to its demonstrated high specificity for serology of other CoVs such as MERS-CoV (6). The secondary goal is to assess the potential added value of IgA in patients recently infected with SARS-CoV-2.

Study population

Negative control serum samples (n=176) were collected for various serological testing in our routine laboratory and stored for analytical validation. These sera were collected in 2013, 2014 and 2018 before the start of the outbreak and thus have not been exposed to SARS-CoV-2. Sera (n=181) of PCR-confirmed COVID-19 patients were collected at the University Hospitals of Geneva from hospitalized patients (n=91) as well as from patients from the outpatient clinic (n=90). Ethical approval for all sera used in this study was waived by the local ethics committee of the HUG that approves usage of leftover of patient serum collected for diagnostic purposes. The number of days from symptom onset to blood collection was based on patient history whenever this information was available or could be retrieved in a reliable way; otherwise, we used the date of RT-PCR positivity as a surrogate for onset of symptoms. Serum samples from unmatched PCR-confirmed COVID-19 hospitalized patients were collected for routine diagnostic purposes under a general informed consent and outpatients were asked if they were willing to

return to the hospital after their symptoms had subsided and to donate a serum sample under written general informed consent.

Blood sample collection

We used serum samples collected on patient admission, during hospitalization or, when needed, left over sera used for other routine investigations. Samples were immediately processed and then frozen and stored at −20°C until ELISA, recombinant immunofluorescence analyses, and pseudoneutralization were performed (see below). Patients were sampled at different times after onset of symptoms (dpos) or according to days post RT-PCR diagnosis (dpd) if the onset was not known. To compare the seropositivity between different time points, we grouped patients in the following dpos/dpd categories: 0-10 (n=8, 4.4%), 11-20 (n=112, 61.9%) and 21-39 (n=61, 33.7%).

SARS-CoV-2 RT-PCR analyses

SARS-CoV-2 RT-PCR was performed according to manufacturers' instructions on various platforms, including initially in house method using eMAG (bioMérieux, France) and Charité RT-PCR protocol(9), then BD SARS-CoV-2 reagent kit for BD Max system (Becton, Dickinson and Co, US) and Cobas 6800 SARS CoV2 RT-PCR (Roche, Switzerland).

Serum IgG and IgA ELISA

Both IgG and IgA enzyme-linked immunosorbent assays (ELISA) used the S1-domain of the spike protein of SARS-CoV-2 as antigen. Sera were diluted at 1:101 and assessed with the IgG and IgA CE-marked ELISA (Euroimmun AG, Lübeck, Germany # EI 2606-9601 G and # EI 2606-9601 A) according to the manufacturer's instructions. They were run on Dynex Agility (Ruwag, Switzerland) according to the manufacturer's protocol. After adding the conjugate, samples' immunoreactivity was measured at an optical density of 450nm (OD450) and then divided by the OD450 of the calibrator provided with each ELISA kit to minimize inter-assay variation(8). The quantitative results obtained were then expressed in arbitrary units and interpreted as follows: OD ratio: <0.8 = negative; ≥0.8 and <1.1 = indeterminate; ≥1.1 = positive. Inter-assay variation was 15.6% for IgG at a ratio of 2.09 (n=17) and 17.7% for IgA at a ratio of 4.85 (n=7).

Recombinant immunofluorescence assay

168 The IgG antibody response against the spike protein of SARS-CoV-2 was assessed by rIFA as
169 described and previously validated for MERS-CoV (6),(7). Briefly, Vero B4 cells were
170 transfected with the mammalian expression vector pCG1-SCoV2-S (kindly provided by M.
171 Hoffmann and S. Pöhlmann, DPZ, Göttingen, Germany) using Fugene HD (Promega, #E2311).
172 After 24h of incubation cells were detached and residual trypsin was removed by centrifugation
173 at 300x g for 5min. 50µl of transfected cells were seeded at a density of $2x10^5$/ml on multi-test
174 glass slides (DUNN Labortechnik GmbH #40-412-05) and incubated for 6h at 37°C, 5% CO_2.
175 Afterwards slides were washed 2x with PBS and fixed for 10min using ice-cold
176 Acetone/Methanol (ratio 1:1). For rIFA staining, slides were rehydrated for 10min using PBS +
177 0.1% Tween20 (PBS-T) and blocked with 5% milk in PBS-T for 30min at RT. Sera were diluted
178 1:40 in blocking buffer, 30µl were applied on each spot and incubated for 60min at 37°C or RT.
179 After 3x washing with PBS-T, secondary goat anti-human-IgG antibody conjugated with
180 Alexa488 (Jackson ImmunoResearch, #109-545-088) was diluted 1:200 in PBS and 25µl were
181 applied to each spot. The secondary antibody was incubated for 45min at 37°C and slides were
182 washed 3x with PBS-T afterwards. Slides were briefly rinsed with dH_2O and mounted using
183 glycerol. rIFA results were judged by 3 observers independently and the inter-observer kappa
184 correlation was 0.59 (95%CI: 0.31-0.87) between observer 1 and 2, 0.62 (95%CI: 0.33-0.91)
185 between observer 2 and 3 as well as 0.75 (95%CI: 0.52-0.99) between observer 1 and 3, which
186 could be considered a substantial level of agreement(10).
187
188 **VSV-based pseudo-neutralization assay**
189 VeroE6 cells were seeded in 96-well plates at 2 x 10^4 cells per well and grown into confluent
190 monolayer overnight. Sera from patients were inactivated at 56°C for 30 minutes and diluted 1:5
191 in 120 µl DMEM 2% FCS in the first column of a 96-well plates (in duplicate). Remaining wells
192 were filled with 60 µl of DMEM 2% FCS. Two-fold dilutions were performed until 1:80 was
193 reached by moving 60 µl from one well column to the following one. VSV-based SARS-CoV-2
194 pseudotypes (generated according to Berger, Rentsch, and Zimmer (11) and Torriani et al. (12))
195 expressing a 19 amino acids C-terminal truncated spike protein (13) (NCBI Reference
196 sequence: NC_045512.2) were diluted in DMEM 2% FCS in order to have MOI=0.01 in 60 µl
197 volume and added on top of serum dilutions (final serum dilutions obtained were from 1:10 to
198 1:160). The virus-serum containing plate was incubated at 37°C, for 2h. Vero E6 were then
199 infected with 100µl of virus-serum mixtures. After incubation at 37°C for 1.5h, cells were washed
200 once with 1X PBS and DMEM 10% FCS was added. After 16-20h of incubation at 37°C, 5%
201 CO_2 cells were fixed with 4% formaldehyde solution for 15 min at 37°C and nuclei stained with

202 1µg/ml DAPI (AppliChem #A4099) solution. GFP positive infected cells were counted with
203 ImageXpress® Micro Widefield High Content Screening System (Molecular Devices) and data
204 analyzed with MetaXpress 5.1.0.41 software.
205
206 **Statistical analyses**
207 Analyses were performed with Graph Pad Prism version 8.3.1 software using Fisher's bilateral
208 exact test and Mann–Whitney *U*-test where appropriate. ROC analyses were performed
209 using ANALYSE-IT™ software for Excel (Microsoft, Redmond, WA, USA) to: (i) confirm the cut-off
210 values, prospectively proposed by the manufacturer, when compared to IFA results as the
211 reference method (samples with very weak fluorescence signal just above background were
212 deemed putative negatives, and were considered as negative for the purpose of this analysis);
213 (ii) compare the diagnostic accuracy of IgG alone, IgA alone, or both combined; and (iii)
214 determine the optimal rule-in cut-off (PPV of 100%), a rule-out cut-off with a NPV above 95%,
215 and an indeterminate interval, taking into account the analytical imprecision-derived least
216 significant change (LSC). The LSC represents the smallest significant detectable difference
217 between two measurements based given the analytical imprecision, and is conventionally
218 defined as $1.96 * \sqrt{2} * VC$ (14) (15). AUC comparisons were performed according to the
219 nonparametric approach proposed by DeLong *et al.* (16). Sensitivity (SE), specificity (SP), PPV
220 and NPV with the respective 95% CIs are given. A value of $p<0.05$ was considered statistically
221 significant.
222
223 **Results:**
224 The baseline demographic characteristics of the participants are summarized in table 1. There
225 was a higher proportion of males (61.3%) vs females (38.7%) in COVID-19 patients compared
226 to controls (Fisher's exact test, p=0.002). Among COVID-19 patients, we included an equal
227 representation of hospitalized patients (n=91, 50.3%) and outpatients (n=90, 49.7%).
228
229 **Prevalence of IgG seropositivity according to rIFA**
230 An in-house developed recombinant immunofluorescence assay (rIFA) using the whole spike
231 protein of SARS-CoV-2 as antigen was used to assess spike-specific serum IgG. Since rIFA
232 was used as a confirmatory assay, we maximized its specificity by interpreting putative
233 negatives as negative to avoid false positive results. Among negative control samples, we found
234 no positive, five putative negative (2.8%) and 171 (97.2%) negative samples indicating a
235 specificity of 100%. Among COVID-19 samples there were 165 (91.2%) positive, two (1.1%)

236 putative negative and 14 (7.7%) negative samples, indicating an overall detection rate of 91.2%.
237 We found that rIFA seropositivity was low at 0-10 dpos/dpd (12.5%) but increased to 92.0% and
238 100% in sera collected at 11-20 and 21-39 dpos/dpd, respectively (table 2). Similar results were
239 found in hospitalized and outpatients. Thus, in our sample of 181 COVID-19 samples, rIFA
240 proved a robust method for the detection of SARS-CoV-2 spike-specific IgG.
241
242 **Diagnostic accuracies of IgG and IgA ELISA against rIFA**
243 Overviews of OD ratios for IgG and IgA are shown in Figure 1 A and B, respectively. In control
244 sera, we found no positive, five (2.8%) indeterminate and 171 (97.2%) negative samples for
245 IgG, and 14 (7.7%) positive, 10 (5.7%) indeterminate and 157 (86.7%) negative samples for IgA
246 according to the manufacturer defined cut-off (EI cut-offs) (table 2). Considering the
247 indeterminate values as positive resulted in a specificity of 97.3% (IgG) and 86.4% (IgA),
248 respectively. Analysis of COVID-19 samples revealed 154 (85.1%) positive, one (0.6%)
249 indeterminate and 26 (14.4%) negative samples for IgG, and 164 (90.6%) positive, four (2.2%)
250 indeterminate and 13 (7.2%) negative samples for IgA. This resulted into an overall significantly
251 higher seropositivity for IgA (90.6%) than IgG (85.1%) in the S1-based ELISA (p= 0.041).
252
253 Next, we analysed the seropositive rates of both ELISAs at different dpos or dpd (Figure 1E)
254 according to the EI cut-offs. Sera collected 21 dpos/dpd had a similarly high seropositivity for
255 both IgG (96.7%) and IgA (96.7%) (p >0.99). A higher seropositivity was observed for IgA
256 compared to IgG for sera collected at 11-20 dpos/dpd (91.1% vs 84.8%, p= 0.0264) but not 0-10
257 dpos/dpd (37.5% vs 0%, p= 0.2). No significant difference was found between hospitalized and
258 outpatients in IgG or IgA ELISA (p= 0.833, and p= 1.000, respectively).
259
260 ROC curve analysis (Fig 1; C and D) indicated that overall, both IgG and IgA had a high
261 diagnostic accuracy with respective AUCs of 0.992 (95%CI: 0.986-0.996) and 0.977 (95%CI:
262 0.963-0.99), respectively. Although modest, this AUC difference was found to be significant
263 according to the de Delong method (p=0.008). Combining IgG and IgA ELISAs together (AUC
264 0.986; 95%CI: 0.976-0.995) tended to decrease the diagnostic accuracy of the model when
265 compared to IgG alone (p=0.080), but outperformed IgA alone (p<0.001). Although these
266 differences were modest, our results indicate that IgG ELISA displays the optimal fit with IgG
267 rIFA and that IgA did not improve diagnostic value. In a subgroup analysis considering only sera
268 harvested before 21 days after symptoms onset (n=120), ROC curve analyses displayed similar

269 results - with an AUC of 0.981 for the IgG ratio and of 0.965 for the IgA ratio. At these early
270 time-points, the AUC difference between IgG and IgA ratio was not significant (p=0.19).
271
272 Thirteen patient samples had discrepant or potentially discrepant results, i.e. were negative or
273 indeterminate in ELISA and putative negative or positive in rIFA. To investigate this
274 discordance, we performed a VSV-based pseudovirus neutralization assay (pseudo-NT) for 1)
275 these 13 samples (n=13), 2) five negative control samples, including one sample that showed
276 putative negative results in rIFA, and 3) seven patient samples that were positive by ELISA IgG,
277 IgA and rIFA (table 3). All samples that showed negative and putative negative rIFA results
278 were confirmed negative, whereas all rIFA positive samples were confirmed as positive by the
279 pseudo-NT assay (table 3). These findings support the accuracy of the rIFA and our decision to
280 count putative negative rIFA results as negative.
281
282 **Optimizing the threshold for IgG serology**
283 Taking into account the variation coefficient (VC) of 15% at a low positivity IgG ratio (2.09), and
284 assuming that such VC could at least be transposed to the 1.1 cut-off ratio, the minimal LSC
285 would be 0.42. Added to the 1.1 ratio, an IgG ratio cut-off of 1.5 (1.1 + 0.42) would be the lowest
286 able to secure 100% PPV while taking into account the analytical imprecision. Furthermore, an
287 LSC of 0.42 also indicates that the indeterminate zone proposed by the manufacturer (between
288 0.8 and 1.1) should be reconsidered, as it falls within the analytical imprecision range. At the 1.5
289 IgG ratio cut-off, ROC curve analyses indicated that the SE was 86%, the SP 100%, and the
290 NPV 89%. Thus, selecting a 1.5 cut-off, rather than the recommended 1.1 cut-off for IgG
291 seropositivity, allows the securing of a PPV of 100% despite a 15% imprecision. Higher VCs
292 would translate into a higher seropositivity cut-off to secure an identical PPV. For rule-out
293 purposes (i.e. the seronegativity lower cut-off), the best trade-off IgG ratio cut-off was found to
294 be < 0.5. At this value, the SE was 97%, the SP 87%, the NPV 97% and the PPV 86% (table 4).
295 This defines an indeterminate range between IgG ratios of 0.5 and 1.5, which represented 43
296 cases (12%) of our samples (including 20 control and 23 COVID-19 samples). In this
297 indeterminate zone, all 20 sera from controls were confirmed as negative (n=19) or putative
298 negative (n=1) by rIFA. For indeterminate samples of COVID-19 patients, five were negative,
299 one was a putative negative and 18 were positive by rIFA.

300 In the subgroup of patients whose samples were taken before 21 dpos, using the manufacturer
301 IgG ratio seropositivity cut-off (1.1), the SE was 91%, the SP 100%, the PPV 100%, and the

302 NPV 62%. At the manufacturer seronegative cut-off (<0.8), the SE was 91%, the SP 100%, the
303 PPV 100%, and the NPV 64% (table 4). Importantly, no patient displayed a ratio between 0.8
304 and 1.1 in this subgroup, preventing us from estimating the importance of the indeterminate
305 cases.
306 Using the aforementioned optimized IgG ratio cut-off for IgG seropositivity (1.5), the SE was
307 82%, the SP 100%, the PPV 100%, and the NPV 46%. At the cut-off for IgG seronegativity
308 (<0.5), the SE was 96%, the SP 69%, the PPV 95%, and the NPV 73% (table 4). The number of
309 undetermined cases between 0.5 and 1.5 IgG ratio values represented 17% (20/120) of the
310 subgroup. Taken together, these results indicate that if the seropositivity cut-off of 1.5 for the
311 IgG ratio would be suitable for patients with sample taken <21 days post symptoms onset, the
312 seronegative cut-off of 0.5 would display a non-optimal NPV for rule-out purposes. The optimal
313 seronegative (NPV 100%) cut-off would have been <0.4, at the cost of a modest increase of
314 indeterminate cases to 20% (24/120). Thus, this assay performs best for convalescent samples
315 taken as of 21 dpos.
316
317 **Discussion:**
318 The key finding of this validation study, derived from a large cohort of laboratory-confirmed
319 SARS-CoV-2 cases and unmatched negative controls, is that the present CE-IVD marked
320 immunoassay for IgG has a good diagnostic accuracy with an AUC of 0.99 and outperformed
321 IgA according to ROC curve comparisons. These results parallel the ones obtained by two other
322 smaller studies (8),(17), which tested the non CE-marked ELISA version and showed potentially
323 better diagnostic performances of IgG compared to IgA ELISA but lower AUCs compared to this
324 study. Our improved results might result from the higher number of negative controls and
325 COVID-19 patient sera tested, and from the decreased non-specific background relative to that
326 observed in the earlier studies. Despite the greater performance of the IgG based ELISA, it was
327 reassuring to see that the IgA results correlated well with the IgG results and gave very similar
328 AUCs upon ROC analyses. However, knowing if and how IgA could be used in clinical practice
329 to further refine rule-in our rule-out strategies still remains elusive.
330
331 The second notable finding of this study is that the current manufacturer cut-offs are prone to
332 misinterpretations and should not be used without proper evaluation before routine testing.
333 Keeping in mind the challenges of developing a serological assay for a new viral disease in an
334 emergency situation; securing both rule-in and rule-out cut-offs is key to mitigate the
335 unavoidable risk of false positive and false negative results due to the combination of a highly

336 dynamic pre-test probability variation, with a suboptimal seroconversion time when studies are
337 undertaken at the peak of an epidemic. Our analyses revealed the following limitations of the
338 manufacturer's seropositivity cut-off. First, with an inter-assay imprecision of 15% assessed at
339 an IgG ratio of 2.09 (above than the 1.5 two cut-off value selected) translating into a LSC of 0.42
340 IgG ratio, our results indicate that the analytical imprecision is higher than the range of the
341 indeterminate zone proposed by the manufacturer, which encompasses a delta of 0.3 IgG ratio.
342 This implies that any result within the 0.8-1.1 IgG ratio range could be randomly either above,
343 within or below these values just because of analytical imprecision. Secondly, with a LSC of
344 0.42 our results indicated that a higher IgG ratio cut-off value was needed to secure an optimal
345 specificity and PPV. Adding the 0.42 LSC to the 1.1 cut-off yielded a 1.5 ratio as the IgG
346 seropositivity cut-off with a PPV of 100%, the lower end of the 95%CI still being compatible with
347 a 97% rule-in strategy. Notably, using this cut-off in the subgroup of patients under 21 dpos/dpd,
348 the SP and PPV were still 100%, however, with broader confidence intervals. Similarly, in an
349 attempt to maximize the negative predictive value at the rule-out cut-off, the cut-off had to be
350 decreased from 0.8 to 0.5 IgG ratio in order to reach an overall NPV of 97%, with a 93% at the
351 lower end of the 95CI. In the subgroup of patients under 21 dpos/dpd this interval was found to
352 be substantially larger (95%CI: 45-91). Taken together, these results indicate that at this stage
353 the optimal rule-in cut-off should be set at >1.5 of IgG ratio for seropositivity and at <0.5 of IgG
354 ratio for rule-out purposes (seronegativity). With these optimized "Geneva's" cut-offs, the
355 indeterminate zone represented 12% of the cases overall (20 controls and 23 COVID-19
356 samples), representing a volume easily amenable to further confirmation tests like rIFA.
357
358 In this respect, the 13 discordant cases between ELISA and rIFA were tested using the pseudo-
359 NT assay. This indicated that all non-positive samples (negative and putative negative) by rIFA
360 were negative in the pseudo-NT assay, and that all positive samples by rIFA (but ELISA
361 negative) were positive in the pseudo-NT assay. One sample that was indeterminate in ELISA
362 and positive in rIFA was also positive in pseudo-NT. These findings may indicate an earlier IgG
363 antibody response, which could better mediate virus neutralization, against the S2 domain of the
364 spike protein, as previously suggested for SARS-CoV-1 neutralizing antibodies targeting the S2
365 domain (18),(19),(20),(21). Although based upon a limited number of observations which
366 prevents us from drawing final conclusions, these results suggest that rIFA may be an
367 appropriate confirmatory assay in the future.

368 Regarding potential limitations, we need to highlight several additional points. We evaluated an
369 ELISA assay measuring antibodies against the S1 domain of the spike protein and not against

370 the full protein, which may contain other highly relevant epitopes from the S2 domain. Such
371 factors could potentially explain why some samples were negative by ELISAs but positive by
372 rIFA, as the whole-spike protein is used in rIFA. Secondly, we strongly emphasize two important
373 cut-off limitations related to the analytical imprecision. At the level of the analytical imprecision,
374 the SARS-CoV-2 IgG intra-individual biological variation being unknown, we had to use the LSC
375 instead of the reference change value (22), which most likely would have translated into a
376 higher seropositivity cut-off. Along the same line, because the intra-lot imprecision of the
377 reagents is still undetermined, but expected to be higher than 15%, this could have a similar
378 impact on cut-off determination. Nevertheless, these cut-offs implemented in routine testing at
379 the Geneva University Hospitals (GE-cut-offs) proved useful in the management of a number of
380 clinically compatible COVID-19 patients with negative PCR results.
381 Importantly, this study is a diagnostic accuracy validation study and not a seroprevalence study.
382 This implies that the current seropositivity cut-off has to be considered with caution in population
383 studies. Indeed, the 100% PPV achieved in this study was due to combined effect of a 100%
384 specificity and a 1:1 distribution of control and samples form patients with positive SARS-CoV-2
385 PCR. Therefore, in population seroprevalence studies with a lower expected proportion of
386 COVID-19, the PPV at the 1.5 cut-off will likely decrease. In this context, increasing the rule-in
387 IgG cut-off to a higher value or using a secondary specific confirmatory assay may become
388 necessary. Due to this limitation, at this stage we recommend the confirmation of all positive
389 ELISA results (including ratios above 1.5) using a second serological assay such as rIFA for
390 seroprevalence studies with a low pre-test probability, and the confirmation of doubtful ELISA
391 results in settings with a pre-test probability around 50%. A summary of our proposed testing
392 strategy is given in figure 2.
393
394 In conclusion, in this validation study performed on 357 sera, of which 50.7% came from
395 patients with COVID-19, we demonstrate a close to optimal diagnostic accuracy of IgG SARS-
396 CoV-2 serology of the Euroimmun assay, without any obvious gains from IgA serology. Taking
397 analytical imprecision into account, we propose optimized cut-offs allowing a PPV of 100% and
398 a NPV of 97% to be secured with an indeterminate zone comprising about 12% of the results,
399 for which additional rIFA analyses are currently necessary. Ongoing seroprevalence studies will
400 be instrumental to further refine the optimal rule-in and rule-out cut-offs, as well as the optimal
401 testing strategy, which may require a highly specific confirmatory assay. For the time being, this
402 assay seems to be fit for the purpose of enabling authorities to make informed decisions
403 regarding measures to be taken in order to manage the SARS-CoV-2 pandemic. Using the GE

cut-offs could also allow the determination of whether individuals have been exposed or not to SARS-CoV-2 with high confidence. As a final caution, at this time simple IgG levels determined by ELISA or rIFA cannot be considered as a surrogate of protection for individual patients. This implies that risk mitigation decisions may not yet be based only on SARS-CoV-2 ELISA/rIFA serology.

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486 **Figure legends:**

487 **Figure 1: A** and **B**: OD ratio of negative control samples (n=176) and PCR-confirmed CoVID-19
488 patients (n=181) were determined using an IgG and IgA ELISA. **C** and **D**: ROC curve analysis
489 for IgG and IgA OD ratio results including the area under the curve (AUC) and 95% confidence
490 intervals. **E** IgG OD ratios of COVID-19 patients at different days post onset of symptoms (dpos,
491 dots) or days post diagnosis (dpd, triangles). Red dots or triangles show samples that are
492 confirmed by whole spike recombinant immunofluorescence analysis. Dotted and dashed lines
493 indicate the Euroimmun- (EI) or Geneva- (GE) cut-offs for negative, indeterminate and positive
494 samples, respectively.

495

496 **Figure 2:** Graphical overview or serological testing strategies in low (<10%) and high (>50%)
497 pre-test probability settings. PPV: positive predictive value; NPV: negative predictive value;
498 rIFA: recombinant immunofluorescence assay; 95%CI: 95% confidence intervals.

499

500 **Tables**

Table 1

	Negative Controls			COVID-19 Patients		
	No (%)	Median (IQR)	Range	No (%)	Median (IQR)	Range
Age (years)						
All	176 (100)	39.2 (23.8)	18.0-89.5	181 (100)	53.1 (29.2)	20.1-86.9
18-40	100 (56.8)			61 (33.7)		
41-65	61 (34.7)			70 (38.7)		
66+	15 (8.5)			50 (27.6)		
Gender						
Female	97 (55.1)			70 (38.7)		
Male	79 (44.9)			111 (61.3)		
Hospitalized				91 (50.3)		
Outpatient				90 (49.7)		
DPOS/DPD						
0-10				8 (4.4)	7.0 (1.75)	0-8
11-20				112 (61.9)	15.0 (2.0)	11-20
21-39				61 (33.7)	28.0 (5.0)	21-39

DPOS days post onset of symptoms, DPD days post diagnosis, IQR inter quartile range

501

Table 2

Samples	Total No. (%)	S-rIFA IgG No. (%)			ELISA IgG No. (%) EI cut-offs[1]			ELISA IgG No. (%) GE cut-offs[2]		
		Negative	Putative Neg.	Positive	Negative	Indeterminate	Positive	Negative	Indeterminate	Positive
Negative Controls	176 (100)	171 (97.2)	5 (2.8)	0 (0)	171 (97.2)	5 (2.8)	0 (0)	156	20 (11.4)	0 (0)
COVID-19 Patient										
All	181 (100)	14 (7.7)	2 (1.1)	165 (91.2)	26 (14.4)	1 (0.6)	154 (85.1)	15 (8.3)	23 (12.7)	143 (79.0)
0-10 DPOS / DPD	8 (4.4)	6 (75.0)	1 (12.5)	1 (12.5)	8 (100)	0 (0)	0 (0)	7 (87.5)	1 (12.5)	0 (0)
11-20 DPOS / DPD	112 (61.9)	8 (7.1)	1 (0.9)	103 (92.0)	17 (15.2)	0 (0)	95 (84.8)	7 (6.3)	19 (17.0)	86 (76.8)
21-39 DPOS / DPD	61 (33.7)	0 (0)	0 (0)	61 (100)	1 (1.6)	1 (1.6)	59 (96.7)	1 (1.6)	3 (4.9)	57 (93.4)
COVID-19 Outpatients										
All	90 (100)	8 (8.9)	0 (0)	82 (91.1)	14 (15.6)	0 (0)	76 (84.4)	7 (7.8)	14 (15.6)	69 (76.6)
0-10 DPOS / DPD	5 (5.6)	4 (80)	0 (0)	1 (20)	5 (100)	0 (0)	0 (0)	4 (80.0)	1 (20.0)	0 (0)
11-20 DPOS / DPD	41 (45.6)	4 (9.8)	0 (0)	37 (90.2)	8 (19.5)	0 (0)	33 (80.5)	2 (4.9)	11 (26.8)	28 (68.3)
21-39 DPOS / DPD	44 (48.8)	0 (0)	0 (0)	44 (100)	1 (2.3)	0 (0)	43 (97.7)	1 (2.3)	2 (4.6)	41 (93.2)
COVID-19 Hospitalized										
All	91 (100)	6 (6.6)	2 (2.2)	83 (91.2)	12 (13.2)	1 (1.1)	78 (85.7)	8 (8.8)	9 (9.9)	74 (81.3)
0-10 DPOS / DPD	3 (3.3)	2 (66.7)	1 (33.3)	0 (0)	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)
11-20 DPOS / DPD	71 (78.0)	4 (5.6)	1 (1.4)	66 (93.0)	9 (12.7)	0 (0)	62 (87.3)	5 (7.0)	8 (11.3)	58 (81.7)
21-39 DPOS / DPD	17 (18.7)	0 (0)	0 (0)	17 (100)	0 (0)	1 (5.9)	16 (94.1)	0 (0)	1 (5.9)	16 (94.1)

DPOS days post onset of symptoms, DPD days post diagnosis, rIFA recombinant immunofluorescence assay

1: EI cut-offs: <0.8 = negative; ≥0.8 and <1.1 = indeterminate; ≥1.1 = positive

2: GE cut-offs: <0.5 = negative; ≥0.5 and <1.5 = indeterminate; ≥1.5 = positive

Table 3

Sample Code	DPOS/ DPD	Type	Result			Reciprocal Endpoint Titre	
			ELISA IgG	S-rIFA IgG	ELISA IgA	Pseudo-NT 90	Pseudo-NT 50
8	NA	Neg. Ctrl.	0.79	NEG	1.29	<10	<10
9	NA	Neg. Ctrl.	0.31	NEG	0.56	<10	<10
10	NA	Neg. Ctrl.	0.57	NEG	0.79	<10	<10
11	NA	Neg. Ctrl.	0.97	NEG	2.72	<10	<10
56	NA	Neg. Ctrl.	0.81	Putative NEG	0.47	<10	<10
209	8	COVID-19 Pat.	0.73	POS	3.66	20	≥160
30181704	22	COVID-19 Pat.	1.08	POS	0.77	20	≥160
30189617	16	COVID-19 Pat.	0.60	POS	9.99	20	80
30186069	14	COVID-19 Pat.	0.55	POS	2.94	20	80
30193397	11	COVID-19 Pat.	0.56	POS	4.21	10	80
30193613	11	COVID-19 Pat.	0.51	POS	0.92	80	≥160
30193717	11	COVID-19 Pat.	0.41	POS	1.02	10	40
30197451	14	COVID-19 Pat.	0.57	POS	3.85	≥160	≥160
30197527	16	COVID-19 Pat.	0.46	POS	0.67	<10	20
30202986	18	COVID-19 Pat.	0.58	Putative NEG	1.53	<10	<10
30208864	14	COVID-19 Pat.	0.47	POS	0.33	20	80
30208885	7	COVID-19 Pat.	0.39	Putative NEG	0.48	<10	<10
30251875	27	COVID-19 Pat.	0.36	POS	0.51	10	80
202	16	COVID-19 Pat.	6.02	POS	10.70	160	≥160
205	11	COVID-19 Pat.	13.08	POS	10.93	≥160	≥160
30175134	15	COVID-19 Pat.	14.64	POS	10.41	≥160	≥160
30175147	15	COVID-19 Pat.	13.90	POS	10.41	≥160	≥160
30175152	18	COVID-19 Pat.	13.67	POS	10.41	≥160	≥160
30175157	15	COVID-19 Pat.	9.74	POS	9.65	≥160	≥160
30193442	11	COVID-19 Pat.	1.40	POS	6.48	20	80

DPOS days post onset of symptoms, DPD days post diagnosis, rIFA recombinant immunofluorescence assay,
NA not applicable, Pseudo-NT 90 and 50: pseudovirus neutralization test using 90% or 50% reduction as cut-off

502

Table 4

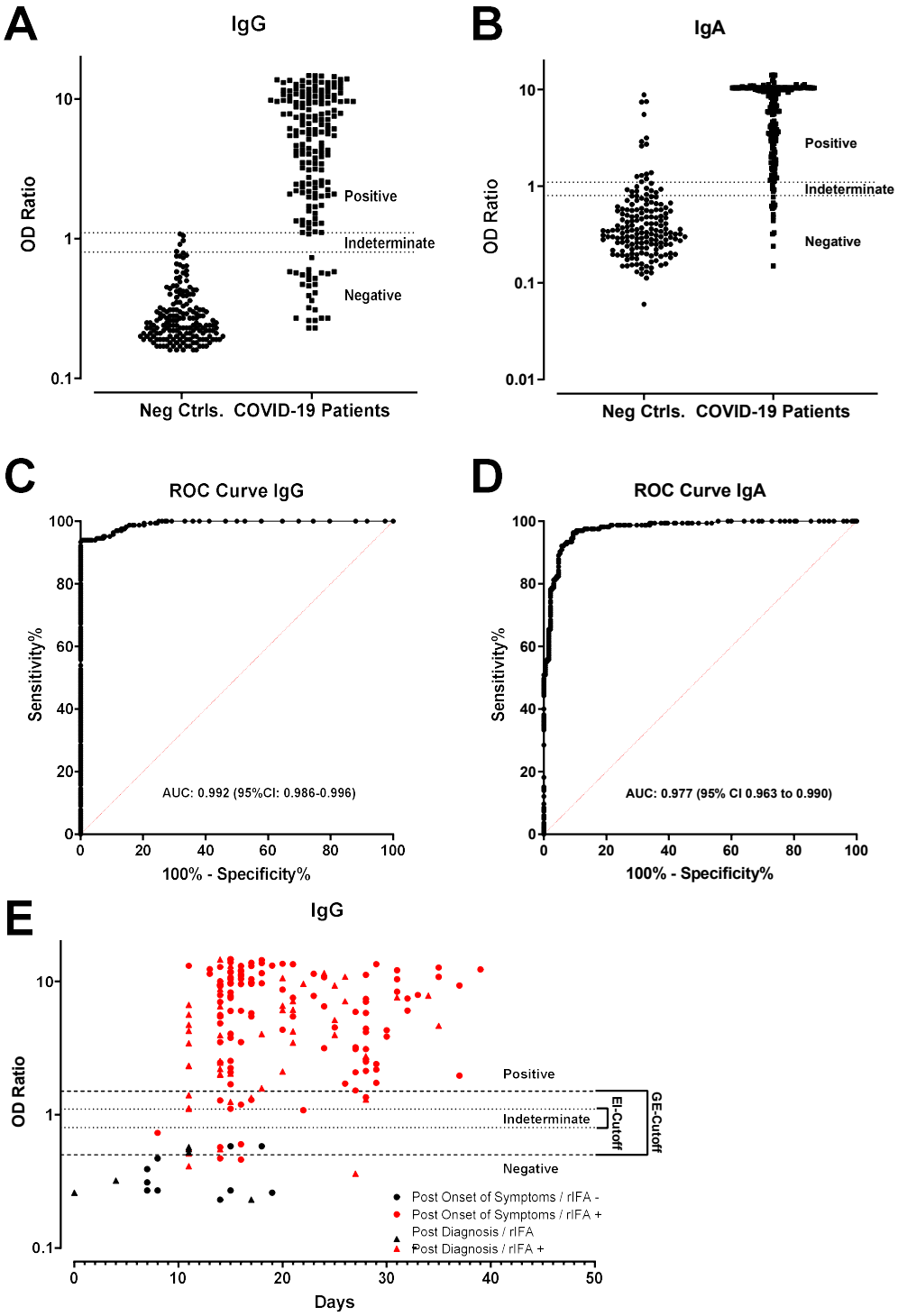
	Cut-Offs	*Sensitivity (95%)*	*Specificity (95%)*	*Positive predictive value (95%)*	*Negative predictive value (95%)*
All samples (n=357) **IgG ratio** **AUC: 0.992** **(95%CI: 0.986-0.996)**	GE cut-offs[1]				
	>1.5 (rule-in)	86% (80-91)	100% (98-100)	100% (97-100)	89% (84-93)
	<0.5 (rule-out)	97% (93-99)	87% (81-91)	86% (81-90)	97% (93-99)
	EI cut-offs[2]				
	>1.1 (rule in)	93% (87-96)	100% (98-100)	100% (97-100)	94% (90-97)
	<0.8 (rule out)	94% (88-94)	98% (94-99)	97% (92-99)	95% (91-97)
<21DPOS/DPD(n=120) **IgG ratio** **AUC: 0.981** **(95%CI: 0.961-1.00)**	GE cut-offs[1]				
	>1.5 (rule-in)	82% (73-88)	100% (76-100)	100% (95-100)	46% (29-63)
	<0.5 (rule-out)	96% (90-99)	69% (41-88)	95% (89-98)	73% (45-91)
	EI cut-offs[2]				
	>1.1 (rule in)	91% (83-95)	100% (76-100)	100% (95-100)	62% (41-79)
	<0.8 (rule out)	91% (84-96)	100% (76-100)	100% (95-100)	64% (43-81)

DPOS days post onset of symptoms, DPD days post diagnosis, AUC area under the curve
1: GE cut-offs: <0.5 = negative; ≥0.5 and <1.5 = indeterminate; ≥1.5 = positive
2: EI cut-offs: <0.8 = negative; ≥0.8 and <1.1 = indeterminate; ≥1.1 = positive

503

504 **Figure 1**

505



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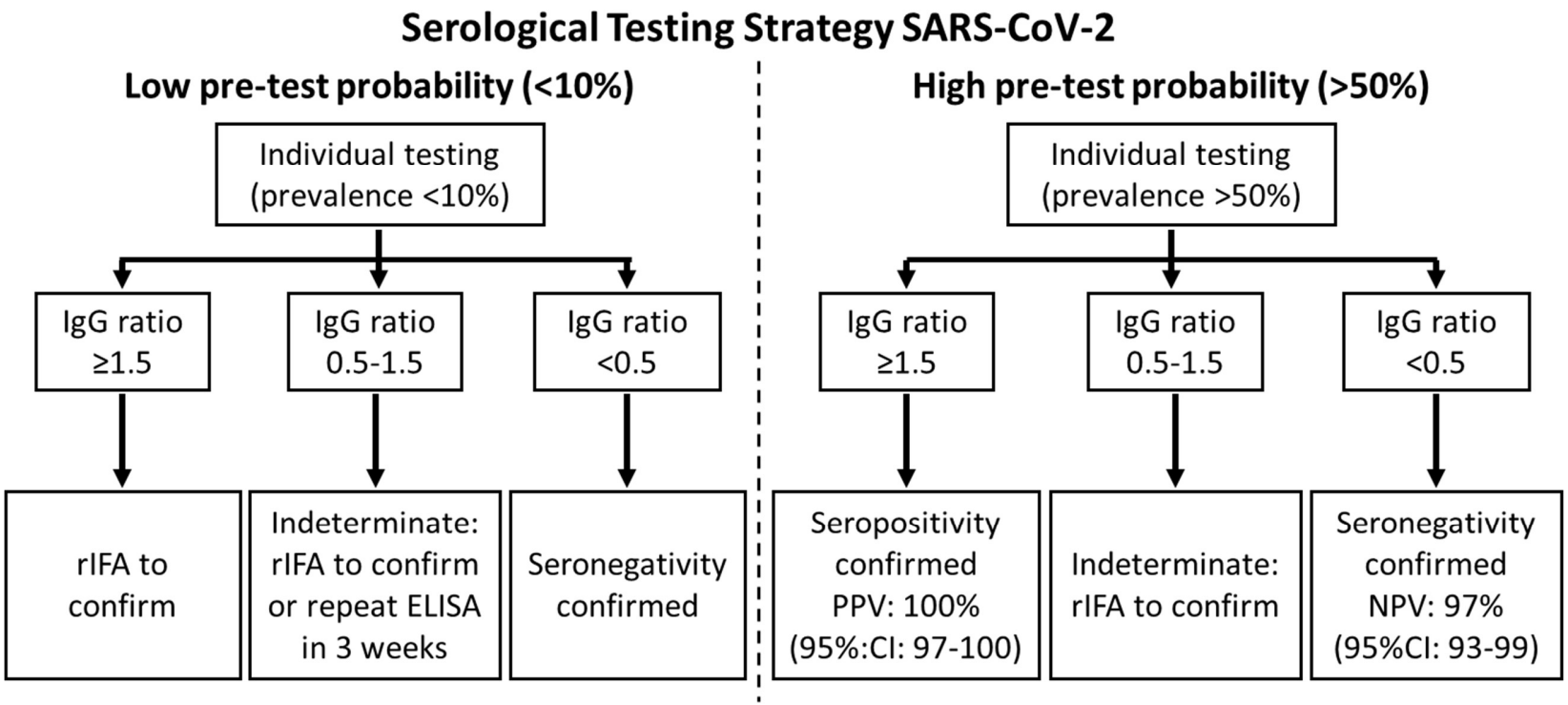
508 **Figure 2**



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