Highly sensitive and specific multiplex antibody assays to quantify immunoglobulins M, A and G against SARS-CoV-2 antigens

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ABSTRACT

Reliable serological tests are required to determine the prevalence of antibodies against SARS-CoV-2 antigens and to characterise immunity to the disease in order to address key knowledge gaps in the context of the COVID-19 pandemic. **Quantitative suspension array technology (qSAT)** assays based on the **xMAP Luminex** platform overcome the limitations of rapid diagnostic tests and ELISA with their higher precision, dynamic range, throughput, miniaturization, cost-efficacy and multiplexing capacity. We developed three qSAT assays to detect **IgM, IgA and IgG** to a panel of **eight SARS-CoV-2 antigens** including spike (S), nucleoprotein (N) and membrane (M) protein constructs. The assays were optimized to minimize processing time and maximize signal to noise ratio. We evaluated the performance of the assays using 128 plasmas obtained before the COVID-19 pandemic (negative controls) and 115 plasmas from individuals with SARS-CoV-2 diagnosis (positive controls), of whom 8 were asymptomatic, 58 had mild symptoms and 49 were hospitalized. Pre-existing IgG antibodies recognizing N, M and S2 proteins were detected in negative controls suggestive of cross-reactive to common cold coronaviruses. The best performing antibody isotype/antigen signatures had specificities of 100% and sensitivities of 94.94% at ≥14 days since the onset of symptoms and 96.08% at ≥21 days since the onset of symptoms, with AUC of 0.992 and 0.999, respectively. Combining multiple antibody markers as assessed by qSAT assays has the highest efficiency, breadth and versatility to accurately detect low-level antibody responses for obtaining reliable data on prevalence of exposure to novel pathogens in a population. Our assays will allow gaining insights into antibody correlates of immunity required for vaccine development to combat pandemics like the COVID-19.

STUDY SAMPLES & ANTIGENS

Positive samples were **115** plasmas from individuals with a confirmed past/current diagnosis of COVID-19. One hundred and eleven had SARS-CoV-2 infection confirmed by real time reverse-transcriptase polymerase chain reaction (rRT-PCR). Fifty-five were recruited in a study of health care workers in Hospital Clínic in Barcelona, most of them with mild symptoms, 1 of them hospitalized and 6 without symptoms, all rRT-PCR positive. Fifty-seven were COVID-19 patients recruited at the Clínica Universidad de Navarra in Pamplona (Spain), of which 48 had severe symptoms and were hospitalized and 9 had mild symptoms (one clinically diagnosed with positive radiology and serology, and negative rRT-PCR); 3 were asymptomatic health workers with positive diagnosis confirmed by four serological tests but no rRT-PCR data.

Negative controls were plasmas from **128** healthy European donors collected before the COVID-19 pandemic, and were used individually.

Antigens were **RBD** (Krammer Lab), **S** (CHO expression), **S1** (GenScript), S2 (Sino Biologicals), **M** (*E. coli* ISGlobal), **N** (*E. coli* ISGlobal), **N C-terminal** & **N-terminal** (*E. coli* CRG).

Table 1. Characteristics of individuals from whom positive samples were tested

Continuous variable		Median (IQR)
Age		49.30 (26.24)
Categorical variables	**Category**	**N (%)**
Sex	Female	71 (61.74)
	Male	44 (38.26)
Symptoms	No	8 (6.96)
	Yes	107 (93.04)
Hospitalized patients	Yes	49 (42.61)
Days since onset of symptoms	0-6	13 (12.26)
	7-13	14 (13.21)
	14-20	28 (26.42)
	21-27	24 (22.64)
	≥28	27 (25.47)
Days since first positive rRT-PCR	0-6	22 (19.13)
	7-13	25 (21.74)
	14-20	33 (28.70)
	≥21	18 (15.64)

RESULTS

Figure 1. Levels (median fluorescence intensity, MFI) of IgM, IgA and IgG antibodies to RBD antigen of SARS-CoV-2 in singleplex using samples from positive and negative individuals at different dilutions after overnight incubation at 4ºC.

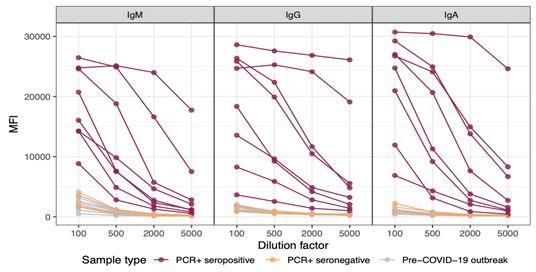

Figure 2. Levels of plasma IgM, IgA and IgG antibodies to the SARS-CoV-2 antigens S and RBD at different dilutions. Comparison of antibody levels (MFI) in **singleplex** (orange) versus **multiplex** (burgundy); the first 10 samples from left to right are from individuals who were positive by rRT-PCR at different time periods since diagnosis, and the last two samples on the right are from individuals pre-COVID-19 pandemia**.**

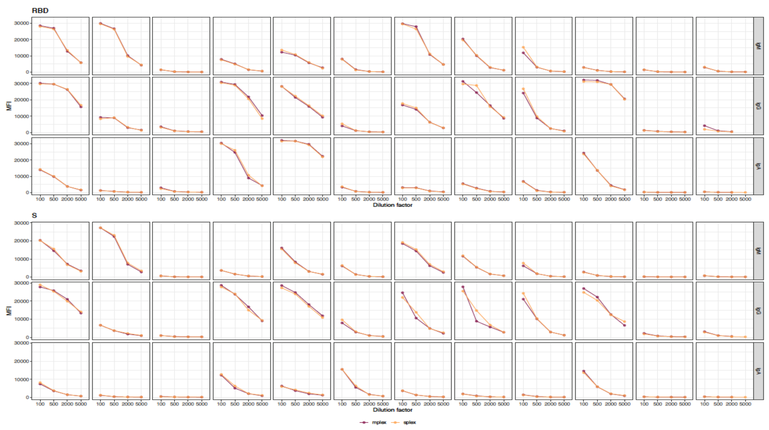

Figure 3. Correlation of IgG and IgM antibody levels against RBD versus S at different dilutions showing the benefit of including multiple antigens in the panel to maximize the detection of seropositives**.** Cutoff values are indicated by dashed lines. Spearman test was used to assess the correlations.

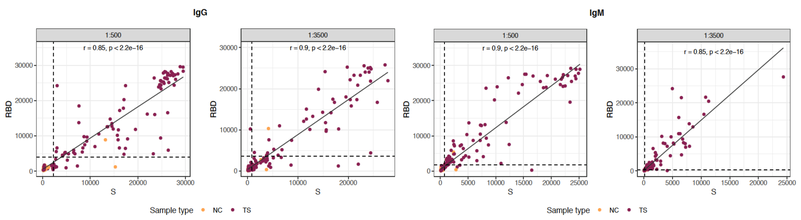

Figure 4. Correlations between antibody levels measured using secondary antibodies conjugated to biotin and SAPE versus PE, for 1 h and 2 h sample incubations. The blue fitting curve was calculated using the LOESS (locally estimated scatterplot smoothing) method and the black line by linear regression. Spearman test was used to assess the correlations.

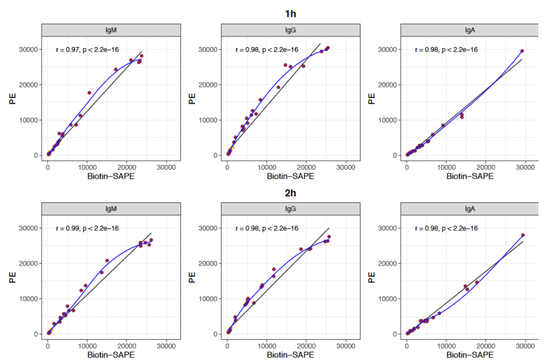

Figure 5. Antibody Luminex assays performance. Receiver operating characteristic (ROC) curve and area under the curve (AUC) using samples from pre-pandemic negative controls plus either all participants with positive COVID-19 diagnosis or participants with positive diagnosis at different times since onset of symptoms, comparing combinations of multiple immunoglobulin isotypes to different antigens with top performances **(A)** versus those of single isotype/antibody markers **(B).**

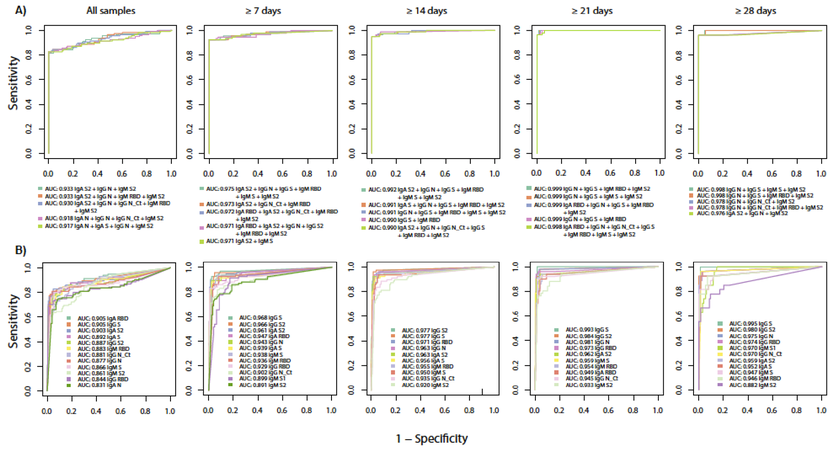

Table 2. Performance of the assays at different thresholds targeting specificities of 100%, 99% and 98%. The top 5 performing signatures are shown.

≥14 days since onset symptoms	AUC	Specificity	Sensitivity
IgA S2 + IgG N + IgG S + IgM RBD + IgM S + IgM S2	0.992	100%	94.94%
IgA S + IgG N + IgG S + IgM RBD + IgM S2	0.991	100%	94.94%
IgG N + IgG S + IgM RBD + IgM S + IgM S2	0.991	100%	94.94%
IgG S + IgM RBD	0.990	100%	94.94%
IgA S2 + IgG N + IgG N Ct + IgG S + IgM RBD + IgM S2	0.990	100%	94.94%
IgG RBD + IgG S	0.984	99.22%	96.20%
IgG N + IgG S2 + IgM S	0.979	99.22%	96.20%
IgG N + IgG S + IgG S2	0.979	99.22%	96.20%
IgG N + IgG N Ct + IgG S + IgG S2	0.978	99.22%	96.20%
IgG N + IgG S	0.978	99.22%	96.20%
IgG N + IgG N Ct + IgG RBD + IgG S + IgG S2 + IgM S	0.990	98.44%	97.47%
IgG N + IgG RBD + IgG S + IgM S	0.989	98.44%	97.47%
IgA S + IgG N + IgG S + IgG S2 + IgM S	0.988	98.44%	97.47%
IgG N + IgG S + IgG S2 + IgM S	0.988	98.44%	97.47%
IgG N + IgG N Ct + IgG S + IgG S2 + IgM S	0.988	98.44%	97.47%

≥21days since onset symptoms	AUC	Specificity	Sensitivity
IgG N + IgG S + IgM RBD + IgM S2	0.999	100%	96.08%
IgG N + IgG S + IgM S + IgM S2	0.999	100%	96.08%
IgA RBD + IgG N + IgG S + IgM RBD + IgM S2	0.999	100%	96.08%
IgG N + IgG S + IgM RBD	0.999	100%	96.08%
IgA RBD + IgG N + IgG N Ct + IgG S + IgM RBD + IgM S + IgM S2	0.998	100%	96.08%
IgG N + IgG S2 + IgM S	0.988	99.22%	98.04%
IgG N + IgG S2	0.984	99.22%	98.04%
IgG N + IgG S + IgM RBD + IgM S2	0.999	99.22%	96.08%
IgG N + IgG S + IgM RBD + IgM S	0.999	99.22%	96.08%
IgG N + IgG S + IgM S + IgM S2	0.999	99.22%	96.08%
IgG N + IgG S + IgM RBD + IgM S2	0.999	98.44%	100%
IgG S + IgG S2 + IgM RBD + IgM S	0.999	98.44%	100%
IgA RBD + IgG RBD + IgG S + IgM S + IgM S2	0.999	98.44%	100%
IgG N + IgG S + IgG S2 + IgM RBD + IgM S2	0.999	98.44%	100%
IgG N + IgG S + IgG S2 + IgM RBD + IgM S + IgM S2	0.999	98.44%	100%

CONCLUSIONS

We developed three novel multiplex immunoassays for quantifying IgM, IgA and IgG to eight SARS-CoV-2 protein constructs and evaluated by machine learning Random Forest classification algorithms the performance of several isotype/antigen combinations to detect any positive antibody response to infection, obtaining specificities of 100% and sensitivities of 94.94% (≥14 days since symptoms onset) or 96.08% (≥21 days since symptoms onset), and very high predictability (AUC ≥0.99). We substantially increased the sensitivity of the assay when combining isotypes/antigens compared to using only one isotype/antigen. Our qSAT assays, based on the xMAP technology, provide the best precision, accuracy and widest range of detection compared to classical qualitative (RDT) or quantitative (ELISA) assays, suited to detect low-level antibody responses expected in asymptomatic children, immunosuppressed individuals, or long-term decaying antibodies. The assays performed equally well in multiplex format, with no interference noted between antigens, even if they had overlapping epitopes. An added advantage of multiplexing is the reduced usage of sample volume, resources and time.

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