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

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Production of recombinant proteins in *Escherichia coli* expression systems

Escherichia coli codon optimized versions of full-length N and M antigens were cloned at ISGlobal into a pET22b expression vector, fusing an in-frame C-terminal 6xHis-tag. Recombinant N and M proteins were expressed in *E. coli* BL21 DE3 by pET22b-N and pET22b-M transformation and induction with 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG) when OD_{600} reached 0.6-0.8, followed by 5 h incubation at 37ºC or 25ºC, respectively. Bacterial pellets were resuspended in binding buffer containing 20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, 0.2 mg/mL Lysozyme, 20 µg/mL DNAse, 1 mM PMSF and 1 mM $MgCl_2$, and lysed by sonication. Lysates were centrifuged at 14,000 rpm and proteins purified by affinity chromatography using a Ni^{2+} column (1 mL GE Healthcare HisTrap HP) and imidazole gradient elution in an AKTA Start protein purification system. M and N proteins were concentrated and buffer changed to phosphate buffered saline (PBS) using Microcon-10 KDa centrifugal filter units (Millipore). For N-terminal (residues from 43 to 180) and C-terminal fragments (residues from 250 to 360) of N, two constructs were designed at CRG depending on secondary structure predictions. The encoding sequences were synthesized and inserted into a plasmid pETM14 with the N-terminal 6xHis-tag under the control of a T7 promoter, and recombinant plasmids transformed into *E. coli* BL21 DE3 competent cells. Briefly, *E. coli* containing the plasmid was grown and the protein expression was induced by addition of IPTG 0.2 mM for 16 h at 18°C. Pelleted cells were resuspended in Buffer A (Tris 20 mM, 250mM NaCl, 10mM Imidazole) supplemented with 0.5% Triton-X100 Substitute (Sigma) and complete mini EDTA-free protease (Roche), sonicated, and centrifuged (30 min, 4°C, 30000 g). The N-terminal, and the C-terminal recombinant proteins containing a N-terminal 6xHis-tag were purified from the resulting supernatant using Hitrap Ni-NTA column (GE Healthcare, Uppsala, Sweden) according to the manufacturer instructions. After washing with Buffer A, the antigen was eluted using linear gradient with buffer B (Buffer A supplemented with 500 mM Imidazol). The fractions of interest were dialyzed against PBS 1x

and concentrated by Vivaspin 5 KD (Millipore, France). The antigens produced were quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce) and their purity controlled by Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE).

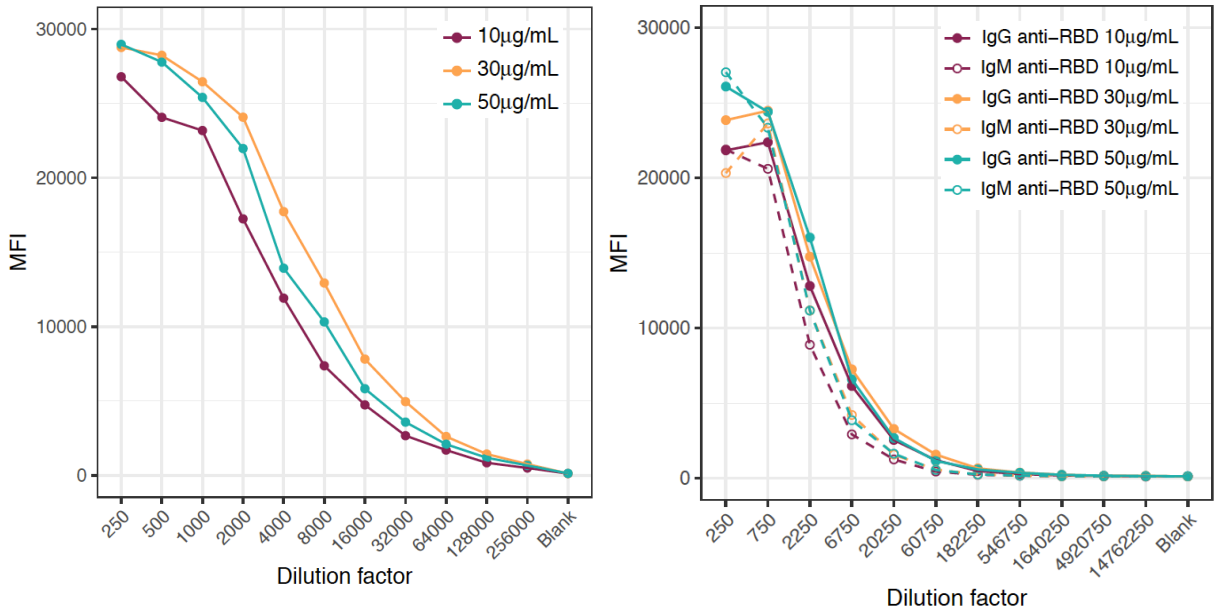
Antigen coupling to microspheres

Magnetic MAGPLEX 6.5 µm COOH-microspheres from Luminex Corporation (Austin, TX) were washed twice with 62.5 µl of distilled water using a magnetic separator (Life Technologies, 12321d), and resuspended in 80 µl of activation buffer, 100 mM monobasic sodium phosphate (Sigma, S2554), pH 6.2. To activate the beads for cross-linking to proteins, 10 µl of 50 mg/mL sulfo-N-hydroxysulfo-succinimide (Thermo Fisher Scientific, 24525) and 50 µL of 50 mg/mL 1-ethyl-3-[3-dimethyl-aminopropyl]-carbodiimidehydrochloride (Thermo Fisher Scientific, 22981) were simultaneously added to the reaction tubes, mixed and incubated at room temperature (RT) for 20 min in a rotatory shaker and protected from light. Next, beads were washed twice with 62.5 µl 50 mM morpholineethane sulfonic acid (MES) (Sigma, M1317) pH 5.0, in a 10,000 beads/ µl concentration. After beads activation, antigen were added to the reaction tubes at three different concentrations and left at 4ºC overnight (ON) on a rotatory shaker protected from light. On the following day, coupled-beads were brought to RT for 20 min in agitation, and blocked by incubating them with 62.5 µl PBS (Sigma) + 1% bovine serum albumin (BSA, Biowest) + 0.05% sodium azide (Sigma, S8032) (PBS-BN) in agitation during 30 min at RT and protected from light. Beads were washed twice with PBS-BN using the magnetic separator. To determine the percentage recovery of beads after the coupling procedure, coupled beads were resuspended in 62.5 µL PBS-BN and counted on a Guava PCA desktop cytometer (Guava Technologies, Automated cell counter, PC550IG-C4C/0746-2747). In all washing and resuspension steps, beads were softly vortexed and sonicated for 30 sec.

SUPPLEMENTARY FIGURES

Figure S1. Selection of the optimal RBD antigen coupling concentration. **A)** Comparison of titration curves with an anti-histidine tag biotinylated antibody at different protein concentrations (left panel) and with a positive plasma pool for IgG and IgM (right panel). **B)** Comparison of IgG and IgM titration curves with a positive plasma pool against 5 different batches of 30 μg/mL RBD-coupling reactions (A-E) for IgM **(C)** and IgG **(D)** showing highly reproducible titration curves.

A)



B) **IgM** **IgG**

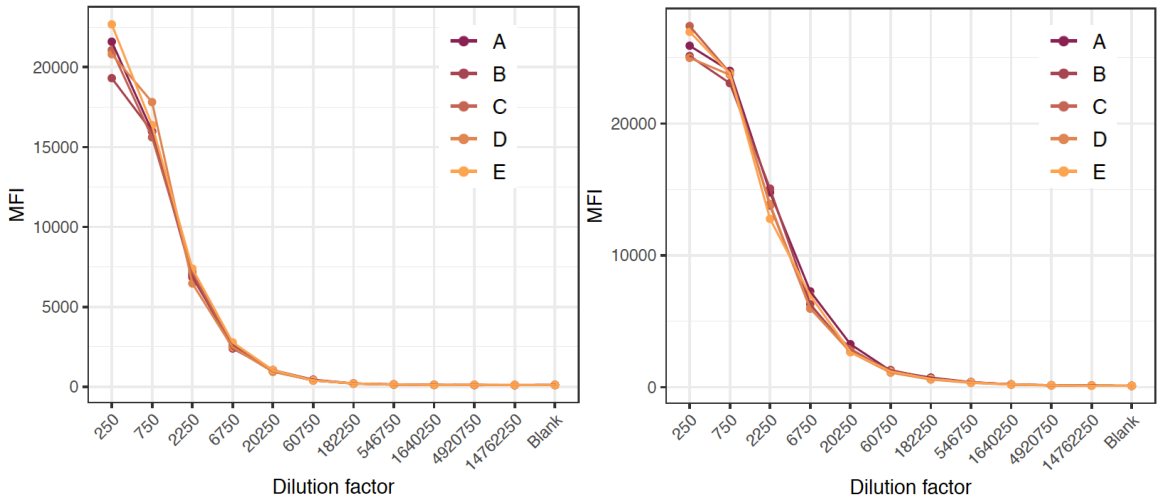
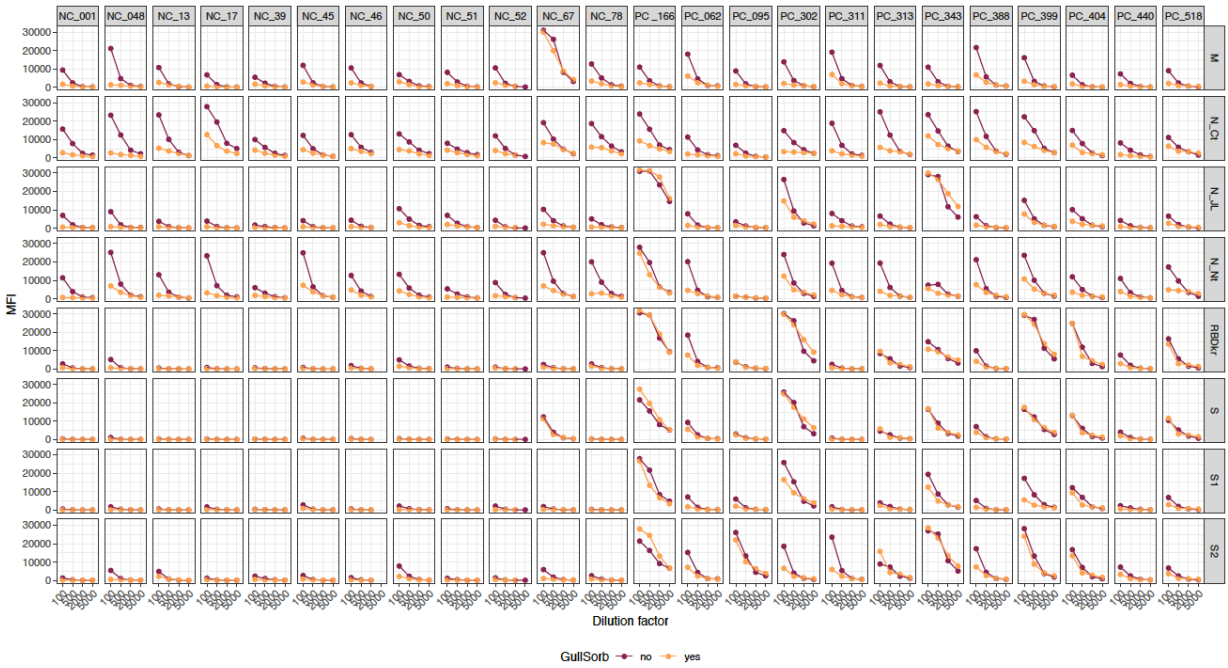


Figure S2. Effect of GullSORB™ treatment on IgM antibody levels to negative (NC) and positive (PC) control samples in a multiplex antigen panel at different plasma dilutions. GullSORB™ reduced or did not change the MFI signal, depending on the sample, antigen and dilution **(A**). This additional incubation generally increased the signal to noise ratio and thus sensitivity and number of seropositive IgM responses among the PC, particularly at the lower dilutions, therefore the GullSORB™ incubation was adopted for this assay **(B)**. IgM reactivity in NC was lower against S-based antigens than against M- or N-based antigens and thus GullSORB™ treatment benefited the most in these later proteins.

A)



B)

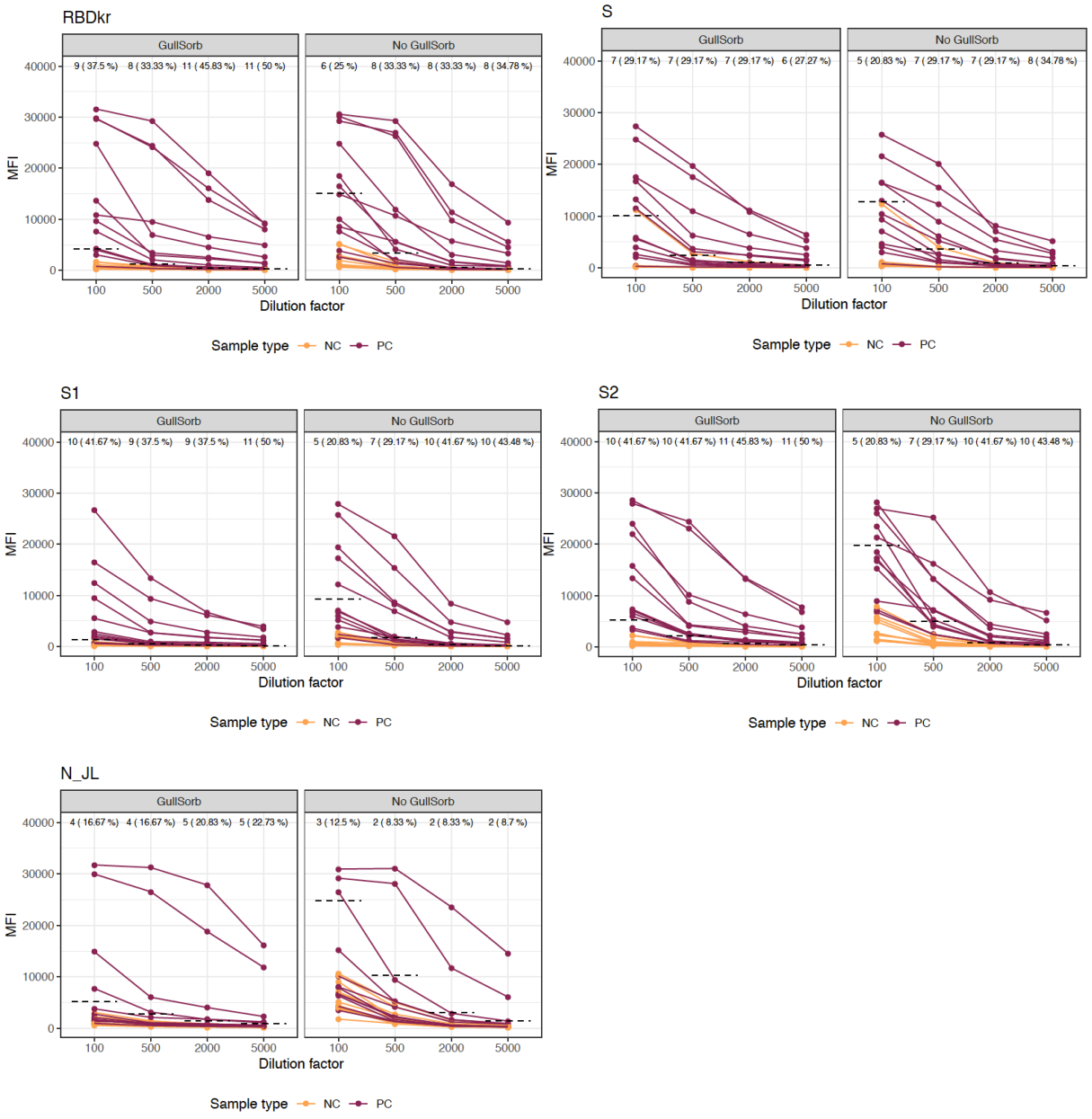

RBDkr
GullSorb
No GullSorb
9 (37.5 %) 8 (33.33 %) 11 (45.83 %) 11 (50 %)
6 (25 %) 8 (33.33 %) 8 (33.33 %) 8 (34.78 %)
MFI
Dilution factor
Sample type NC PC
S
7 (29.17 %) 7 (29.17 %) 7 (29.17 %) 6 (27.27 %)
5 (20.83 %) 7 (29.17 %) 7 (29.17 %) 8 (34.78 %)
S1
10 (41.67 %) 9 (37.5 %) 9 (37.5 %) 11 (50 %)
5 (20.83 %) 7 (29.17 %) 10 (41.67 %) 10 (43.48 %)
S2
10 (41.67 %) 10 (41.67 %) 11 (45.83 %) 11 (50 %)
5 (20.83 %) 7 (29.17 %) 10 (41.67 %) 10 (43.48 %)
N_JL
4 (16.67 %) 4 (16.67 %) 5 (20.83 %) 5 (22.73 %)
3 (12.5 %) 2 (8.33 %) 2 (8.33 %) 2 (8.7 %)

Figure S3. Levels of IgM, IgA and IgG antibodies (median fluorescence intensity, MFI) to RBD in positive (TS) and negative samples (NC), and % seropositivity among TS, comparing 45 min versus 30 min incubation times of the secondary antibody conjugated to PE after 1 hour incubation of plasma samples with antigen beads.

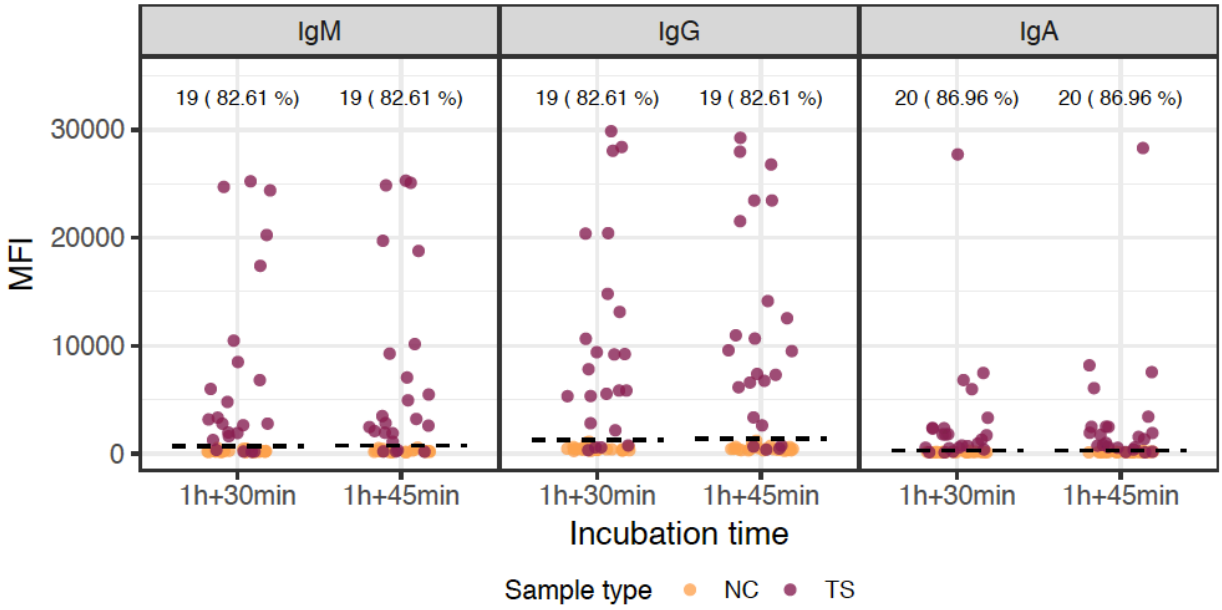


Figure S4. Ranking of isotype/antigen markers by Random Forest models for all negative controls plus either all positive samples or positive samples at different periods since onset of symptoms.