🌐 Quantitative Estimation of IgM and IgG Antibodies Against SARS-CoV-2

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MATERIALS TEXT

ELISA procedure:

Clear Flat-Bottom Immuno Non-sterile 96-Well Plates (3855, Thermo Fisher Scientific, USA)
96 Well Polypropylene Storage Microplates (AB0796, Thermo Fisher Scientific, USA)
Sealing plate for 96-well plates (15036, Thermo Fisher Scientific, USA)
Non-fat dry milk powder (1706404, Bio-Rad, USA)
1x phosphate-buffered saline (PBS; 10010-023, Gibco, USA)

Polysorbate™ 20 (BP337-500, Fisher Scientific, USA)
10X PBS (46-013-CM, Corning, USA)
Deionized water
IgM - CR3022 (Ab01680-15.0, Absolute Antibody, USA)
IgG1- CR3022 (Ab01680-10.0, Absolute Antibody, USA)
Anti-human IgM (Fc5µ)-peroxidase antibody produced in rabbit (SAB3701404, Sigma-Aldrich, USA)
Anti-human IgG (Fc specific) -peroxidase antibody produced in goat (A0170, Sigma-Aldrich, USA)
Phospho-citrate buffer (P4809, Sigma-Aldrich, USA)
3,3',5,5'-Tetramethylbenzidine dihydrochloride (T3405, Sigma-Aldrich, USA)
Hydrogen peroxide (H1009, Sigma-Aldrich, USA)
3M hydrochloric acid (S25856, Fisher Scientific, USA)
1,4-Dithiothreitol (10708984001, Millipore Sigma, USA)
15 mL centrifuge tubes (339651, Thermo Fisher Scientific, USA)
50 mL centrifuge tubes (339653, Thermo Fisher Scientific, USA)
1.5 mL microcentrifuge tubes (T6649, Millipore Sigma, USA)
2.0 mL cryogenic vials (V5007, Millipore Sigma, USA)
Micropipette tips (0.5-10 µL: 76322-132, VWR; 20-200 µL: 76322-150, VWR; 100-1000 µL: 76322-154)
Sterile reservoirs (07-200-127, Fisher Scientific, USA)
ELISA plate washer (Biotek 50 TS or equivalent)
ELISA plate reader (Biotek ELx808 or equivalent, compatible to record absorbance @ 450 nm)
Micropipettes (single channel and multi-channel)
Class II biological safety cabinet
Incubator (37°C)
Ultra-Low Freezer (-80°C)
Refrigerator at 2-8°C
Vortex mixer
Sterile 0.5-2.0 L glass bottles
Timer

<u>Production and Purification of SARS-CoV-2 Spike RBD:</u>

Transfections of plasmid pSL1510 (pCAGGS-RBD from Florian Krammer, Mount Sinai, USA) (prepared using the Qiagen HiSpeed Maxiprep Kit) were performed using the Expi293 Expression System from ThermoFisher (Catalog # 14524, Expi293F cells, Expi293 Media, and the ExpiFectamine 293 Transfection Kit). Cells were cultured according to manufacturer's instructions, (37°C, 8% CO_2, in shaker flasks at 120-130 rpm). The cells were resuspended to 3E6/mL prior to transfection; ExpiFectamine 293 Transfection Enhancer 1 & 2 were added to the transfection culture ~20 h post transfection. The supernatant was harvested by simple centrifugation (273.5x g, 5 min, room temperature) on the third day for downstream processing. Cell viability and concentration were monitored throughout the transfection to ensure that the culture remained in log phase growth.

Culture supernatant was incubated with pre-equilibrated Ni-NTA (ThermoSci HisPur, VWR catalog #: PI88223) resin in 1X PBS (0.5 mL of equilibrated Ni-NTA for every 50 mL of supernatant) at 4°C for 1 h on a nutator. The resin was applied to a gravity column and washed four times with 10 column volumes of wash buffer (57 mM NaH2PO4, 30 mM NaCl, 20 mM Imidazole). Protein was eluted from the resin with 4 column volumes of elution buffer (57 mM NaH2PO4, 30 mM NaCl, 235 mM Imidazole). Eluted protein was dialyzed to completion in 1xPBS and snap frozen for storage at -80°C.

ELISA procedure

1 **Coating of ELISA plates**
   - Coat the ELISA plates with 50 µL (per well) of the receptor-binding domain (RBD) of SARS-CoV-2 spike antigen at a concentration of 2 µg/mL diluted in phosphate-buffered saline (PBS, pH 7.3±0.1). A summary of recombinant RBD antigen production protocol is included in the Materials section.
   - Seal the coated plates with adhesive plate sealers and stored at 5 ± 3°C. The coated plates can be stored at 5±3°C and can be used until 1 week post-coating for ELISA (Stadlbauer et al., 2020).

2 **Blocking**
   - Use freshly prepared 1x PBS with 0.05% Polysorbate™ 20 as wash buffer.
   - Use 5% non-fat dry milk powder in wash buffer as the blocking buffer. Vortex samples briefly to obtain a homogenous solution, and place in a 37°C incubator for 5-10 min.
   - Note that all wash steps are performed with 200 µL/well wash volume; if using an ELISA washer, include program to

aspirate well contents as the first step prior to dispensing of wash buffer to wells
- After overnight incubation of coated plates, wash plates three times with wash buffer.
- Next, add 200 μL per well of the freshly prepared blocking buffer.
- Seal plates with adhesive plate sealers and incubate at 37°C for 1 h.

3   Sample preparation

- Place serum/plasma samples at 56°C for 30 min in a dry/water bath to heat inactivate. The heat inactivated samples can be stored at 5±3°C until tested, or stored long-term at -80°C (samples can also be heat-inactivated earlier, stored appropriately, and then thawed on the day of the test).
- Prepare 3% non-fat milk in wash buffer as a dilution buffer.
- Dilute heat inactivated serum/plasma samples to 1:50 with dilution buffer in sterile U-bottom plates. Perform serial three-fold dilutions starting from 1:50 up to 1:4050.
- Prepare desired concentrations of commercially available monoclonal antibodies (typically 1 μg to 0.01 μg) in PBS. Use 50 μL of each dilution as a positive control.

4   Sample addition to the ELISA plates

- After incubating with blocking buffer (step 2), wash the ELISA plates three times with wash buffer.
- Transfer 50 μL (per well) of the diluted samples and appropriate controls (positive controls: monoclonal antibodies; negative controls: dilution buffer) from the U-bottomed plate to the ELISA plate.
- Seal plates with adhesive plate sealers and incubate at 37°C for 1 h.

5   Addition of secondary antibody

- After incubation, wash ELISA plates three times with wash buffer.
- Add 50 μL (per well) of the following diluted secondary antibodies:
- **For IgM ELISA**: Peroxidase-conjugated anti-human IgM (Fc5μ) antibody, diluted 1:12,000 with 3% non-fat milk.
- **For IgG ELISA**: Peroxidase-conjugated anti-human IgG (Fc specific) antibody, diluted 1:10,000 with 3% non-fat milk.
- Seal plates with adhesive plate sealers and incubate at 37°C for 1 h.

6   Developing the reaction

- Dilute chromogenic visualization solution with 1 mg (tablet) of 3,3',5,5'-Tetramethylbenzidine (TMB) in 10 mL of phospho-citrate buffer with 3 μL of hydrogen peroxide prepared just before use.
- After incubation with secondary antibody (step 5), wash three times with wash buffer.
- Add 100 μL (per well) of the prepared TMB solution and incubate at room temperature (22±3°C) for 10 min in light-protected conditions.

7   Stopping the reaction and recording the results

- Stop the reaction by adding of 50 μL per well 3 M hydrochloric acid.
- Read ELISA plates at 450 nm wavelength and record optical densities (OD).

8   Interpreting the results

- The reciprocal of the highest dilution of the tested sample with the OD just above the cut-off is interpreted as the titer of the sample.

9   Determining cut-off OD

- Run an appropriate number (≥75) of negative (preferably pre-COVID-19) serum/plasma samples.
- Determine a suitable cut-off OD by calculating the value representing three- or six-times the standard deviation over the mean ODs of the negative samples.

Checking class specificity of the ELISAs

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- The isotype specificity of the ELISA methods can be tested by treating the serum/plasma samples with 1,4-Dithiothreitol (DTT).
- Prepare 1M DTT in 10X PBS.
- Dilute the samples to be tested to 1:50 in 1x PBS.
- Add DTT to the diluted sample such that the final concentration of DTT is 0.005 M.

- Mix well and incubate at 37°C for 30 min.
- Dilute the treated samples three-fold in 3% non-fat milk.
- Test the samples as described in the ELISA method above.

Expected result:
DTT inactivates IgM rapidly (compared to IgG), and therefore a significant drop in ODs and/or titers is expected in the IgM titers post-treatment. The IgGs do also get inactivated. However, the drop in ODs and/or titers is not as significant as with IgM.