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

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ELISA procedure:

Coating of ELISA plates

1. 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 (Fc5) 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 phosho-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
Checking class specificity of the ELISAs

1. The isotype specificity of the ELISA methods can be tested by treating the serum/plasma samples with 1,4-Dithiothreitol (DTT).
2. Prepare 1M DTT in 10X PBS.
3. Dilute the samples to be tested to 1:50 in 1x PBS.
4. Add DTT to the diluted sample such that the final concentration of DTT is 0.005 M.
5. Mix well and incubate at 37°C for 30 min.
6. Dilute the treated samples three-fold in 3% non-fat milk.
7. 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.