COVID-19 (SARS-CoV-2) IgG antibody test kit

Test Principle: The IONTOX SARS-CoV-2 Antibody Test is an indirect ELISA performed in a 96 well microplate. A microplate reader capable of measuring absorbance of 450 or 490 nm is required. The specimens tested are venous plasma or serum. The ELISA assay consists of antigenic proteins specific for SARS-CoV-2 which are transferred to protein binding 96 well microwell plates. During the test procedure, antibodies to SARS-CoV-2, if present in the human serum sample, will bind to the antigens coated onto the wells forming antigen-antibody complexes. Excess antibodies are removed by washing. Anti-human IgG antibodies conjugated with horseradish peroxidase are then added which bind to the antigen-antibody complexes. Excess conjugate is removed by washing. This is followed by the addition of a chromogenic substrate, tetramethylbenzidine (TMB). If specific antibodies to the antigen are present in the patients’ serum, a blue color will develop. The enzymatic reaction is stopped with an acidic solution causing the contents of the well to turn yellow. The wells are read photometrically with a microplate reader at 450nm.

This procedure was developed as a two-stage indirect Enzyme-Linked Immunosorbent Assay (ELISA) in which the first stage includes relatively high-throughput screening of samples in a single serum dilution against the SARS-Related Coronavirus 2, Wuhan-Hu-1 Spike Glycoprotein Receptor Binding Domain (RBD) or full length SARS-Related Coronavirus 2, Wuhan-Hu-1 Spike Glycoprotein (spike protein). This is followed by a second stage in which positive samples from the first stage are subjected to a confirmatory ELISA in which dilution curves are performed.

The procedure requires a plate reader and other listed laboratory equipment. A plate washer is optional but not necessary if care is taken during manual washing. The protocol can be also adapted to use with an automated liquid handler.

The assay can be adjusted to detect different antibody types (IgG or IgM can be detected with this kit, IgA detection antibodies can be substituted) and can be run with human serum or plasma.

The IONTOX SARS-CoV-2 Antibody Test Kit uses the following:

Antigens bound to the ELISA plates are SARS-CoV-2 Wuhan-Hu-1 spike protein receptor binding domain (RBD) or full length spike protein.

IgG Kit components
<table>
<thead>
<tr>
<th>Components required but not included with the test:</th>
</tr>
</thead>
<tbody>
<tr>
<td>● Class II biological safety cabinet (for handling human blood or serum) The Centers for Disease Control &amp; Prevention and the National Institutes of Health recommend that potentially infectious agents should be handled at the Biosafety Level 2 facility.</td>
</tr>
<tr>
<td>● Automated 96 well microplate washer (optional but recommended)</td>
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<tr>
<td>● Multichannel pipettor(s) capable of pipetting 50 to 250 µl</td>
</tr>
<tr>
<td>● (optional) Multichannel pipettor(s) capable of pipetting 10 to 20 µl</td>
</tr>
<tr>
<td>● Sterile reservoirs for multichannel pipettor</td>
</tr>
<tr>
<td>● Timer</td>
</tr>
<tr>
<td>● 96 well low-protein binding (i.e. polypropylene or polyethylene) plates (BD Falcon 353263 or equivalent) or 1.5-ml microcentrifuge tubes</td>
</tr>
<tr>
<td>● Microplate reader capable of measuring absorbance of 450 or 490 nm</td>
</tr>
<tr>
<td>● Polypropylene sterile conical tubes: 15-ml, 50-ml</td>
</tr>
<tr>
<td>● Pipet-Aid or equivalent</td>
</tr>
<tr>
<td>● Sterile, serological pipettes: 5-ml, 10-ml, 25-ml</td>
</tr>
<tr>
<td>● Micropipettors</td>
</tr>
<tr>
<td>● Micropipette tips: 20-µl barrier tips, 200-µl barrier tips, 200-µl tips, 1000-µl barrier tips</td>
</tr>
</tbody>
</table>
Reagent Stability
Store the test kit at 2-8°C. The shelf life of all components is shown on each respective label. The components are good for one month once opened provided it has not yet reached its expiration date. Do not freeze kit components. Do not use the kit reagents after their expiration date. Do not expose reagents to strong light during storage or incubation. The substrate solution (TMB) is light sensitive and must be stored in the dark. Unused microtiter wells can be resealed in foil pouch with desiccant and stored at 2-8°C for up to one month.

Use properly collected patient sera samples. Do not use hyperlipaemic, hemolytic, contaminated, or turbid sera. Serum samples can be held at 2-8°C for at least 7 days and can withstand up to 3 freeze and thaw cycles. It is the responsibility of the individual laboratory to determine specific stability criteria.

Stability of the patient samples: The Clinical and Laboratory Standards Institute (CLSI GP44-A4) recommends the following storage conditions for samples: Samples should be stored at room temperature no longer than 8 hours. If the assay will not be completed within 8 hours, the samples should be refrigerated at +2°C to +8°C. If the assay will not be completed within 48 hours, or if the samples will be stored beyond 48 hours, samples should be frozen at -20°C or lower. Samples should not be repeatedly frozen and thawed. Frozen samples must be mixed well after thawing and prior to testing. Diluted samples should be incubated within 8 hours. It is the responsibility of the individual laboratory to use all available references and/or its own studies to determine its own specific stability criteria.

Protocol
Heat inactivation of IgG assay samples:
(this is a general safety precaution for work with human serum). CAUTION: We have not tested if this procedure completely inactivates SARS-CoV-2; please consult with your local biosafety officer to discuss proper safety precautions.

1. Set the heating unit to 56°C.
2. Once temperature is reached, place the serum/plasma samples in the heating unit and incubate for 30 minutes. Do not leave samples at 56°C for longer than 1 hr.
3. Store at 4°C for imminent use or keep frozen at -20°C or lower.

Initial testing – Single dilution screen
The procedure will require one Pre-Dilution plate per sample set, and one Dilution plate per antigen coated ELISA plate. 96 well Polypropylene plates are recommended (not supplied). Polystyrene plates or microcentrifuge tubes can be used.

Pre-diluting samples for ELISA:
In a biological safety cabinet, set up Pre-Dilution plate or tubes to pre-dilute samples and negative controls at a 1:10 ratio. Use low-protein binding (i.e. polypropylene or polyethylene) plates.

Add 90 µl of sterile phosphate buffered saline (PBS) to all wells of the pre-dilution plate. Gently mix each serum sample and add 10 µl to the pre-dilution vessel, mixing once more. Do this for all remaining samples including the negative controls.

The volume of unused pre-diluted samples from the initial screen will be stored and used for the confirmatory assay steps, below.

Set up Dilution plates:

1. Prepare at least 25 ml of phosphate buffered saline with 0.1% Tween-20 (PBS-T) + 1% (w/v) milk powder.
2. Using a new polypropylene or polyethylene plate, prepare one Dilution plate per antigen coated ELISA plate.
3. Add 145 µl of PBS-T containing 1% w/v milk powder to all wells of the dilution plate (including blank wells).
4. Leaving columns 1 and 12 as blanks, add 5 µl of pre-diluted sample (or negative controls) to the designated wells. This results in a final serum dilution of 1:300.
5. Positive control: Add 5 µl of 30 µg/ml Positive control to the 145µl PBS-T,1% milk powder in designated wells (1µg/ml final concentration).
6. Continue until all samples and controls have been added to the designated wells. See reference plate layout in Figure 1.

![Figure 1](image)

Figure 1. Suggested Screening ELISA reference plate layout. The layout in which samples could be prepared in a 96-well cell culture plate (dilution plate) is shown. Wells designated for positive (+) and negative (−) controls are indicated.
Transfer serum dilutions to coated ELISA plate(s):

7. Using a multichannel pipettor, pipette up and down four to six times in the wells of the first row of the dilution plate to mix. Transfer 100 µl from each well of the first row of the dilution plate to the corresponding wells in the ELISA plate. Change tips and continue to transfer the second row of the dilution plate to the ELISA plate in the same manner.

8. Start the timer for 2 hours as soon as the contents of all the rows have been transferred to the first ELISA plate. Cover the plates at room temperature or place plates in a 20°C (RT) incubator. Do not exceed 4 hours of incubation at RT.

Incubating with secondary antibody:

9. After 2 hours incubation at RT, wash the ELISA plates three times with 200µl PBS-T. Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

10. For IgG ELISA: Dilute the 20µg/ml anti-human IgG HRP-labeled secondary antibody 1:100 in PBS-T containing 1% milk (0.2µg/ml final concentration). Prepare at least 5 ml per plate.

11. Add 50 µl of the secondary antibody solution to all wells of the plate using a multichannel pipettor. Be sure to avoid touching the walls of the wells with the pipette tips, to avoid carry-over and high background signals.

12. Start the timer for 1 hour (stay in a range of 50 to 65 min) as soon as the secondary antibody has been added to the first plate. Incubate plates at room temperature or in a 20°C (RT) incubator.

Develop and read plates:

13. After 1 hour, wash plates three times with 200µl PBS-T.

14. Prepare HRP substrate solution and calculate amount needed. Add 100 µl HRP substrate solution to all wells of the plate. Begin the timer for as soon as solution has been added to the first row of the first plate. Shake plates gently.

15. After 10 min, add 100 µl of 1 M HCl (caution: corrosive) to all wells to stop the reaction.

16. Prior to measuring, carefully shake the microplate to ensure a homogeneous distribution of the solution. Read ELISA plates photometric absorbance of the color intensity in a plate reader at a wavelength of 450 nm within 30 minutes of adding the stop solution and record data.

Samples that exceed a certain optical density cutoff value (proposed cutoff: optical density = 0.15 to 0.2, are assigned as presumptive positives and will be tested in the confirmatory ELISA (below).

The optical density cutoff must be experimentally determined and depends on assay background and noise as well as plate reader performance variability.

Interpretation of Results

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>FINAL RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigle dilution screen</td>
<td>NEGATIVE</td>
<td>No further testing</td>
</tr>
<tr>
<td>POSITIVE*</td>
<td></td>
<td>Presumptive positive: test by dilution curve assay</td>
</tr>
</tbody>
</table>
Confirmatory assay – dilution curve ELISA

Dilute samples:
Retrieve the 1:10 pre-diluted samples to be tested and confirmed (samples that are above proposed threshold in the Screening ELISA).

Perform serial dilutions:
1. Prepare at least 20 ml of PBS-T + 1% (w/v) milk powder per plate.
2. Using a new polypropylene or polyethylene plate, add 120 µl of PBS-T containing 1% milk to all wells of each plate.
3. Leaving column 1 as blanks, add an extra 54 µl of PBS-T containing 1% milk to wells in columns 2 and 7 except for well A7.
4. Add 6 µl of the selected 1:10 pre-diluted samples (final dilution 1:300 on the plate) to the desired wells in column 2 and column 7 as needed. Reserve row A for positive controls. Plate layout can be rearranged as needed.
5. With the multichannel pipettor, pipette up and down four to six times in column 2 to mix. Discard these tips. With new tips, transfer 60 µl (3-fold dilution) from column 2 to column 3, and pipette up and down four to six times to mix. Repeat serial dilutions until column 6.
6. Taking fresh tips, mix column 7 by pipetting up and down four to six times. Repeat the same process of transferring, mixing, and discarding tips from columns 7 to 11.
7. Transfer negative controls into wells (see suggested reference plate layout in Figure 2).
8. Positive control dose response dilutions: Add 6 µl of 30 µg/ml Positive control to row A, column 2 (1µg/ml final concentration). Mix and move 60µl of row A, col 2 to col 3, Repeat through to column 10.

Figure 2. Suggested Confirmatory dilution curve ELISA reference plate layout. The sample layout on the ELISA plate is shown, including the serial dilution steps that need to be performed. Wells designated for positive (STD) and negative (−) controls are indicated.

<table>
<thead>
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<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
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<th>9</th>
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<tbody>
<tr>
<td>A</td>
<td>Blank</td>
<td>Stnd 0.003 µg/ml</td>
<td>Stnd 0.001 µg/ml</td>
<td>Stnd 0.003 µg/ml</td>
<td>Stnd 0.001 µg/ml</td>
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<td>Stnd 0.001 µg/ml</td>
<td>Stnd 0.003 µg/ml</td>
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<td>B</td>
<td>Blank</td>
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<td>sample 1</td>
<td>sample 1</td>
<td>sample 1</td>
<td>sample 1</td>
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<td>sample 1</td>
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<td>sample 1</td>
<td>sample 1</td>
<td>sample 1</td>
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<tr>
<td>C</td>
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<td>sample 2</td>
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<td>sample 2</td>
<td>sample 2</td>
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</tr>
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<td>sample 7</td>
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<td>sample 7</td>
<td>sample 7</td>
<td>sample 7</td>
<td>sample 7</td>
<td>sample 7</td>
</tr>
</tbody>
</table>
9. Transfer dilutions to the coated ELISA plate. Start the timer for 2 hours once the first ELISA plate has been transferred.

10. Place plates in a 20°C (RT) incubator. Do not exceed 4 hours of incubation at RT.

**Incubate with secondary antibody:**

11. After 2 hours of incubation at RT, wash the plates three times with PBS-T. Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

12. Dilute anti–human IgG (Fab-specific) HRP-labeled secondary antibody 55µl in 5.5ml PBS-T containing 1% milk.

13. Add 50 µl of the secondary antibody solution to all wells of the plate using a multichannel pipettor. Avoid touching the tips of the pipette to the walls of the well.

14. Start the timer for 1 hour (stay in a range of 50 to 65 min) as soon as the secondary antibody has been added to the first plate. Place plates in a 20°C (RT) incubator.

**Develop and read plates:**

15. After 1 hour, wash plates three times with PBS-T either manually or with an automated plate washer.

16. Prepare HRP substrate solution and calculate amount needed. Add 100 µl HRP substrate solution to all wells of the plate. Begin the timer for as soon as solution has been added to the first row of the first plate.

17. After 10 min, add 100 µl of 1 M HCl (caution: corrosive) to all wells to stop the reaction.

18. Prior to measuring, carefully shake the microplate to ensure a homogeneous distribution of the solution. Read ELISA plates photometric absorbance of the color intensity in a plate reader at a wavelength of 450 nm within 30 minutes of adding the stop solution and record data.

True positive samples will have a signal higher than the negative control plus 3 standard deviations of the negative controls in at least two consecutive dilutions.

**Interpretation of Results**

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>FINAL RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single dilution screen</td>
<td>NEGATIVE</td>
<td>No further testing</td>
</tr>
<tr>
<td></td>
<td>POSITIVE*</td>
<td>Presumptive positive:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>test by dilution curve assay</td>
</tr>
<tr>
<td>Dilution curve assay</td>
<td>NEGATIVE</td>
<td>No further testing</td>
</tr>
<tr>
<td></td>
<td>POSITIVE*</td>
<td>Confirmed positive for IgG antibody.</td>
</tr>
</tbody>
</table>

* > 0.2 at an absorbance of 450 nm

**IgG assay performance characteristics**
The test was validated against a panel of previously frozen samples consisting of 60 SARS-CoV-2 positive serum samples and 110 antibody-negative serum samples. Each of the 60 positive samples was confirmed with a nucleic acid amplification test (NAAT).

<table>
<thead>
<tr>
<th>IgG test Performance</th>
<th>Test method</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Comparative method</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
<td>109</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>109</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>100.00%</td>
<td>94.04% to 100.00%</td>
</tr>
<tr>
<td>Specificity</td>
<td>99.10%</td>
<td>95.08% to 99.98%</td>
</tr>
</tbody>
</table>

Data was analyzed with MedCalc® Statistical Software version 19.5.3 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020)

LIMITATION OF DETECTION METHOD
The IONTOX SARS-CoV-2 Antibody Test has completed the Section IV.D notification process under FDA’s “Policy for Coronavirus Disease-2019 Tests During the Public Health Emergency (Revised)” and has not been reviewed by FDA.

Negative results do not rule out SARS-CoV-2 infection in those who have been in contact with the virus. If acute infection is suspected, direct testing for SARS-CoV-2 is necessary.

Results from antibody testing should not be used as the sole basis to diagnose or exclude SARS-CoV-2 infection or to inform infection status.

Weak Positive or equivocal positive results may be due to past or present infection with non-SARS-CoV-2 coronavirus strains, such as coronavirus HKU1, NL63, OC43, or 229E.

The product is designed only for use with human serum or plasma samples for the detection of novel coronavirus (SARS-CoV-2) antibodies. This test is not for the screening of donated blood.

Coronavirus may not be detected even though coronavirus antibodies are present in the sample, leading to a false negative. This may occur if the amount of coronavirus antibodies is below the detection level of the kit. If the product is stored improperly prior to use it may cause incorrect results.

A negative serologic result could indicate that an individual has not developed detectable antibodies at the time of testing. This could be due to a variety of factors such as testing too early in the course of infection, the absence of the tested antibody isotype as opposed to other isotypes, or the lack of adequate immune response which can be due to conditions or treatments that suppress immune function. It is recommended the patient be re-tested with the Anti-SARS-CoV-2 ELISA assay one to two weeks later with freshly drawn sample.
Current literature suggests that detectable antibodies against SARS-CoV-2 develop approximately 8 to 11 days following onset of symptoms. A positive serologic result is not diagnostic but indicates that an individual has likely been infected with SARS-CoV-2 and produced an immune response to the virus. It is not known at this time whether detectable antibody correlates with immunity.

IgM antibodies are typically formed initially by the patient at about the time that symptoms first appear (after a 2 to 14-day incubation period). The IgM antibodies will dissipate after approximately one month. IgG antibodies are often second to appear, about one week after symptoms appear. They continue to be present in the blood stream for longer periods of time. If the results show a positive for IgM antibodies it is indicative of an active infection. A positive for IgG antibodies indicates that the patient either has an active case or has previously been infected with the SARS-CoV-2 virus and has produced an immune response to the virus. It is not known at this time whether detectable antibody correlates with immunity. If the patient has symptoms and the antibody kit is negative, then an additional molecular test for the viral RNA should be considered.
Protocol Check list

Initial testing – Single dilution screen
SARS-CoV-2 IgG Screen ELISA check list

Samples:

- Heat inactivate 30min to 1 hr 56°C.
- Dilute 10X PBS-T to 1X.
- Add 25ml PBS-T, to 1% milk powder (mp) bottle.

Sample dilutions (after heat inactivation):

- Pre-Dilute samples and negative control 1:10 in PBS (10 µl+90 µl PBS) in pre-dilution plate or tubes. Use low-protein binding (i.e. polypropylene or polyethylene) plates.
- Dilution plate: dilute Pre-Dilutions 1:30 in PBS-T, 1% milk powder in a new plate (5 µl pre-dilution +145 µl PBS-T,1% milk powder).
- Negative controls: Add negative control pre-dilutions to Dilution plate designated wells for a 1:300 dilution.
- Positive control: Add 5 µl of 30 µg/ml Positive control to the 145µl PBS-T,1% milk powder in designated wells (1µg/ml final concentration).

Incubations:

- Add 100µl of dilutions to ELISA plate. Incubate 2 hours at room temperature (RT) or 25°C.
- 2°-HRP antibody: dilute anti-human IgG in PBS-T 1% milk: (55 µl in 5.5 ml PBS-T 1% milk).
- Wash plate 3X with 200µl per well PBS-T (use aspirator or plate washer).
- Add 50µl/well of 2°-HRP antibody (5ml/plate). Incubate for 1 hour at RT.
- Wash plate 3X with 200µl per well PBS-T.
- Add 100µl TMB (HRP substrate). Incubate 10 minutes at RT.
- Stop: add 100µl 1M HCl (caution: corrosive).
☐ Read 450nm
Confirmatory assay – dilution curve ELISA
SARS-CoV-2 IgG dilution curve ELISA check list

- Retrieve previously heat-inactivated and pre-diluted samples (1:10 in PBS).

  Dilution plate:
  - Add 120 µl PBS-T 1% milk powder to all wells.
  - Add 54 µl more PBS-T 1% milk powder to columns 2, column 12, and column 7 rows B through H.
  - Add 6 µl of selected 1:10 Pre-Diluted samples to designated wells in Dilution plate columns 2 and 7.
  - Mix and move 60 µl of col 2, rows B-H to col 3. Repeat up to col 6.
  - Repeat process for appropriate rows in columns 7-11.

- Positive control dose response dilutions: Add 6 µl of 30 µg/ml Positive control to column 2, row A (1 µg/ml final concentration). Mix and move 60 µl of col 2, row A to col 3, Repeat through to column 10.

- Negative controls: Add 6 µl negative control to rows H.

  Incubations:
  - Add 100 µl of sample dilutions to plate. Incubate 2 hours room temperature (RT) or 25°C.

  - Wash plate 3X with 200 µl per well PBS-T. Use aspirator or plate washer.

  - 2°-HRP antibody: dilute anti-human IgG 1:100 in PBS-T 1% milk: 55 µl in 5.5 ml PBS-T 1% milk.

  - Add 50 µl/well (5 ml/plate). Incubate for 1 hour at RT.

  - Wash plate 3X with 200 µl per well PBS-T.

  - Add 100 µl TMB (HRP substrate). Incubate 10 minutes at RT.
- **Stop:** add 100µl 1M HCl
- **Read 450nm**