Bethyl Laboratories, Inc.
SARS-CoV-2 IgG ELISA Kit

For Research Use Only. Not For Use in Clinical or Diagnostic Procedures.

Cat. No. E88-301

Shelf Life: 2 months from date of sale

Intended Use

The Bethyl SARS-CoV-2 IgG ELISA is an Enzyme-Linked Immunosorbent Assay (ELISA) intended for semi-quantitative detection of IgG antibodies to SARS-CoV-2 in human serum or plasma collected in Potassium EDTA, Sodium Citrate or Lithium Heparin. The Bethyl SARS-CoV-2 IgG ELISA is intended for use as an aid in identifying individuals with an adaptive immune response to SARS-CoV-2, indicating recent or prior infection. Understanding the timing, duration and effectiveness of humoral immune responses in individuals previously infected with SARS-CoV-2 will be important for conducting vaccine and epidemiological research. At this time, it is unknown for how long antibodies persist following infection and if the presence of antibodies confers protective immunity.

Results are for the detection of SARS-CoV-2 antibodies. IgG antibodies to SARS-CoV-2 are generally detectable in blood several days after initial infection, although the duration of time antibodies are present post-infection is

not well characterized. Individuals may have detectable virus present for several weeks following seroconversion.

The sensitivity of Bethyl SARS-CoV-2 IgG ELISA early after infection is unknown. Negative results do not preclude acute SARS-CoV-2 infection.

False positive results for Bethyl SARS-CoV-2 IgG ELISA may occur due to cross-reactivity from pre-existing antibodies to SARS-CoV-1 or other possible causes.

Background

Coronavirus Disease 2019 (COVID-19) is a systemic disease caused by the novel Coronavirus designated SARS-CoV-2. The initial disease outbreak was first reported in Wuhan, Hubei province, China in December 2019. Within months the World Health Organization declared a global pandemic. COVID-19 can be a serious and life-threatening disease. "Understanding the number of individuals who are exposed and whether they then develop immunity is absolutely key to managing the epidemic." (Ragon Institute of MGH, MIT and Harvard, n.d.)

Principle of the Assay

This kit provides for an indirect ELISA, in which a recombinant receptor binding domain (RBD) of the Spike1 protein of SARS-CoV-2 is coated on the wells of the microtiter plate. Antibodies to SARS-CoV-2 RBD when present in the test sample bind specifically to the RBD protein. After sample binding, unbound proteins and molecules are washed off, and a HRP-conjugated detection antibody is added to the wells to bind to the captured anti-SARS-CoV-2 IgG antibodies. The chromogenic substrate TMB (3,3',5,5'-tetramethylbenzidine) is then added. This reaction produces a blue product, which turns yellow when the reaction is terminated by addition of dilute sulfuric acid. The absorbance of the yellow product at 450 nm, corrected for plate imperfections by subtracting the absorbance at 570 nm, is proportional to the amount of RBD-specific anti-SARS-CoV-2 IgG present in the sample. After determining that the values for the Positive Control and Negative Control are valid and acceptable by comparing them to the value for the Calibrator, values for samples are compared to the Calibrator to generate a ratio. Ratios above a cutoff indicate positive samples and values below a cutoff indicate negative samples.

Reagents: Storage and Expiration

This kit should be refrigerated upon receipt and stored at 2-8ºC. Do not freeze. Expiration date is 2 months post receipt of the kit.

Materials Supplied

1. 96 well Precoated SARS-CoV-2 S1-RBD plate (E88-S1-RBD-PC), 1 plate
2. Positive Control: Recombinant Human anti-RBD IgG (ready to use) (E-PC88-201). 1 vial -0.3 ml
3. Calibrator: Recombinant Human anti-RBD IgG antibody (ready to use) (E-RC88-201), 1 vial - 0.3 ml
4. Negative Control: Dilute normal human serum (ready to use) (E-NC88-201) 1 vial - 0.3 ml
5. Detection Antibody: Goat Anti-Human IgG-Fc antibody – HRP conjugated (ready to use) (E88-201P), 1 vial - 11 ml
6. Assay Dilution Buffer (ready to use) (E-DBG60), 1 vial – 60 ml
7. TNT Wash Buffer packet for reconstitution (E118) 1 packet.
8. One Component TMB substrate: TMB (ready to use) (E-TMB12), 1 vial – 12 ml
9. Stop solution (0.18 M Sulfuric Acid; ready to use) (E-SS12) 1 vial - 12 ml
10. Sealing Tape:, 3 Sheets
11. SDS
12. Instructions for use

Additional Materials Required

- Deionized water.
- Precision pipettors, with disposable plastic tips.
- Polypropylene or polyethylene tubes or low protein binding 96-well plates to prepare samples. *Do not use polystyrene, polycarbonate or glass tubes.*
- A container to prepare TNT Wash Buffer.
- A 96-well plate washer. *An autoclavable plate washer is recommended, if available.*
- Disposable reagent reservoirs.
- A microtiter plate reader for measuring absorbance at 450 nm and 570 nm.

Safety Precautions

Universal precautions using appropriate personal protective equipment should be observed when handling all reagents. Dispose of all reagents and samples appropriately. Avoid eye and skin contact with reagents and samples. Stop solution contains dilute sulfuric acid. In case of eye or skin contact, rinse area with copious amounts of water. Remove and wash any contaminated clothing. Do not ingest. Individual components may contain preservatives.

Additional Precautions

- Store all reagents at 2-8ºC. *Do not freeze reagents*.
- All reagents must be at room temperature (20-25□C) before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated and result in erroneous data.

- Do not contaminate the TMB Solution. *Do not expose TMB Substrate solution to glass, foil, or metal*. If the solution is blue before use, DO NOT USE IT.

Procedure Overview

1. Allow all kit components to equilibrate to room temperature.
2. Prepare TNT Wash Buffer.
3. Prepare 1:100 patient serum or plasma sample dilutions by diluting 5 µl of sample into 495 µl Dilution Buffer.
4. Once at room temperature, remove the pre-coated microtiter plate from its foil pouch.
5. Add 100 µl each of Positive Control, Negative Control , Calibrator and diluted samples to designated wells on the pre-coated plate.
   Note: It is suggested to run each control or sample in duplicate.
6. Cover the plate with sealing tape and incubate at room temperature (20-25ºC) for 30 minutes.
7. Wash the plate 5 times with TNT Wash Buffer. Wash volume 250 µl per well, each wash.
8. Add 100 µl of anti-human IgG-Fc Detection Antibody to each well.
9. Cover the plate with fresh sealing tape and incubate at room temperature for 30 minutes.
10. Wash the plate 5 times with TNT Wash Buffer. Wash volume 250 µl per well, each wash.
11. Add 100 µl of TMB Substrate Solution to each well.
12. Incubate the plate in the dark at room temperature for 15 minutes.
13. Stop the reaction by adding 100 µl of Stop Solution to each well.
14. Measure absorbance on a plate reader at 450 nm and at 570 nm.

Handling and Preparation of Reagents, Standards, and Samples

Sample Handling

- This ELISA assay can be used for human serum or plasma.
- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing of infectious agents.
- 100 µl of diluted sample or standard is required per well.
- It is recommended that samples be assayed in duplicate each time the assay is performed.
- Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at or below -70°C. Avoid repeated freeze-thaw cycles when storing samples.
- If particulates are present in samples, centrifuge prior to use.
- If blood samples are clotted, grossly hemolyzed, lipemic, or the integrity of the sample is of concern, make a note on the Plate Template and interpret results with caution.

Sample Preparation

- Serum and plasma – Recommended dilution is 1:100. For example, add 5 µl of plasma/serum into 495 µl of 1X Dilution Buffer to give a 1:100 dilution. Mix thoroughly.

Preparation of TNT Wash Buffer

- Prepare Wash Buffer by reconstituting TNT packet in 1L of deionized water. Mix well.

Assay Procedure

Sample Incubation

- Use a Plate Template to record the well locations of the Positive Control, Calibrator, Negative Control, and unknown samples.

1. Add 100 µl of controls and diluted samples to the appropriate wells. Run each control, and the samples in duplicate if appropriate.
2. Carefully cover the wells with a new sealing tape and incubate for 30 minutes at room temperature, 20-25°C.

3. Carefully remove the adhesive plate cover and wash 5 times with TNT Wash Buffer, as described in the Plate Washing section below.

Plate Washing

- Using a manual or automated plate washer, perform the following steps.

1. Rinse the tips of the plate washer by dispensing the TNT Wash Buffer into the wash trough and aspirating the solution. Repeat this step 5 times. For automated plate washers, program the rinse step accordingly.

   Note: This initial rinse step is necessary especially if the plate washer has been idle for several days or longer. Automated plate washers are susceptible to microbial growth in the fluid lines and cavities.

2. Aspirate the solutions from the wells. Fill the wells to about 90% (250 µl) full with TNT Wash Buffer and then aspirate the wash solution. Repeat this wash step 4 more times. For automated plate washers, program 5 washes at 300 µl per wash, according to the manufacturer's instructions.

Incubation with Detection Antibody

1. Add 100 µl of Detection Antibody to each well. Mix by gently tapping the plate several times.
2. Carefully attach a new adhesive plate cover and incubate the plate for 30 minutes at room temperature, 20-25°C.
3. Carefully remove the adhesive plate cover and wash 5 times with TNT Wash Buffer (see Plate Washing section above).

Incubation with TMB Substrate and Stopping the Reaction

- Do NOT use glass pipette to measure the TMB Substrate Solution. Do NOT cover the plate with aluminum foil or metalized mylar. If the solution is blue before use, DO NOT USE IT!

1. Add 100 µl of TMB Substrate Solution into each well and incubate at room temperature (20-25ºC) in the dark for 15 minutes. Do NOT cover plate with a plate sealer.
2. Stop the reaction by adding 100 µl of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should immediately change color from blue to yellow.

Absorbance Measurement

Note: Wipe the underside of the wells with a lint-free tissue.

Measure the absorbance on an ELISA plate reader set at 450 nm and 570 nm within 30 minutes of stopping the reaction.

Calculations:

A. For each well, derive the corrected A450 value by subtracting the value measured at 570 nm from the value measured at 450 nm.
B. For calibrator, each control and each sample
    a. Calculate the mean of the corrected A450 values.
    b. Calculate the standard deviation (StdDev) of the corrected A450 values.
    c. Calculate the Percent Coefficient of Variation (%CV) of the corrected A450 values:

        $\%CV = StdDev \div mean \times 100$
C. Derive the ratio of the Positive Control to the Calibrator by dividing the mean of the corrected A450 values of the Positive Control by the mean of the corrected A450 values of the Calibrator.
D. Derive the ratio of the Negative Control to the Calibrator by dividing the mean of the corrected A450 values of the Negative Control by the mean of the corrected A450 values of the Calibrator.
E. For each sample, calculate the ratio for the sample by dividing the mean of the corrected A450 values of the sample by the mean of the corrected A450 values of the Calibrator.

Validity and Acceptability:

See “Validity and Acceptability” on enclosed insert.

Interpretation of Results:

See “Interpretation of Results” on enclosed insert.

Limitations of the Assay

1. This test is for the semi-quantitative assessment of seroconversion. Higher absorbance values correlate with higher relative concentration of specific antibody. This assay detects antibody specific to the RBD protein only and does not reflect total antibody response to other SARS-CoV-2 associated proteins.
2. Microbial contamination of samples or reagents, cross contamination of samples and/or kit reagents or extreme temperature excursions may yield erroneous results.
3. Previous infection with SARS-CoV-1 may result in a positive test result.

Performance Characteristics

Analytical Sensitivity, Specificity and Cross Reactivity

Samples procured from a commercial source from 34 distinct patients that had tested positive by molecular test (Roche Swab or Abbot RealTime) and tested as reactive by the Siemens ADVIA Centaur Sars-CoV-2 Total assay performed by an independent clinical laboratory were evaluated with the Bethyl SARS-CoV-2 IgG IVD ELISA.

		Bethyl SARS-CoV-2 IgG ELISA Results	
Days from Symptom Onset	Number of Samples Tested	IgG Positive results	IgG PPA
0-7 days	0	0	n/a
8-14 days	0	0	n/a
≥15 days	34	34	100%

Serum samples collected prior to December 2019 from 161 distinct donors, from two commercial sources were evaluated. One hundred fifty-eight samples (98.1%) were negative and three (1.9%) were not negative for IgG to SARS-CoV-2.

	Bethyl SARS-CoV-2 IgG ELISA Results	
Number of Samples Tested	IgG Negative results	IgG NPA (95% CI)
161	158	98.1% (96.0% – 100.2%)

Class Specificity

Human serum samples previously identified as either Negative, Equivocal, or Positive in the SARS-CoV-2 IgG ELISA were incubated with either diluent alone (Untreated), diluent + goat polyclonal anti-human IgG (Bethyl A80-104) to deplete IgG, or diluent + goat polyclonal anti-human IgM (Bethyl A80-100) to deplete IgM, and then RBD-specific IgG binding was measured in the SARS-CoV-2 IgG assay. All samples were also evaluated in an assay for anti-SARS-CoV-2 IgM (E88-302) binding.

	IgG Ratio Range	IgM Ratio Range	% Untreated Ratio (95% CI)	
			IgG-Depleted	IgM-Depleted
Neg (n=5)	0.09 - 0.38	0.17 - 0.51	21.3 (9.8 - 32.8)	96.4 (93.2 - 99.6)
Equiv (n=1)	0.83	0.72	4.1	102.1
Pos (n=10)	2.45 - 9.84	1.86 - 6.40	0.6 (0.2 - 1.0)	102.6 (100.2 - 105.0)

	IgG Ratio Range	IgM Ratio Range	IgG-Depleted			IgM-Depleted		
			Neg.	Equiv	Pos	Neg.	Equiv	Pos
Neg (n=5)	0.09 - 0.38	0.17 - 0.51	5	0	0	5	0	0
Equiv (n=1)	0.83	0.72	1	0	0	0	1	0
Pos (n=10)	2.45 - 9.84	1.86 - 6.40	10	0	0	0	0	10

The results demonstrate that depletion of IgG (>99% reduction in IgG assay ratio) from Positive samples converts them to Negatives and that depletion of IgM (>97% reduction in IgM assay ratio, data not shown) from Positive samples has no effect on their disposition in the assay. Similarly, the Negative and Equivocal samples saw no change in assay disposition with IgM depletion, while the Equivocal sample was converted to Negative with depletion of IgG.

These data show that signal in the SARS-CoV-2 IgG ELISA is due specifically to the IgG component of a patient's immune response to SARS-CoV-2 without significant interference from SARS-CoV-2-specific IgM.

Matrix Equivalency

Serum, citrate plasma, EDTA plasma, and heparin plasma were each collected from five individuals testing negative in both Bethyl's SARS-CoV-2 IgG ELISA and SARS-CoV-2 IgM ELISA. Convalescent serum from an individual who tested positive in Bethyl's SARS-CoV-2 IgG ELISA was spiked into a 1:100 dilution of each negative matrix at four different concentrations intended to yield negative, low positive (two different concentrations), and moderate positive IgG ELISA outcomes. 1:100 negative matrices were also tested without any spike. Each combination of negative matrix and spike was tested in duplicate.

Deming regression ($\alpha = 0.05$) was performed for the comparison of plasma to serum and showed the following results:

	Citrate	EDTA	Heparin
n	25	25	25
Assay Ratio Range (Serum)	0.17 to 2.76	0.17 to 2.76	0.17 to 2.76
Assay Ratio Range (Plasma)	0.13 to 2.80	0.16 to 2.61	0.19 to 2.88
Regression Equation (y = plasma, x = serum)	-0.041 + 1.047 x	0.016 + 0.979 x	-0.016 + 1.061 x
95% CI of intercept	-0.092 to 0.009	-0.024 to 0.056	-0.064 to 0.033
95% CI of slope	0.985 to 1.109	0.939 to 1.019	0.999 to 1.123
H_o: slope = 1, H_1: slope ≠ 1; p-value	0.132	0.282	0.054
Variance ratio X/Y (λ)	1.335	1.555	1.040

For each spike amount, there was 100 % agreement of assay outcome across all matrices, all individuals, and all replicates, thus demonstrating robust matrix equivalency.

	No Spike	Negative Spike	Low Positive Spike #1	Low Positive Spike #2	Moderate Positive Spike
Ratio Range	0.16 - 0.39	0.48 - 0.67	1.3 - 1.6	1.58 - 2.12	2.14 - 2.66
Assay Outcome	NPA		PPA		
Serum, n=5	100%	100%	100%	100%	100%
Citrate Plasma n=5	100%	100%	100%	100%	100%
EDTA Plasma n=5	100%	100%	100%	100%	100%
Heparin Plasma n=5	100%	100%	100%	100%	100%

References

1. Ragon Institute of MGH, MIT and Harvard. n.d. <http://www.ragoninstitute.org/the-ragon-institute-of-mgh-mit-and-harvard-receives-gift-from-nancy-zimmerman-to-help-fund-development-of-early-response-covid-19-diagnostic/>.
   "The Ragon Institute of MGH, MIT and Harvard Receives Gift from Nancy Zimmerman to Help Fund Development of Early Response COVID-19 Diagnostic." March 2020. *Ragon Institute of MGH, MIT and Harvard.*

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