AKSTON BIOSCIENCES

Akston Biosciences

AntiCoV-ID™ IgG ELISA

Instructions for Use

600016

Reagents for 80 Determinations

RUO for research use only

Manufactured by

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Explanation of Symbols Used on Labels

 Σ = 80	Reagents for 80 determinations
	Expiry Date
	Store between 2—8°C
	Lot Number
	For research use only

Intended Use

Akston Biosciences AntiCoV-ID™ ELISA is a method for the quantitative determination of IgG in human serum, plasma, or heat-inactivated (56°C; 1 hour) human serum or plasma.

Summary and Explanation of the Assay

The SARS-CoV-2 virus is a highly contagious pathogen, and it has been implicated in a worldwide pandemic. The SARS-CoV-2 virus is responsible for the disease it causes, namely the Coronavirus Disease 2019 (COVID-19), and as such the virus is also known as the COVID-19 virus or the 2019 Novel Coronavirus. Covid-19 is associated with symptoms such as fever, tiredness, dry cough, aches and pains, nasal congestion, runny nose, and sore throat. In some cases, the host may exhibit mild symptoms or may be asymptomatic. However, more severe cases are associated with severe respiratory distress, pneumonia, and even death.

SARS-CoV-2 is of the genus Betacoronavirus. Coronavirus contains four protein structures, including the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. Among them, S protein is principally involved in the attachment of the virus and entry into cells. Entry is thought to be accomplished by binding to human ACE2 receptor via the S protein receptor-binding domain (RBD). Therefore, due to its critical role on cell entry, the SARS-CoV-2 spike protein RBD has emerged as a strong target for the development of virus attachment inhibitors, neutralizing antibodies, and vaccines (Jun Lan, et al. *Nature* 2020).

There is an urgent need to understand which proportions of the population may already have developed strong immunity to SARS-CoV-2 via seroconversion to produce endogenous IgG-type antibodies against the virus, to support convalescent plasma clinical trials by identifying those with IgG-type antibodies, and to identify which therapies perform well in vaccination trials against SARS-CoV-2. Moreover, due to the vast scale of the pandemic, there is an urgent need to obtain this data in a quantitative and high-throughput fashion in order understand differences in the antibody titers between patients.

Underlying Principle of the Procedure

Akston Biosciences AntiCoV-ID™ IgG ELISA is an indirect, enzyme linked immunosorbent assay (ELISA) designed to measure anti-spike protein RBD antibodies against the SARS-CoV-2 virus (COVID-19 virus, 2019 Novel Coronavirus) in human patient serum and plasma samples, including heat-inactivated serum or heat-inactivated plasma. The indirect immunoassay uses a recombinant SARS-CoV-2 spike protein RBD immobilized on ELISA plates as the capture antigen to bind the SARS-CoV-2 specific anti-spike RBD antibodies in the serum samples when incubated in the microplate wells. A simple wash step removes all unbound proteins, leaving the anti-SARS-CoV-2 spike protein RBD antibodies stuck to the plate. A second incubation is performed where the anti-spike protein RBD antibodies are detected by an anti-human IgG antibody (not cross-reactive to IgM) that is conjugated to horseradish peroxidase (HRP). After a second simple wash step to remove the unbound enzyme-conjugate, the assay plate wells are incubated with 3,3',5,5'-trimethylbenzidine which causes a colorimetric change that is proportional to the amount of bound enzyme conjugate in each well. The color development is stopped by adding acid that halts development, and the color density of each well is measured using a spectrophotometric microplate reader.

Warnings, Safety and Other Considerations

- For Research Use Only. None of the kit reagents are for internal or external use in animals or people.
- Follow all precautions for storing, using, and disposing of hazardous materials at your site. This kit Stop Solution contains 1% sulfuric acid.
- Use universal precautions when handling patient blood, serum, plasma, and other biospecimens. All blood, serum, and plasma samples should be treated as potentially infectious.
- The kit is designed such that each well is only used one time.

Required and Optional Materials That Are Not Provided

- Pipettes and pipette tips of appropriate sizes. Use of multichannel pipettes is encouraged for decreasing the overall assay time
- Items for preparation of reagents (graduated cylinders, mixing containers, and tubes)
- Microplates or tubes for performing dilutions of serum samples before addition to the Assay Plate
- Magnetic stirrer
- Deionized purified water
- Vortexer
- Microplate reader equipped to measure at 450 nm
- Water bath for heat-inactivating virus potentially present in samples (optional, if desired)
- Microplate shaker (optional)

Reagents

Each Akston Biosciences AntiCoV-ID™ IgG ELISA (Part No. 600016) contains reagents for 96 wells, sufficient for 38 samples, 2 controls, and 1 calibration curve in duplicate. When running multiple assay plates at once, best results may be obtained by pooling identical reagents from identical lots. Expiry dates may be found on the outer packaging of the kit. A storage temperature of 2—8°C is recommended.

Assay Plate	1 plate	96 wells 8-well strips	Ready for use	
IgG Calibrator pre-coated strips (with **GOLD** ● markings)				x 2
Recombinant SARS-CoV-2 RBD coated strips				x 10
Assay Control 1 **GRAY** ●	1 vial	0.25 mL	Ready for use	
Assay Control 2 **GREEN** ●	1 vial	0.25 mL	Ready for use	
10X Enzyme Conjugate **BLACK** ●	1 bottle	1.5 mL	Preparation required, see below	
Enzyme Conjugate Diluent **BLUE** ●	1 bottle	15 mL	Ready for use	
Sample Dilution Buffer YELLOW ○	1 bottle	30 mL	Ready for use	
10X Wash Buffer **VIOLET** ●	2 bottles	60 mL	Dilute 100 mL with 900 mL of deionized water to give 1X Wash Buffer	
TMB Substrate **WHITE** ○	1 bottle	15 mL	Ready for use	
Stop Solution 1% sulfuric acid **RED** ●	1 bottle	15 mL	Ready for use	

SPECIMEN COLLECTION AND HANDLING

Serum

With the help of a trained phlebotomist, collect a blood sample via venipuncture in a serum separator tube (SST) or an equivalent tube that allows the blood to clot. Separate the blood from serum by centrifugation according to the blood collection or SST tube manufacturer's instructions. Store samples at 2-8°C for shorter times, or take serum to freeze and store at -20°C for longer timeframes. Repeated freeze-thaw cycles should be avoided.

Plasma

With the help of a trained phlebotomist, collect a blood sample via venipuncture in a sodium citrate collection tube that prevents clotting. Separate the blood from plasma by centrifugation according to the blood collection tube manufacturer's instructions. Store samples at 2-8°C for shorter times, or take plasma to freeze and store at -20°C for longer timeframes. Repeated freeze-thaw cycles should be avoided.

Heat-inactivated Serum and Heat-inactivated Plasma

In some cases when dealing with potentially infectious samples, heat treatment may lead to the inactivation of pathogens in a biospecimen. Collect serum or plasma as described above and aliquot a small volume into a small microtube with a tight lid. Incubate the sample in a water bath or dry heat block at 56°C for a minimum of 1 hour. Treatment in this manner may help inactivate pathogens in the biospecimen. Heat-inactivation is not a substitute for proper laboratory safety; even with heat-inactivation, use universal precautions and proper personal protective equipment when working with potentially infectious samples. Consult your facility's biosafety officer with questions.

Preparation of samples

Samples may be diluted in Sample Dilution Buffer for use in this assay from 1:50 to 1:400 or higher. However, based on experience, a 1:100 dilution will be optimal for assay performance. See the detailed Test Procedure for more details.

AntiCoV-ID™ IgG ELISA Test Procedure

Precautions: Human serum/plasma samples should be treated as potentially infectious biological samples and all precautions for blood borne pathogens, including use of appropriate personal protective equipment such as lab coats, gloves, face shield/eye goggles, and masks. Use of biosafety level 2 cabinets are highly recommended. Additional precautions include the inactivation of virus by heat treatment at 56°C for 1 hour prior to use is encouraged.

1. Leave the assay kit at room temperature for not less than 1 hour before conducting the assay (all reagents must be brought to room temperature prior to the assay).
2. Dilute serum or plasma samples before adding to the Assay Plate wells as shown on Page 13 on the Assay Plate Layout. Each sample dilution should be performed by diluting the sample into Sample Dilution Buffer (YELLOW) at a ratio of 1:100. A two-step dilution is suggested as follows:
   - *1:10 dilution: 10 µL serum/plasma + 90 µL Sample Dilution Buffer, mix well*
   - *1:100 dilution: 25 µL of 1:10 dilution + 225 µL Sample Dilution Buffer, mix well*
3. Open the sealed pouch and unpack the Assay Plate containing the calibrator IgG coated 8-well strips and the spike protein RBD coated 8-well strips. Orient the plate as shown in the Assay Plate Layout on Page 13.
4. Load 100 µL Sample Dilution Buffer into each well of the two calibrator IgG standard strips. The Calibrator IgG standard strips are marked with **GOLD** ● dots, and they are precoated at the following nominal concentrations:

Calibrator Strip Well	Concentration (µg/mL)
A1,A2	0.800
B1,B2	0.400
C1,C2	0.200
D1,D2	0.100
E1,E2	0.050
F1,F2	0.025
G1,G2	0.012
H1,H2	0.000

5. Gently mix or vortex Assay Controls.
6. Load 100 µL Assay Control 1 (**GRAY**) into wells A3 and A4.
7. Load 100 µL Assay Control 2 (**GREEN**) into wells B3 and B4.
8. Add 100 µL of the 1:100 diluted patient samples from Step 2 into each well within the sample area of the plate (see non-shaded area of Assay Plate Layout), and record the sample IDs in the plate layout.

9. Place the Microplate Adhesive Film on top of the plate. Incubate the plate for 1 hour at room temperature.
10. Dilute the 10X Wash Buffer (VIOLET) with deionized water as follows:
    - 100 mL 10X Wash Buffer concentrate + 900 mL deionized or distilled water to give 1X Wash Buffer.
    - NOTE: Two (2) x 60 mL bottles of 10X Wash Buffer (VIOLET) are provided.
11. Dilute the 10X Enzyme Conjugate (BLACK) with Enzyme Conjugate Diluent (BLUE) as follows, then set aside and keep in the dark:
    - 1.2 mL 10X Enzyme Conjugate + 10.8 mL of Enzyme Conjugate Diluent to give 1X Enzyme Conjugate.
12. Wash the plate 5X in a plate washer or manually using a multichannel pipettor as follows:
    - 300 µL wash buffer per well for each wash cycle. Aspirate all wash buffer after the last wash cycle, pat dry on an absorbent paper towel.
13. Add 100 µL of diluted 1X Enzyme Conjugate solution per well to all wells. Incubate the plate at room temperature in dark for 1 hour.
14. Wash the plate 5X in a plate washer or manually using a multichannel pipettor as follows:
    - 300 µL wash buffer per well for each wash cycle. Aspirate all wash buffer after the last cycle, pat dry on an absorbent paper towel.
15. Wash the plate 1X further using a multichannel pipettor as follows:
    - 300 µL deionized water per well. Aspirate all wash buffer after the last cycle, pat dry on an absorbent paper towel.
16. Add 100 µL of TMB Substrate (WHITE) per well to all wells column by column. Incubate the plate in dark for 25 minutes (or until sufficient color development is achieved in the standard strips). A faint to dark blue color will develop.
17. Add 100 µL of Stop Solution (RED) to all wells to stop the reaction, in the same order and pace as how the TMB Substrate was added in the above step. The blue color will turn to a faint to dark yellow color. Gently tap plate slightly to ensure good mixing of Stop Solution in all wells.
18. Read the plate at 450 nm wavelength in a microplate reader within 30 minutes. (Serum samples that have anti-spike protein antibodies will develop a yellow color).
19. Using appropriate software on the computer attached to a plate reader (e.g. SoftMax Pro or Gen 5), create a standard curve using a 4-PL model curve fit to the first two IgG calibrator standard strip concentrations (see Step 4 for IgG calibrator well concentrations needed to construct the standard curve) and interpolate the anti-spike protein RBD antibody concentrations in the serum samples to obtain antibody titers expressed as human IgG units in µg/mL. The sample values obtained should be multiplied by the sample dilution factor used to obtain the final antibody titer values (e.g. for samples diluted 1:100, the obtained values should be multiplied by 100).

20. Results of the assay are valid if the reported Assay Controls meet both of the following criteria:

Validity Check	**Value obtained is within +/- 30% of the following values**
Assay Control 1 (GRAY)	<Value reported on Assay Control 1 vial label>
Assay Control 2 (GREEN)	<Value reported on Assay Control 2 vial label>

21. If both of the Assay Control criteria are not met, then the assay results are NOT valid.
22. When finished, dispose of all reagents, microplate, and unused kit materials according to your facility's environmental, health, and safety protocols.
23. Notes:
    - Samples demonstrating very high antibody titers (e.g. reading at or above the top of the calibrator standard curve) may be re-tested with a higher dilution (e.g. 200-fold or 400-fold or higher) to obtain more reliable antibody titer levels.
    - Be careful not to contaminate the TMB Substrate with enzyme conjugate solution.

CALCULATION OF RESULTS

See Step 19 of the assay Test Procedure.

LIMITATIONS OF THE PROCEDURE

As with all tests, a definitive clinical diagnosis should not be based on the results of a single test but should be made by the physician after all clinical findings have been evaluated. Highly lipemic or hemolyzed samples may result in a degradation of the antigen coated on the plate which could give falsely low values, thereby contributing to higher interassay variation. Time and temperature may exacerbate this assay variation. Keep hemolyzed samples cold or on ice to prevent protein degradation.

ASSAY PERFORMANCE CHARACTERISTICS

Akston recommends interpreting a potential POSITIVE/NEGATIVE cutoff for demonstrating clinically relevant levels of IgG-class antibodies against SARS-CoV-2 spike protein RBD as follows, after back multiplying the sample values by the sample dilution factor to obtain a numeric titer on the assay:

1. Locate the POSITIVE/NEGATIVE CUTOFF VALUE located on the label affixed to Page 16 (back cover) of this Instructions for Use.
2. If a sample demonstrates a numeric value LESS that the CUTOFF VALUE, then that sample should be reported as NEGATIVE.
3. If a sample demonstrates a numeric value that is EQUAL OR GREATER than that of the CUTOFF VALUE, then that sample should be reported as POSITIVE, and the sample should be reported as POSITIVE and reported with the absolute numeric value in micrograms per mL ("µg/mL").

Limit of Detection

Based on pre-validation work, the limit of detection is 0.010 µg/mL.

Spike and Recovery

Recovery upon spiking N=30 independent human serum samples taken from Covid-19 negative patients with an anti-SARS-CoV-2 spike protein RBD antibody was found to be 100% ± 5% SD.

Precision

- Using Assay Control 1, a 6.6% Coefficient of Variation (CV) was obtained for 6 replicates conducted between 2 operators.
- Using Assay Control 2, a 10.4% CV was obtained for 6 replicates conducted between 2 operators.

Specificity

Determining assay specificity is ongoing, but the antibodies measured in this assay are not expected to cross react with other viruses, since the SARS-CoV-2 spike protein RBD has low amino acid sequence homology with other non-beta coronaviruses. Also, cross reactivity to other coronaviruses is expected to be minimal due to the low sequence homology between the SARS-CoV-2 spike protein RBD region and other coronavirus spike protein sequences. However, there could be some degree of cross-reactivity to antibodies present in serum or plasma from patients who were exposed to the original SARS-CoV virus that occurred ~15 years ago. More studies are warranted to experimentally measure the cross reactivity of the AntiCoV-ID IgG ELISA to serum containing antibodies against other viral pathogens.

ASSAY PLATE LAYOUT

AntiCoV-ID™ IgG ELISA Plate Layout

	Calibrator IgG Standards (GOLD ●)		Sample and Assay Controls Area									
	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1		Assay Control 1		ID:		ID:		ID:		ID:	
B	Std 2		Assay Control 2		ID:		ID:		ID:		ID:	
C	Std 3		ID:		ID:		ID:		ID:		ID:	
D	Std 4		ID:		ID:		ID:		ID:		ID:	
E	Std 5		ID:		ID:		ID:		ID:		ID:	
F	Std 6		ID:		ID:		ID:		ID:		ID:	
G	Std 7		ID:		ID:		ID:		ID:		ID:	
H	Std 8		ID:		ID:		ID:		ID:		ID:	

Warranty

The performance of the assay and data provided were obtained following the assay test procedure outlined as indicated and at the recommended sample dilution levels and the product. If the test procedure is performed as described the Product will perform as intended for a period of at least 3 months from the date of manufacture. This Warranty shall not apply to any product that shall have been altered in any way, nor to any product that shall have been used or applied contrary to the printed instructions. NO OTHER WARRANTY EXPRESSED OR IMPLIED, WHETHER OF FITNESS FOR A PARTICULAR PURPOSE OR OF MERCHANTABILITY OR OF ANY OTHER KIND, SHALL EXIST IN RESPECT TO SUCH PRODUCT.

References

Jun Lan, et al. “Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor”, *Nature* 2020.

OVERVIEW OF TEST PROCEDURE
Akston Biosciences AntiCoV-ID™ IgG ELISA

Prepare diluted patient samples, suggested 1:100 dilution in Sample Dilution Buffer (SDB)	1st dilution: 10 µL sample + 90 µL SDB (Sample Dilution Buffer); 2nd dilution: 25 µL + 225 µL of SDB to achieve 250 µL at 1:100 dilution for each sample to be tested
Add Sample Dilution Buffer to Calibrator Strips	100 µL
Add Assay Controls 1 and 2, add diluted Samples	100 µL
Incubate	1 hour, at room temperature
Wash plate with 1X Wash Buffer	300 µL, 5 times
Add 1X Enzyme Conjugate	100 µL
Incubate	1 hour, at room temperature, protect from light
Wash plate with 1X Wash Buffer	300 µL, 5 times
Wash plate with Deionized Water	300 µL, once only
Add TMB Substrate	100 µL
Incubate	25 minutes at room temperature, protect from light
Add Stop Solution	100 µL
Measure absorbance at 450 nm	Calculate results

AKSTON BIOSCIENCES

For technical assistance, please email: techsupport@akstonbio.com

Affix Positive/Negative
Label Here

Akston Biosciences Corporation
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#MASXXXX-RYY