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Antibodies & Engineered Cell Lines™

MANUAL

SARS-CoV-2 Neutralizing Antibodies Detection Kit (P.1 Variant, Gamma)

For research use only. Not for diagnostic use

Cat. No. 90-7010

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1. Intended Use

The **SARS-CoV-2 Neutralizing Antibodies Detection Kit (P.1 Variant, Gamma)** contains key reagents required to test the presence of functional neutralizing antibodies against SARS-CoV-2 (P.1 Variant, Gamma) present in the serum or plasma independently of the species and isotypes. It is an easy and fast alternative *in vitro* to the classical neutralization assay using Vero E6 cells. Before using this Kit to detect neutralizing antibodies against SARS-CoV-2, we recommend using Abeomics **SARS-CoV-2 (Spike RBD) IgG Serological ELISA Kit with false positive control** (Cat. No. 90-7004) to detect the presence of IgG against SARS-CoV-2.

2. Introduction

Corona viruses (CoVs) are enveloped non-segmented positive-sense single-stranded RNA viruses and can infect respiratory, gastrointestinal, hepatic and central nervous system of human and many other wild animals (1). Recently, a new severe acute respiratory syndrome b-corona virus called SARS-CoV-2 (or 2019-nCoV) has emerged, which causes an epidemic of acute respiratory syndrome (called corona virus human disease 2019 or COVID-19) (2).

SARS-CoV-2 contains 4 structural proteins, including Envelope (E), Membrane (M), Nucleocapsid (N) and Spike (S), which is a transmembrane protein, composed of two subunits S1 and S2 (3). The S1 subunit contains a receptor binding domain (RBD), which binds to the cell surface receptor Angiotensin-Converting Enzyme 2 (ACE2) present at the surface of epithelial cells, causing mainly infection of human respiratory cells (4). Following an infection with SARS-CoV-2, patients develop specific IgG, IgA and IgM immuneresponse. A subset of these antibodies against Spike (RBD) can block virus infection / entry into cells by a process that is called *neutralization*. A strong correlation between the levels of RBD-binding antibodies and levels of SARS-CoV-2 blocking / neutralizing antibodies in patients has been observed (5). Recently, a new variant of SARS-CoV-2, called P.1 was detected in Brazil. The P.1 variant carries three mutations in the RBD at the positions 417, 484 and 501 (K417T, E484K, N501Y). The P.1 or Brazilian variant is a form of the SARS-CoV-2 corona virus that might have contributed to a surge in cases in the northern city of Manaus.

3. General References

- (1) Coronaviridae. Positive Sense RNA Viruses. Available online: https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/222/coronaviridae
- (2) A pneumonia outbreak associated with a new coronavirus of probable bat origin: P. Zhou, et al.; *Nature* **579**, 270 (2020)
- (3) The spike protein of SARS-CoV - a target for vaccine and therapeutic development: L. Du, et al.; *Nat. Rev. Microbiol.* **7**, 226 (2009)
- (4) SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor: M. Hoffmann, et al.; *Cell* **181**, 271 (2020)
- (5) The receptor binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients: L. Premkumar, et al.; *Science Immunol.* **48**, eabc 8413 (2020)

4. Assay Principle

This **SARS-CoV-2 Neutralizing Antibodies Detection Kit (P.1 Variant, Gamma)** is a colorimetric kit, which measures the neutralizing/blocking activity of antibodies present in human serum/plasma on the binding of the SARS-CoV-2 Spike (RBD) (P.1 Variant, Gamma) protein to its human receptor ACE2. SARS-CoV-2 Spike (RBD) (P.1 Variant, Gamma) recombinant protein has been precoated onto the 96-well microtiter plate. The serum or plasma containing antibodies against SARS-CoV-2 Spike are pipette into the wells for binding to the coated viral protein and for blocking the interaction to ACE2. After washing to remove unbound components, ACE2 (human) protein coupled to HRP is added. Following a final washing, peroxidase activity is quantified using the substrate 3, 3', 5, 5'- tetramethylbenzidine (TMB). The presence of neutralizing antibodies in the samples are detected by reduction of Optical Density (OD) indicating the inhibition of the Spike (RBD) (P.1 Variant) - hACE2 binding.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use
- The validity period is 12 months.
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

- | | | |
|---|-------------------|------------------------|
| • 1 plate coated with Spike (RBD) (P.1 Variant) | (6x16-wellstrips) | |
| • ACE2 (human) (rec.) (HRP) (lyophilized) | (1 vial) | (ACE2-HRP) |
| • 1 vial Positive Control | (480µl) | (Positive) |
| • 1 vial Negative Control | (480µl) | (Negative) |
| • 3 bottles Wash Buffer 10x | 3 x30ml | (WashBuffer10x) |
| • 1 bottle TMBK-Blue Aqueous | 12 ml | (TMB) |
| • 1 bottle Stop Solution | 12 ml | (STOP) |
| • 2 plate Covers (plastic film) | | |
| • 2 silica Gel Mini bags | | |

7. Materials Required but Not Supplied

- Microtiter plate reader at 450nm
- Calibrated precision pipettes. Disposable pipette tips
- Deionized or distilled water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General Assay Protocol

Preparation and Storage of Reagents

NOTE: Prepare just the appropriate amounts of buffers necessary for the assay.

- **Wash Buffer 10X** has to be diluted with deionized or distilled water 1:10 before use (e.g. 30 ml Wash Buffer 10X + 270 ml water) to obtain **Wash Buffer 1X**.
- **ACE2 linked to HRP (ACE2-HRP)** has to be reconstituted with 120µl of Wash Buffer 1X.
 - Mix the ACE2-HRP to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes **at room temperature**. Mix well prior to making dilutions.
 - After reconstitution of ACE2-HRP, prepare aliquots if needed and store them at -20°C.
Avoid freeze/thaw cycles.
 - Dilute the reconstituted ACE2-HRP to the working concentration (e.g. 100µl in 10ml of Wash Buffer 1X).
- **Negative Control** should be tested in duplicate. It contains diluted (1/10) human serum negative for SARS-CoV-2 antibodies and screened for viral markers.
- **Positive Control** should be tested in duplicate. It contains diluted (1/10) human serum positive for SARS-CoV-2 (including P.1 Variant, Gamma) antibodies and screened for viral markers.

Sample Collection, Storage and Dilution

Serum: Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at $\leq -20^{\circ}\text{C}$ for later use. Avoid repeated freeze/thaw cycles.

Plasma: Collect plasma using heparin, citrate or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at $\leq -20^{\circ}\text{C}$ for later use. Avoid repeated freeze/ thaw cycles.

Serum and Plasma have to be diluted in Wash Buffer 1X. Each serum/plasma sample should be tested at 1/10 as a start by mixing 25 μl of serum / plasma in 250 μl of Wash Buffer 1X. If needed, higher dilutions ($>1/10$) can be tested.

NOTE:

- *Diluted Serum or Plasma in Wash Buffer 1X should be used fresh.*
- *Vortex serum or plasma samples at room temperature to ensure homogeneity. Then centrifuge samples at 10,000 rpm for 5 minutes prior to assay to remove particulates. Please do not omit this centrifugation step if samples are cloudy and contain particles.*
- *Serum/Plasma from animals immunized with SARS-CoV-2 antigens can be tested using this kit.*
- *Heat inactivation at 56°C for 30 minutes is optional, but serum/plasma used should all treated the same way (either heat inactivated or not).*
- *Severe hemolytic samples should not be used.*
- *Sample safety: All samples are regarded as potentially infectious and strictly handled in accordance with relevant national standards and guidelines.*
- *This kit can measure neutralizing antibodies against SARS-CoV-2 (P.1 Variant, Gamma) from different isotypes and species.*

Assay Procedure (Checklist)

<input type="checkbox"/>	<p>1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the aluminium foil bag with 2 silica gel mini bags and stored at 4°C.</p> <p><i>Remaining 16-well strips coated with Spike (RBD) (P.1 Variant) when opened can be stored in the presence of 2 silica gel mini bags at 4°C for up to 1 month.</i></p>
<input type="checkbox"/>	<p>2. Add 100µl of Negative control (Negative) and 100µl of Positive control (Positive) In duplicate (see 8.1. Preparation and Storage of Reagents).</p>
<input type="checkbox"/>	<p>3. Add 100µl of diluted (1/10) serum or plasma samples in duplicate (see 8.1. Preparation and Storage of Reagents and 8.2 Collection, Storage and Dilution).</p>
<input type="checkbox"/>	<p>4. Cover the plate with plastic film and incubate for 1 hour at 37°C.</p>
<input type="checkbox"/>	<p>5. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.</p>
<input type="checkbox"/>	<p>6. Add 100µl to each well of the diluted ACE2 (human)-HRP (ACE2-HRP) (see 8.1. Preparation and Storage of Reagents).</p>
<input type="checkbox"/>	<p>7. Cover the plate with plastic film and incubate for 1 hour at 37°C.</p>
<input type="checkbox"/>	<p>8. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.</p>
<input type="checkbox"/>	<p>9. Add 100µl to each well of TMB substrate solution (TMB).</p>
<input type="checkbox"/>	<p>10. Allow the color reaction to develop at Room Temperature in the dark for 5-10 minutes. Do not cover the plate.</p>
<input type="checkbox"/>	<p>11. Stop the reaction by adding 100µl of Stop Solution (STOP). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (STOP) is added.</p>
<input type="checkbox"/>	<p>! CAUTION: CORROSIVE SOLUTION!</p>
<input type="checkbox"/>	<p>12. Measure the OD at 450 nm in an ELISA reader.</p>

9. Typical Data

The presence of neutralizing antibodies against SARS-CoV-2 (P.1Variant, Gamma) in the serum/ plasma is measured by calculating the percent inhibition of each sample using the following formula:

$$= (1-(OD \text{ of the sample}) / OD \text{ of Negative Control}) \times 100$$

Samples	OD	Inhibition (%)*
Negative Control	2.65	0
Positive Control	1.44	45.67
Serum Healthy Patients	2.60	1.67
	2.57	2.66
Serum COVID-19 Patients (infected with SARS-CoV-2 before appearance of P.1 variant)	1.43	41.39
	1.51	38.00
	1.53	46.31

Table1: Examples of Optical Density (OD) and inhibition (%) of the controls and samples

10. Performance Characteristics

- i) **Intra-assay:** Three serum samples were assayed in replicates 4 times to test precision within an assay.
- ii) **Inter-assay:** Three serum samples were assayed in 4 separate assays to test precision between assays.

Intra-assay(n=6)	
Samples	CV (%)
1	7.72
2	8.02
3	5.88
Inter-assay (n=4)	
Samples	CV (%)
1	6.34
2	8.37
3	10.21

Table2: Examples of intra and inter-assays of different samples.

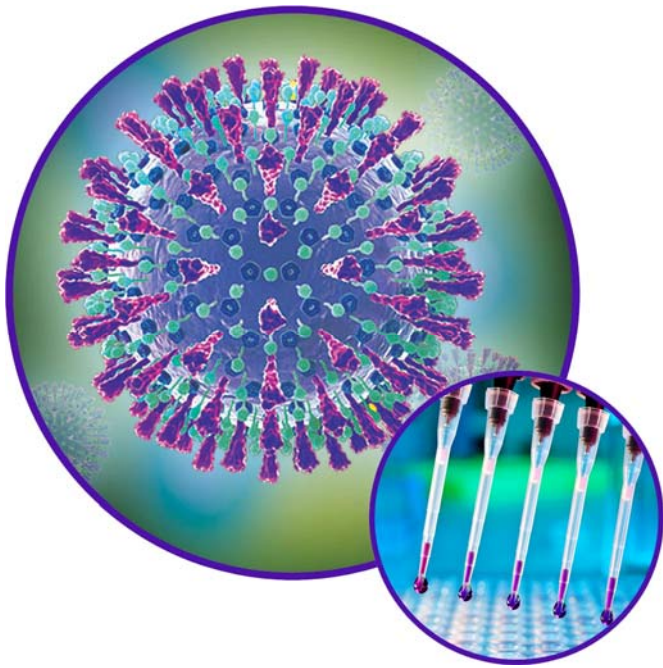
- iii) **Cut off suggestion:** With the panels of samples tested, we can propose the following cut off values for the presence of neutralizing antibodies in the serum / plasma of COVID-19 positive patients:
 - For samples without neutralizing antibodies, inhibition (%) should be < 20%
 - For samples with neutralizing antibodies, inhibition (%) should be >20%.

11. Technical Hints, Precautions and Safety

- It is recommended that samples to be run in duplicate.
 - Do not combine left over reagents with those reserved for additional wells.
 - The kit should not be used beyond the expiration date on the kit label.
 - Reagents from the kit with a volume less than 100µl should be centrifuged.
 - Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
 - Crystals could appear in the 5X or 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solution.
 - Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at anytime during the assay.
 - Keep TMB Solution protected from light.
 - When reading the results, ensure that the plate bottom is dry and there are no air bubbles inside the wells.
 - The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.
 - All specimens from human origin should be considered as potentially infectious. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety.
 - This test is only for qualitative detection.
 - Bacterial or fungal contamination of serum specimens or reagents, or cross-contamination between reagents may cause erroneous results.
- If the result for neutralizing antibodies is negative while the patient was confirmed positive for COVID-19 by PCR, it is recommended to collect a new sample from the patient a few days later and test it again.

12. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of ACE2-HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.



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