Instructions for Use

SARS-CoV-2 Neutralization Assay Development Kit (RBD-ACE2)

Reagents for the identification and qualitative measurement of neutralizing antibodies.

10 x 96 well plates

NTRL-RBD-10

Read the documentation

For Research Use Only

CONTENTS

1. INTRODUCTION

SARS-CoV-2 is a respiratory virus, which causes coronavirus disease 2019 (COVID-19). It spreads primarily through contact with an infected person via respiratory droplets generated when a person coughs or sneezes, or through droplets of saliva or discharge from the nose. The incubation period is thought to range from 2-11 days. Infection with SARS-CoV-2 can cause mild symptoms including a runny nose, sore throat, cough, and fever. Indeed, the majority of COVID 19 cases (about 80%) are asymptomatic or show mild symptoms. However, it can be more severe for some people and may lead to pneumonia or breathing difficulties. The elderly, and people with pre-existing medical conditions (such as, diabetes and heart disease) are especially vulnerable to becoming severely ill with the virus.

The SARS-CoV-2 genome encodes four major structural proteins, which are the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein and the envelope (E) protein, each of which is essential to produce a viral particle. The SARS-CoV-2 spike (S) glycoprotein is a class I viral fusion protein on the outer envelope of the virion that plays a critical role in viral infection by recognizing host cell receptors and mediating fusion of the viral and cellular membranes. The S glycoprotein is synthesized as a precursor protein which is then cleaved into an amino (N)-terminal S1 subunit and a carboxyl (C)-terminal S2 subunit. Three S1/S2 heterodimers assemble to form a trimer spike protruding from the viral envelope. The S1 subunit contains a receptor-binding domain (RBD), while the S2 subunit contains a hydrophobic fusion peptide and two heptad repeat regions that mediates the fusion of the viral and host cell membranes. SARS-CoV-2 S protein initially binds to the ACE2 receptor on the host cell via the RBD. The S1 domain is then shed from the viral surface, allowing the S2 domain to fuse to the host cell membrane. This process is dependent upon activation of the S protein, by cleavage at two sites (S1/S2 and S2') via the proteases Furin and TMPRSS2. Furin cleavage at the S1/S2 site may lead to conformational changes in the viral S protein that exposes the RBD and/or the S2 domain. TMPRSS2 cleavage of the SARS-CoV-2 S protein is believed to enable the fusion of the viral capsid with the host cell to allow viral entry. This essential pathway makes these proteins key targets for drug development and viral inhibition.

2. INTENDED USE

These reagents are intended for the detection of SARS-CoV-2 neutralizing antibodies which block the interaction between the receptor binding domain (RBD) of the viral spike glycoprotein with the ACE2 cell surface receptor.

3. PRINCIPLE OF THE ASSAY

The qualitative immunoenzymatic determination of neutralizing antibodies is based on the ELISA (Enzyme-linked Immunosorbent Assay) technique. Microplates are coated with RBD to bind corresponding ACE2 or blocking antibodies of the sample. After washing the wells to remove all unbound sample material, sample containing the antibody of interest is added and allowed to bind. After incubation a horseradish peroxidase (HRP) labelled ACE2 conjugate is

added and incubated. This conjugate binds to the captured RBD which has not been bound by the antibody sample. In a second washing step unbound conjugate is removed. ACE2-HRP conjugate which has bound (and therefore represents the absence of neutralizing antibody), is visualized by adding Tetramethylbenzidine (TMB) substrate which gives a blue reaction product. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450nm is then read using a suitable microwell plate reader.

4. MATERIALS

4.1. Reagents supplied

This kit contains 4 reagents comprising a positive control anti SARS-CoV-2 RBD antibody, a negative control anti SARS-CoV-2 antibody, a recombinant SARS-CoV-2 RBD protein and a recombinant ACE2 protein, conjugated to horseradish peroxides (HRP):

(1) Negative Control Antibody (MAB12440-NTRL-5): 1 vial containing 100µl monoclonal antibody at 50µg/ml. Total quantity/volume required per 96-well plate is 0.2µg in 1.0 ml.

(2) Positive Control Antibody (MAB12443-NTRL-5): 1 vial containing 100µl monoclonal antibody at 50µg/ml. Total quantity/volume required per 96-well plate is 0.2µg in 1.0 ml.

(3) SARS-CoV-2 RBD Protein (REC31882-NTRL-150): 1 vial containing 150µl (0.15mg at 1mg/ml) recombinant receptor binding domain (RBD) protein. Total quantity/volume required per 96-well plate is 15µg in 15 ml (1µg/ml).

(4) HRP-labelled ACE2 Protein (REC31876-NTRL-10.5) 100X: 1 vial containing 1.5ml recombinant angiotensin converting enzyme-2 (ACE2) protein with BSA and Proclin. Total quantity supplied sufficient for 150ml at working strength.

4.2. Materials and Equipment needed

- High-binding flat bottom microtitre plate – Greiner 762071 or equivalent
- BSA – Sigma A7906 or an equivalent grade
- Tween 20 – Sigma P1379 or equivalent grade
- DPBS pH 7.2 – GIBCO Catalogue no. 14190-136 or equivalent
- TMB High Kinetic – Catalogue no. MO701D, Europa Bioproducts or equivalent
- Stop solution (1M HCl or 1N H_2SO_4).
- Microwell plate reader, equipped for the measurement of absorbance at 450nm

5. STABILITY AND STORAGE

Store the kit frozen at -20°C or below. Freeze in small aliquots to minimize damage due to freezing and thawing.

6. ASSAY PROCEDURE

6.1. Reagent Preparation

Please read the instruction for use carefully before performing the assay. Result reliability depends on strict adherence to the instruction for use as described. The following test procedure is only validated for manual procedure. Perform all assay steps in the order given and without any delays. A clean, disposable tip should be used for dispensing each standard/control and sample.

Required buffers and diluents: The following buffers and diluents are not included and must be prepared fresh by the user (prepared buffers & diluents may be stored frozen -20°C for re-use):

- Coating buffer: Dulbecco's Phosphate Buffered Saline (DPBS)
- Blocking solution: DPBS + 1%BSA (w/v)
- Wash buffer: DPBS + 0.05% Tween20 (v/v)
- Dilution buffer: DPBS + 1%BSA (w/v) + 0.05% Tween20 (v/v)

Prior to use, thaw all the reagents completely at ambient temperature (18-28°C). Mix liquid reagents by repeated gentle inversion or using a roller mixer prior to use.

HRP labelled ACE2 conjugate 100X concentrate:

Dilute the 100X conjugate to 1X working concentration using dilution buffer:

No. of strips	**2**	**4**	**6**	**8**	**10**	**12**
100X ACE2-HRP conjugate (µl)	20	40	60	80	100	120
Dilution buffer (µl)	1980	3960	5940	7920	9900	11880

Mix thoroughly by roller mixing for 10 minutes.

Negative and positive controls: Prepare a working concentration of 0.2µg/ml using dilution buffer.

Samples: Samples that show a precipitate or are highly lipaemic should be centrifuged to clarify the sample as far as possible before use. Perform a 1:20 dilution of the samples (for example, 25µl sample + 475µl dilution buffer); ensure that the diluted samples are thoroughly mixed.

6.2. Assay Protocol

Prepare a plate plan to include duplicate wells for B0, negative, positive and substrate blank controls, plus all samples required (see suggested plate layout, below).

1. Coat the required wells of a 96-well plate with 1ug/ml of RBD antigen using DPBS coating buffer. Add 100µl of the prepared coating solution in all the wells. Seal and incubate the plate for exactly 1 hour static at ambient temperature (18-28°C). Do not extend the coating time.
2. Aspirate the plate contents. Gently tap once on the paper towel to blot off any remainder of the coating solution. Add 1%BSA/PBS blocking solution, 300µl/well to block the plate. Incubate for an hour static at ambient temperature (18-28°C).
3. Aspirate the plate contents, add wash buffer 300µl per well and perform 3 wash/empty cycles, then invert the plate onto an absorbent paper and pat away any excess wash buffer.
4. Add 100µl of dilution buffer in B0 wells and 100µl of diluted controls/samples as per the plate plan. Seal and incubate the plate for 1 hour at ambient temperature (18-28°C), shaken at 800rpm. *Do not add any reagent in the substrate blank wells*.
5. After an hour of incubation DO NOT EMPTY/WASH THE PLATE, add 100µl of diluted ACE2-HRP conjugate (1X) in every well, *except the substrate blank wells*; seal the plate and incubate for 1 hour at ambient temperature (18-28°C) at 800rpm. The total volume of reagent per well on completion of this step is 200µl.
6. After an hour of co-incubation, aspirate the plate contents, taking any safety precautions as required by sample type. Add wash buffer 300µl per well and perform 4 wash/empty cycles, then invert the plate onto an absorbent paper and pat away any excess wash buffer.
7. Add 100µl of TMB substrate in all wells (including the blank wells). Cover the plate with a new plate sealer and incubate the plate for 15 minutes in the dark at ambient temperature (18-28°C). The plate should be protected from direct sunlight.
8. After 15 minutes, add 100µl of stop solution to every used well. Read the plate at 450nm using an ELISA plate reader within 10 minutes of stopping the reaction.

6.3. Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Blank**	**Blank**	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
B	**B0**	**B0**	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
C	**NEG**	**NEG**	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
D	**POS**	**POS**	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
E	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
F	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
G	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
H	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44

*POS – Positive antibody control, NEG – Negative antibody control

7. RESULTS

7.1. Result Calculations

Average the duplicate readings for each control and sample, and subtract the average optical density of Substrate blank wells.

Calculate the binding rate (B/B0%) using the equation:

$$\frac{\text{Average optical density of sample or control bound (B)}}{\text{Average optical density of maximum bound (B0)}} \times 100$$

7.2. Assay Performance Criteria

In order for an assay to be considered valid, the following criteria must be met:

- Substrate Blank: Absorbance value < 0.1
- B0 (Maximum binding): Absorbance value of B0 must be greater than absorbance value of positive control
- Negative Control: Binding rate (B/B0%) for negative control should be > 60%
- Positive Control: Binding rate (B/B0%) for positive control should be < 50%

If these criteria are not met, the test is not valid and must be repeated.

7.3. Interpretation of Results

Cut off (%B/B0) value	Result	Interpretation
≤ 50	Strong positive	SARS-COV-2 neutralizing antibody present
> 50 but < 60	Positive	SARS-COV-2 neutralizing antibody present
≥ 60	Negative	SARS-COV-2 neutralizing antibody absent

7.4. Example Data

This data is an example of the data typically produced with this assay procedure. However, the results obtained by users may not be identical to these. This data is intended as an example only, and must not be used to calculate your sample values.

Sample I.D/Control	O.D. 450nm	Substrate Blank Reduced	B/B0%	Result
Substrate Blank	0.035	0		
B0 (Maximum Binding)	1.324	1.289		
Negative control (MAB12440), 0.2µg/ml	1.388	1.353	105	Negative
Positive control (MAB12443), 0.2µg/ml	0.496	0.461	36	Positive
SARS sample nnnnn13	1.040	1.005	78	Negative
SARS sample nnnnn15	0.920	0.885	69	Negative
SARS sample nnnnn62	0.870	0.835	65	Negative
SARS-COV-2 sample nnnnn81	0.711	0.677	52	Positive
SARS-COV-2 sample nnnnn78	0.483	0.448	35	Positive
SARS-COV-2 sample nnnnn13	0.692	0.657	51	Positive
Pre-pandemic normal serum HSR7849	1.044	1.009	78	Negative
CDC Zika p23-I	0.830	0.795	62	Negative
Chikungunya 465 positive sample	1.085	1.050	81	Negative
Dengue nnnnn16	1.141	1.106	86	Negative
Dengue nnnnn21	0.879	0.844	65	Negative
Dengue nnnnn13	0.985	0.951	74	Negative
Dengue nnnnn20	0.913	0.878	68	Negative
Dengue nnnnn09	0.922	0.887	69	Negative
Dengue nnnnn19	0.930	0.895	69	Negative
West Nile nnnnn176	0.929	0.894	69	Negative

8. PRECAUTIONS AND WARNINGS

- Products are for Research Use or for Further Manufacturing Use only. Not for Diagnostic or Therapeutic Use.
- This kit is only designed for qualified personnel who are familiar with good laboratory practice.
- For potential hazardous substances, please check the safety data sheets.

9. ORDERING INFORMATION

Prod. No.: NTRL-RBD-10 SARS-CoV-2 Neutralization Assay Development Kit (RBD-ACE2)

10. BIBLIOGRAPHY

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