RayBio® COVID-19 Spike Variant-ACE2 Binding Assay Kit I

SARS-CoV-2 inhibitor screening ELISA kit for
COVID-19 drug and antibody screening

User Manual Version 1.1
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Catalog numbers: **CoV-SM2ACE2-1 (1 plate)**
CoV-SM2ACE2-2 (2 plates)
CoV-SM2ACE2-5 (5 plates)

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RayBio® COVID-19 Spike Variant-ACE2 Binding Assay Kit I Protocol

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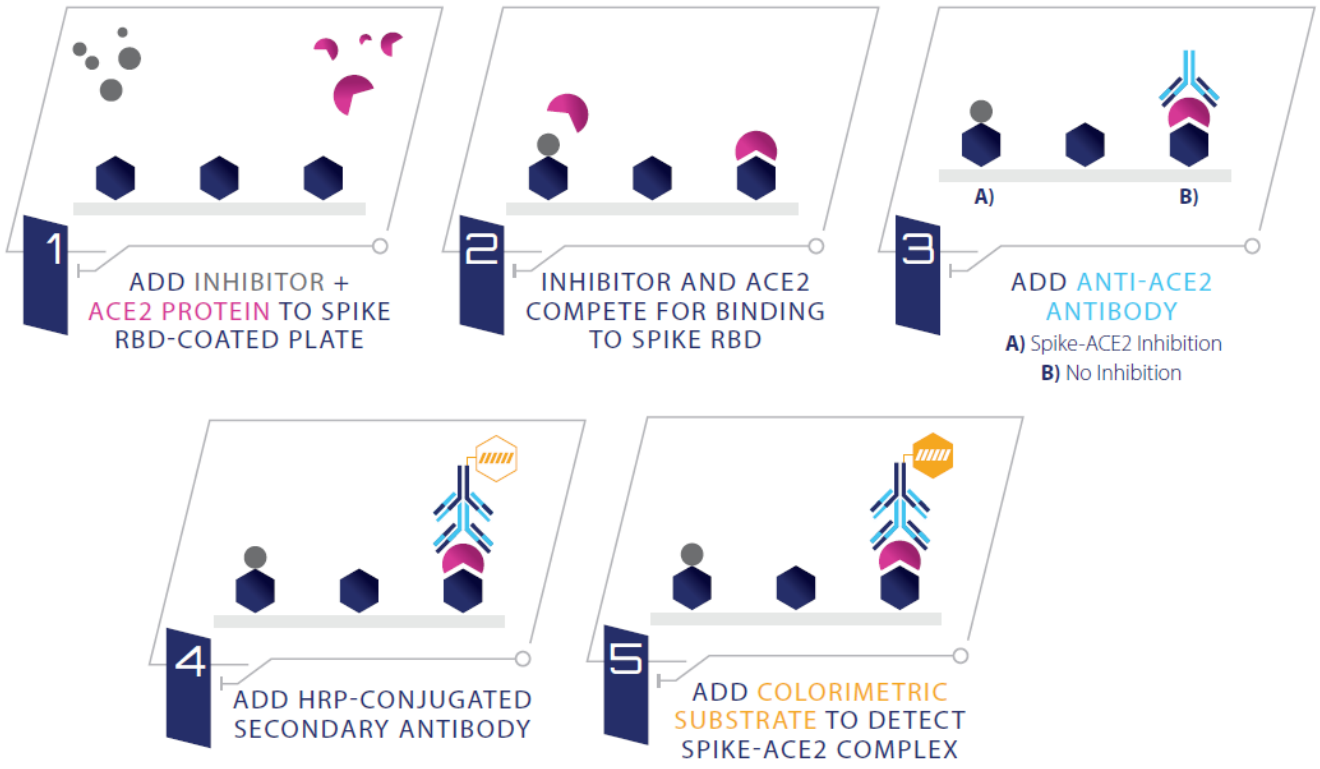
I. INTRODUCTION

The coronavirus disease 2019 (COVID-19) is caused by the SARS-CoV-2 virus. A critical step of infection is when the virus enters human host cells, which is enabled by the interaction between the SARS-CoV-2 Spike (S) protein's receptor binding domain (RBD) on the surface of the viral particle and the Angiotensin Converting Enzyme 2 (ACE2) receptor on the surface of human cells. Thus, the identification of small molecules, antibodies, or other biological molecules that interfere with the formation of the S-ACE2 complex could help develop drugs to prevent or treat COVID-19.

The RayBio® COVID-19 Spike Variant-ACE2 binding assay kit is a rapid, simple, and sensitive method to characterize the binding affinity of the S-ACE2 complex in the presence of potential inhibitors within 5 hours. The kit is compatible with various inhibitor types, including small molecules, peptides, antibodies, and patient serum. For example, this kit can be used to screen inhibitor activity, help develop COVID-19 vaccines, and test potential therapeutic drugs to treat COVID-19.

The RayBio® COVID-19 Spike Variant-ACE2 binding assay uses a 96-well plate coated with recombinantly-expressed S-RBD. The testing reagent-of-choice is then added to the wells in the presence of recombinant human ACE2 protein. Unbound ACE2 is removed with washing, and a goat anti-ACE2 antibody is added that binds to the Spike Variant-ACE2 complex. HRP-conjugated anti-goat IgG is then applied to the wells in the presence of 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The HRP-conjugated anti-goat IgG binds to the ACE2 antibody and reacts with the TMB solution, producing a blue color that is proportional to the amount of bound ACE2. The HRP-TMB reaction is halted with the addition of the Stop Solution, resulting in a blue-to-yellow color change. The intensity of the yellow color is then measured at 450 nm.



A schematic showing how the RayBio® Spike Variant-ACE2 Binding Assay Kit I can measure the inhibition of the Spike RBD and ACE2 in the presence of a potential inhibitor.

II. STORAGE

The kit may be stored at 4°C up to 1 month from the date of shipment.

III. MATERIALS PROVIDED

COMPONENT	SIZE / DESCRIPTION	STORAGE / STABILITY AFTER PREPARATION
S-RBD-coated Microplate (Item A-2)	96 wells (12 strips x 8 wells) coated with recombinant SARS-CoV-2 Spike Mutant RBD (N501Y)	1 month at 4°C*
Wash Buffer Concentrate (20x) (Item B)	25 ml of 20x concentrated solution	1 month at 4°C
5x Assay Diluent (Item E2)	15 ml of 5x concentrated buffer**	1 month at 4°C
ACE2 Protein (Item F)	2 vials of purified recombinant ACE2 Protein (1 vial is enough to assay half of the microplate)	5 days at 4°C
Detection Antibody ACE2 (Item C-1)	2 vials of Goat anti- ACE2 IgG antibody (1 vial is enough to assay half of the microplate)	5 days at 4°C
HRP-Conjugated Anti-Goat IgG (Item D-2)	15 µl of 1000x concentrated HRP-Conjugated Anti-Goat IgG	5 days at 4°C
TMB One-Step Substrate Reagent (Item H)	12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution	N/A
Stop Solution (Item I)	8 ml of 0.2 M sulfuric acid	N/A

**Return unused wells to the pouch containing desiccant pack, reseal along entire edge.*

***This item is used to dilute the "Test Reagent" (i.e., the potential inhibitor), ACE2 Protein (Item F), Detection Antibody ACE2 (Item C-1), and HRP-Conjugated Anti-Goat IgG (Item D-2).*

IV. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450 nm
2. Shaker
3. Pipettes capable of accurately delivering 2 µl to 1 ml volumes
4. Pipettes capable of delivering 1 – 25 ml volumes for reagent preparation
5. Graduated cylinders: 100 ml and 1 liter
6. Distilled or deionized water
7. Tubes to prepare “Test Reagent” sample dilutions

V. REAGENT PREPARATION

1. Bring all reagents and “Test Reagent” samples to room temperature (18 - 25°C) before use.
2. 5x **Assay Diluent** (Item E2) should be diluted 5-fold with deionized or distilled water before use to make a “1x Assay Diluent.”
3. If the **Wash Buffer Concentrate** (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 25 ml of Wash Buffer Concentrate into deionized or distilled water to yield 500 ml of “1x Wash Buffer.”

   Note: *500 ml of 1x Wash Buffer is enough to wash the plate as recommended.*

4. Briefly spin the **ACE2 Protein** (Item F) before use. Add **100 µl** of 1x Assay Diluent into the **ACE2 Protein** vial to prepare a “50x ACE2 Protein Concentrate”. Pipette up and down to mix gently. This protein concentrate should then be diluted 50-fold with 1x Assay Diluent to yield a “1x ACE2 protein” solution and will be used in Part VII, step 6. (see also Part VI, Test Reagent Sample Preparation on page 6)
5. Briefly spin the **Detection Antibody ACE2** (Item C-1) before use. Add 100 µl of 1x Assay Diluent into the vial to prepare a 55x detection antibody concentrate. Pipette this detection antibody concentrate up and down to mix gently. This detection antibody concentrate should then be diluted 55-fold with 1x Assay Diluent to yield a “1x Detection Antibody” solution and will be used in Part VII, step 6.
6. Briefly spin the **HRP-Conjugated Anti-Goat IgG** (Item D-2) before use. HRP-Conjugated Anti-Goat IgG should be diluted 1000-fold with 1x Assay Diluent to yield a “1x HRP-conjugated IgG” solution. Each reaction requires 100µl of 1x HRP-conjugated IgG solution, thus, the researcher should create enough volume to perform the desired number of reactions for each experiment.

VI. TEST REAGENT SAMPLE PREPARATION

General Considerations

Dose Response. When evaluating a potential inhibitor (i.e., the Test Reagent) for its ability to block formation of the Spike Variant-ACE2 complex, it is highly recommended to perform a titration curve. This approach will help the researcher to empirically determine the dose-responsive range of Test Reagent as well as the lowest level that yields detectable inhibition. It will also help confirm whether the inhibition is real; that is, whether the inhibition increases as more Test Reagent is applied.

Since different Test Reagents will inhibit the Spike Variant-ACE2 binding to different extents, there is no recommended starting dilution. Rather, it is encouraged that the researcher chooses a dilution series based on their knowledge of the Test Reagent.

Preparation of Reactions. Since the ACE2 protein putatively competes with the Test Reagent for binding to ACE2, it is critical that ACE2 be present in the same concentration in every well. An example is provided below of how to prepare a Test Reagent serial dilution in which the concentration of ACE2 is held constant while the Test Reagent varies. Note that the researcher should determine an appropriate serial dilution based on the known properties of their Test Reagent. From the result of this dilution series, the best dilution of the Test Reagent can be empirically determined.

Serial Dilution Preparations.

1. Label a series of tubes for preparation of a serial dilution of the Test Reagent. For an example of how to set up this serial dilution, refer to the Serial Dilution Example below.
2. Mix the Test Reagent with the Assay Diluent and ACE2 Protein Concentrate (prepared on page 5 in Part V, step 4) to create the first reaction mix. NOTE: it is recommended that all Test Reagent samples be run at least in duplicate. Therefore, replicates should be taken into account when calculating the volumes to be prepared.
3. Create the remaining reaction mixes according to the dilution series set forth in step 1 of the example below, or as needed for the researchers chosen dilutions.

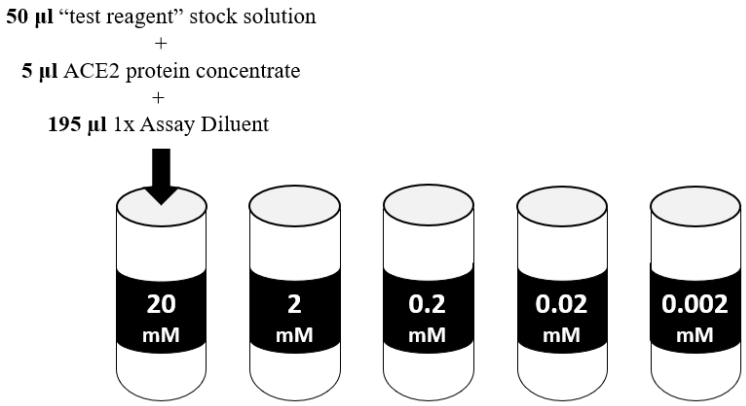
SERIAL DILUTION EXAMPLE

In this example, the Test Reagent stock solution = 100 mM, and the researcher wishes to test 20 mM, 2 mM, 0.2 mM, 0.02 mM, and 0.002 mM dilutions of the Test Reagent.

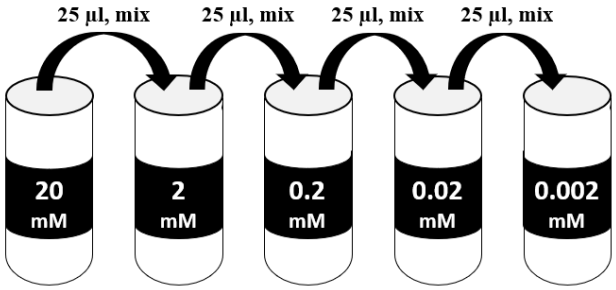
1. *Label 5 serial dilution tubes as follows: 20 mM, 2 mM, 0.2 mM, 0.02 mM, and 0.002 mM.*
2. *Make the first reaction mix:*
    a. *In the tube labeled "20 mM", prepare the first reaction mix by mixing the following components:*
        - *50 µl of the Test Reagent stock solution*
        - *5 µl of ACE2 Protein Concentrate (prepared on page 5 in Part V, 4)*
        - *195 µl 1x Assay Diluent*
    b. *Mix thoroughly. This creates sufficient volume to run duplicate wells.*

NOTE: It is strongly recommended to include a vehicle control to assess effects of the Test Reagent's solvent or buffer on the Spike RBD-ACE2 reaction. For example, if the Test Reagent is in dimethyl sulfoxide (DMSO), a parallel set of tubes should be prepared with 50 µl of DMSO (step 2 above).

3. *Make the remaining reaction mixes:*
    a. *The 50x ACE2 Protein Concentrate prepared in Part V step 4 (page 5) should be diluted 50-fold with Assay Diluent to make a 1x ACE2 Protein Working Solution.*
    b. *Pipette 225 µl of the 1x ACE2 Protein Working Solution into the remaining four empty tubes (2 mM, 0.2 mM, 0.02 mM, 0.002 mM):*



    c. *Pipette 25 µl from the first reaction mix (20 mM tube) into the second serial dilution tube (2 mM). Mix thoroughly.*
    d. *Repeat for each serial dilution, using 25 µl of the prior concentration until the final concentration is reached:*



    e. *Finally, pipette 250 µl of the 1x ACE2 Protein Working Solution into a separate tube labeled "0 mM." Mix thoroughly. This reaction contains no Test Reagent and will serve as the positive control.*

NOTE: You will need to perform this positive control sample, as well as any vehicle control samples for every individual assay performed, even after the best dilution of your Test Reagent is identified.

VII. ASSAY PROCEDURE

1. Bring all reagents to room temperature (18 - 25°C) before use. It is recommended that all controls and Test Reagent samples be run at least in duplicate.

2. Label removable 8-well strips as appropriate for your experiment.

3. As prepared above in Part VI, **add 100 µl of each Test Reagent sample** into an appropriate well.

   Note: For data reliability, it is recommended that all Test Reagent samples should be run in at least duplicate.

4. Cover wells with the provided plate sealing film and incubate for 2.5 hours at room temperature or overnight at 4°C with gentle shaking.

5. Discard the solution and **wash 4 times** with 1x Wash Solution. Wash by filling each well with 1x Wash Buffer (300 µl) using a multi-channel pipette or auto-washer. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining 1x Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 100 µl of prepared **1x Detection Antibody** (Reagent Preparation Step 5) to each well. Incubate for 1 hour at room temperature with gentle shaking.

7. Discard the solution and repeat the wash step as described Step 5 above.

8. Add 100 µl of the prepared **1x Anti Goat HRP-conjugated IgG** (see Reagent Preparation Step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.

9. Discard the solution. Repeat the wash as described in Step 5 above.

10. Add 100 µl of **TMB One-Step Substrate Reagent** (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

11. Add 50 µl of **Stop Solution** (Item I) to each well.

12. Read at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents and Test Reagent samples as instructed.

2. Add 100 µl Test Reagent samples to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.

3. Add 100 µl prepared Detection Antibody to each well. Incubate 1 hour at room temperature.

4. Add 100 µl the prepared 1X HRP-conjugated IgG antibody solution. Incubate 1 hour at room temperature.

5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.

6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

IX. DATA ANALYSIS

1. Determine the average absorbance across the replicate readings for each Test Reagent and positive control samples performed.
2. *Optional:* If running the recommended vehicle control as well, subtract these values from your Test Reagent values.
3. Compare the Test Reagent sample data to the positive control, 0 mM sample (no Test Reagent).

   Note: *A Test Reagent's sample absorbance will decrease if the Spike RBD-ACE2 interaction is inhibited when compared to the positive control, and that reduction can be compared directly by measuring the percent binding inhibition of the interaction against the positive control.*

4. Determine the percent binding inhibition (BI%):

   BI% = [1 – (OD of Test Reagent Well / OD of positive control)] x 100

   Note: *This calculation uses OD's after subtraction of vehicle control. The "positive control" is the "no inhibitor control."*

Need further characterization of your inhibitor? *RayBiotech offers a COVID-19 Pseudovirus Service to analyze Spike Variant-ACE2 neutralization activity in cell culture.*

X. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
Too low sample signal	Sample concentration is too low	Increase sample concentration
Too high sample signal	Sample concentration is too high	Reduce sample concentration
Large CV	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed	Review the manual for proper washing. If using an automated plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low positive control signal	Improper storage of the ELISA kit	Upon receipt, the kit should be stored at -20°C. Store the positive control at -70°C or below after reconstitution.
	Stop solution	Stop solution should be added to each well before measurement and read OD immediately.
	Improper primary or secondary antibody dilution	Ensure correct dilution

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