RayBio® COVID-19 Spike-ACE2 binding assay kit

For COVID-19 drug and antibody screening

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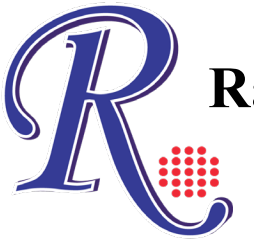
Catalog numbers: **CoV-SACE2-1 (1 plate)**
CoV-SACE2-2 (2 plates)
CoV-SACE2-5 (5 plates)



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RayBio® COVID-19 Spike-ACE2 binding assay kit 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 I 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-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-10 vaccines, and test potential therapeutic drugs to treat COVID-19.

The RayBio® COVID-19 Spike-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-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.

Note: RayBiotech also offers this binding assay in a different format (catalog # CoV-ACE2S2) where the ACE2 is immobilized and the S-RBD protein is added in the presence of the test reagent. The user must determine which format(s) they wish to use.

II. MATERIAL PROVIDED

1. **COVID-19 S-protein Microplate (Item A)**
   96 wells (12 strips x 8 wells) coated with recombinant COVID-19 S-protein RBD domain
2. **Wash Buffer Concentrate (20x) (Item B)**
   25 ml of 20x concentrated solution
3. **5x Assay Diluent (Item E2)**
   15 ml of 5x concentrated buffer
   This item is used to dilute the "test reagent" (that is, the potential inhibitor), ACE2 protein (Item F), detection antibody (Item C) and HRP-conjugated IgG Concentrate (Item D).
4. **ACE2 protein (Item F)**
   2 vials of purified human recombinant ACE2 protein (1 vial is enough to assay half microplate)
5. **Detection Antibody ACE2 (Item C-1)**
   2 vials of goat anti-ACE2 (1 vial is enough to assay half microplate)
6. **HRP-conjugated Anti-goat IgG (Item D-1)**
   25 µl of 1000x concentrated HRP-conjugated anti-goat IgG
7. **TMB One-Step Substrate Reagent (Item H)**
   12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution
8. **Stop Solution (Item I)**
   8 ml of 0.2 M sulfuric acid

III. STORAGE

Upon receipt, the kit should be stored at 4°C. Use within 6 months from the date of shipment.

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 sample dilutions.

V. SAMPLE PREPARATION

1. Mix your "test reagent" (i.e., potential inhibitor) with ACE2 protein concentrate (see "Serial Dilution Example" and "Part VI, step 4")

   Note: *It is recommended that all samples be run in at least duplicate.*

2. Dilute the mixture with 1x Assay Diluent dilute to make a 1x ACE2 protein working concentration. (See "Part VI, step 4")

   Note: *Each "test reagent" sample should have the same 1x ACE2 protein concentration.*

3. Optional: Perform serial dilutions

   Note: *The optimal dilutions must be determined empirically by the researcher. For the initial experiment, we recommend performing a serial dilution (e.g., 5-fold to 5000-fold) to determine the optimal amount of test reagent to use.*

   SERIAL DILUTION EXAMPLE

   1. *Using a 100 mM stock solution of the "test reagent," label and prepare 5 serial dilution tubes labeled: 20 mM, 2 mM, 0.2 mM, 0.02 mM, and 0.002 mM.*

   2. *Prepare the first serial dilution tube by mixing the following together in the tube labeled "20 mM" for duplicate analyses:*

   - *i. 50 µl of the "test reagent" stock solution*
   - *ii. 2.5 µl of ACE2 protein concentrate (prepared in Part VI, 4)*
   - *iii. 197.5 µl 1x Assay Diluent into the first tube labeled "20 mM".*
   - *iv.* ***Note:*** *It is highly recommended that a control be included to account any effects that the "test reagent's" buffer may have on the Spike-ACE2 interaction. If the "test reagent" is in dimethyl sulfoxide (DMSO), for example, a parallel set of tubes should be prepared with 50 µl of DMSO in step 2i.*

   3. *Mix thoroughly.*

4. *Pipette 225 µl of the 1x ACE2 protein working solution (prepared in Part VI, 4) into the remaining five labeled tubes (2 mM, 0.2 mM, 0.02 mM, 0.002 mM).*
5. *Pipette 25 µl from the prepared 20 mM tube into the second serial dilution tube (2 mM). Mix thoroughly.*
6. *Repeat step 5 for each serial dilution, using 25 µl of the prior concentration until the final concentration is reached.*
7. *Pipette 225 µl of the 1x ACE2 protein working solution (prepared in Part VI, 4) into a separate tube labeled "0 mM." Pipette 25 µl from the 1x Assay Diluent into the tube labeled "0 mM." Mix thoroughly. This will serve as the positive control.*

VI. REAGENT PREPARATION

1. Bring all reagents and 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 Concentrate** (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of "1x Wash Buffer."

4. Briefly spin the **ACE2 protein** (Item F) before use.

    a. Add **50 µl** of 1x Assay Diluent into the Item F vial to prepare an ACE2 protein concentrate. Pipette up and down to mix gently.
    (see also "Part V, Sample Preparation" on page 4)

    Note: *The amount of 1x Assay Diluent that is added to Item F differs between the two Spike-ACE2 binding assay kit formats (cat no. CoV-SACE2, CoV-ACE2S2). The volume indicated in this manual is specific to catalog number "CoV-SACE2."*

b. The ACE2 protein concentrate should be diluted 100-fold with 1x Assay Diluent to yield a "1x ACE2 Working Solution" *(see also "Part V, Sample Preparation" on page 4)*

5. Briefly spin the **Detection Antibody** (Item C-1) before use. Add 100 µl of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days or at -80°C for one month). The goat anti-ACE2 antibody concentrate should be diluted 55-fold with 1x Assay Diluent and used in *Part VII, step 4* to yield a "1x Detection Antibody" solution.

6. Briefly spin the **HRP-conjugated anti-goat IgG** (Item D-1) before use. HRP-conjugated anti-goat IgG concentrate should be diluted 1000-fold with 1x Assay Diluent to yield a "1x HRP-conjugated anti-goat IgG" solution.

EXAMPLE: Briefly spin the vial to collect contents to the bottom. Add 5 µl of HRP-conjugated anti-goat IgG concentrate into a tube with 5 mL 1x Assay Diluent, then pipette up and down to mix gently to prepare a 1000-fold diluted HRP-conjugated anti-goat IgG solution. Mix well.

VII. ASSAY PROCEDURE:

1. Bring all reagents to room temperature (18 - 25°C) before use.

2. **Add 100 µl of each sample** into an appropriate well.

   Note: It is recommended that all samples should be run in at least duplicate. (See also Parts V – VI)

3. Cover well with plate holder and **incubate** for 2.5 hours at room temperature or overnight at 4°C with shaking.

4. 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 autowasher. Complete removal of liquid at each step is essential to 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.

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

6. Discard the solution. Repeat the wash as described in Step 3.

7. Add 100 µl of prepared **1x HRP-conjugated anti-goat IgG** (see Reagent Preparation Step 6) to each well. Incubate for 1 hour at room temperature with shaking.

8. Discard the solution. Repeat the wash as described in Step 3.

9. 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 shaking.

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

11. **Read** at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents and samples as instructed.

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2. Add 100 µl sample to each well.
   Incubate 2.5 hours at room temperature or overnight at 4ºC.

⇩

3. Add 100 µl prepared detection antibody to each well.
   Incubate 1.0 hours at room temperature.

⇩

4. Add 100 µl prepared 1X HRP-conjugated 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

Determine the average absorbance across the replicate readings. Compare the "test reagent" sample data to the "0 nM" (no test reagent) positive control. Absorbance will decrease if the Spike-ACE2 interaction is inhibited.

Need further characterization of your inhibitor? *RayBiotech offers a COVID-19 Pseudovirus Service to analyze Spike-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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