



## SARS-CoV-2 Spike-ACE2 Interaction Inhibitor Screening Assay Kit

Item No. 502050

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## GENERAL INFORMATION

### Materials Supplied

Item Number	Item	Quantity/Size	Storage
402056	Spike Inhibitor Screening Reagent	3 vials	-20°C
402057	ACE2 Inhibitor Screening Reagent	3 vials	-20°C
402054	Anti-His-HRP Conjugate (50X)	1 vial/360 µl	-20°C
402055	SARS-CoV-2 Inhibitor Control	1 vial	-20°C
401703	Immunoassay Buffer C Concentrate (10X)	1 vial/10 ml	4°C
400108	Immunoassay Buffer D Concentrate (5X)	1 vial/10 ml	4°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
400004/400006	Mouse Anti-Rabbit IgG-Coated Plate	1 plate	4°C
400074	TMB Substrate Solution	2 vials/12 ml	4°C
10011355	HRP Stop Solution	1 vial/12 ml	RT
400012	96-Well Cover Sheet	1 ea	RT

If any of the items listed on page 3 are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.



**WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.**

## Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

## Precautions

**Please read these instructions carefully before beginning this assay.**

This kit may not perform as described if any reagent or procedure is replaced or modified.

## If You Have Problems

### Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3640

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

## Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

## Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm
2. An orbital microplate shaker
3. Adjustable pipettes and a multichannel or repeating pipetor
4. A source of ultrapure water is recommended. Pure water - glass-distilled or deionized - may not be acceptable *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*

## INTRODUCTION

### Background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped positive-stranded RNA virus and a member of the *Betacoronavirus* genus.<sup>1,2</sup> It is the causative agent of COVID-19, a primarily respiratory illness characterized by fever, cough, and shortness of breath that can lead to life-threatening complications.<sup>3-5</sup> The SARS-CoV-2 genome contains approximately 30 kilobases encoding four structural proteins: surface glycoprotein, envelope, membrane, and nucleocapsid.<sup>1,2</sup> The surface glycoprotein, also known as the spike glycoprotein, is located on the outer envelope of the virion.<sup>1</sup> It is comprised of an S1 and S2 subunit divided by a furin S-cleavage site not found in other SARS-CoVs.<sup>6,7</sup> The S1 subunit contains the receptor binding domain (RBD), which binds to the carboxypeptidase angiotensin-converting enzyme 2 (ACE2) following spike glycoprotein preactivation by furin and TMPRSS2, which cleave it at the S1/S2 and S2' sites, respectively, facilitating viral fusion with the host cell membrane.<sup>8-12</sup> In this way, ACE2 acts as the functional receptor for SARS-CoV-2.

ACE2 is expressed in vascular endothelial cells, as well as in the epithelial cells of the kidney, heart, lung, small intestine, and liver.<sup>13</sup> It acts as a negative regulator of signaling through angiotensin II by converting angiotensin II to the vasodilatory and anti-inflammatory peptide angiotensin 1-7.<sup>14</sup> ACE2 is downregulated by SARS-CoV-2 binding, which disrupts the protective effects of angiotensin 1-7.<sup>14</sup> The SARS-CoV-2-ACE2 interaction is a potential target for reducing viral infection. Recombinant human soluble ACE2 inhibits SARS-CoV-2 attachment to cells, and antibodies in convalescent plasma or those directly raised against the SARS-CoV2 spike glycoprotein reduce viral entry *in vitro*.<sup>8,15</sup>

The broad expression of ACE2 allows SARS-CoV-2 to disrupt function in a wide variety of cells and tissues, but the mechanism of SARS-CoV-2-ACE2 binding provides multiple targets that can potentially be exploited to reduce cellular infection and the severity of COVID-19.

### About This Assay

Cayman's SARS-CoV-2 Spike-ACE2 Interaction Inhibitor Screening Assay Kit provides a robust and easy-to-use platform for identifying novel inhibitors of the SARS-CoV-2 spike and ACE2 interaction. The assay uses a recombinant rabbit Fc-tagged SARS-CoV-2 spike S1 RBD that binds to a plate precoated with a mouse anti-rabbit antibody. A recombinant His-tagged ACE2 protein binds the spike RBD and the complex is detected with an HRP-conjugated anti-His antibody, which is easily quantified by reading the absorbance at 450 nm. A control is included for competition of the SARS-CoV-2 spike RBD-ACE2 interaction.

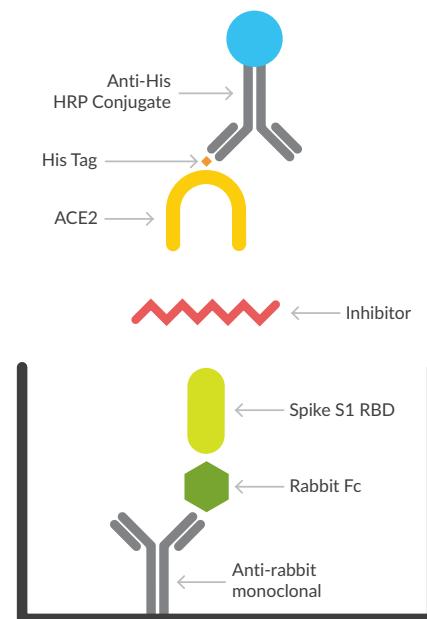


Figure 1. Schematic of the SARS-CoV-2 Spike-ACE2 Inhibitor Screening Assay Kit

### Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for approximately two months.

*NOTE: It is normal for the concentrated buffers to contain crystalline salts after thawing. These will completely dissolve upon dilution with ultrapure water.*

#### 1. Immunoassay Buffer C (1X) Preparation

Dilute the contents of one vial of Immunoassay Buffer C Concentrate (10X) (Item No. 401703) with 90 ml of ultrapure water. Be certain to rinse the vial to remove any salts that may have precipitated.

#### 2. Immunoassay Buffer D (1X) Preparation

Dilute the contents of one vial of Immunoassay Buffer D Concentrate (5X) (Item No. 400108) with 40 ml of ultrapure water. Be certain to rinse the vial to remove any salts that may have precipitated.

#### 3. Wash Buffer Preparation

Dilute the contents of one vial of Wash Buffer Concentrate (400X) (Item No. 400062) with ultrapure water to a total volume of 2 L and add 1 ml of Polysorbate 20 (Item No. 400035). Smaller volumes of Wash Buffer (1X) can be prepared by diluting the Wash Buffer Concentrate (400X) 1:400 and adding Polysorbate 20 to an end concentration of 0.5 ml/L. *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.*

### Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in Immunoassay Buffer C (1X) at a concentration 3X the desired final assay concentration (e.g., for 50 µl of sample with a final volume of 150 µl per well). This solution may contain up to 1% DMSO, DMF, or ethanol. The final concentration of organic solvents in the assay will then be ≤0.33% (see 'Effects of Solvents' on page 23).

## Reagent Preparation

### 1. Spike Inhibitor Screening Reagent

This kit includes three vials of lyophilized Spike Inhibitor Screening Reagent (Item No. 402056). One vial, after reconstitution, is a sufficient amount of reagent for one-third of a 96-well plate. At the time of the assay, reconstitute one of the vials with 2 ml of Immunoassay Buffer C (1X). Use immediately after reconstitution. Prepare and pool additional vials as needed.

### 2. ACE2 Inhibitor Screening Reagent

This kit includes three vials of lyophilized ACE2 Inhibitor Screening Reagent (Item No. 402057). One vial, after reconstitution, is a sufficient amount of reagent for one-third of a 96-well plate. At the time of the assay, reconstitute one of the vials with 2 ml of Immunoassay Buffer C (1X). Use immediately after reconstitution. Prepare and pool additional vials as needed.

### 3. Anti-His-HRP Conjugate (1X)

Dilute the contents of one vial of Anti-His-HRP Conjugate (50X) (Item No. 402054) with 18 ml of Immunoassay Buffer D (1X). It is recommended that the HRP conjugate be diluted immediately prior to performing the assay. If all of the Anti-His-HRP Conjugate (50X) will not be used at one time, store the undiluted conjugate at 4°C where it will be stable for at least 6 months.

### 4. SARS-CoV-2 Inhibitor Control

Reconstitute the SARS-CoV-2 Inhibitor Control (Item No. 402055) with 250 µl of ultrapure water. Mix 50 µl of SARS-CoV-2 Inhibitor Control with 150 µl of Immunoassay Buffer C (1X). If all of the SARS-CoV-2 Inhibitor Control will not be used at one time, aliquot the reconstituted reagent and store at -80°C where it will be stable for at least 3 months.

## ASSAY PROTOCOL

### Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate prior to adding the reagents. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

There is no specific pattern for using the wells on the plate. However, each plate or set of strips must contain a minimum of two blank, two background, and two 100% initial activity wells. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results. Each inhibitor (including the provided control) should be assayed in duplicate. For statistical purposes, we recommend assaying inhibitors in triplicate.*

A suggested plate format is shown in Figure 2, on page 12. The user may vary the location and type of wells present as necessary for each particular experiment. We suggest recording the contents of each well on the template sheet provided (see page 29).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	Blk	Blk	5	5	5	13	13	13	21	21	21
B	Bkg	Bkg	Bkg	6	6	6	14	14	14	22	22	22
C	100%	100%	100%	7	7	7	15	15	15	23	23	23
D	Ctrl	Ctrl	Ctrl	8	8	8	16	16	16	24	24	24
E	1	1	1	9	9	9	17	17	17	25	25	25
F	2	2	2	10	10	10	18	18	18	26	26	26
G	3	3	3	11	11	11	19	19	19	27	27	27
H	4	4	4	12	12	12	20	20	20	28	28	28

Blk = Blank

Bkg = Background

100% = 100% Initial Activity

Ctrl = Inhibitor Control

1-28 = Inhibitors

Figure 2. Sample plate format

### General Information

- The final volume of the assay is 150  $\mu$ l in all the wells.
- Use the diluted assay buffer in the assay.
- All reagents should be prepared as described above and kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- If the appropriate inhibitor concentration is not known, it may be necessary to assay at several concentrations.
- It is recommended to assay the samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at room temperature with shaking.

## Performing the Assay

### Pipetting Hints

- Use different tips to pipette each reagent.
- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

## Addition of the Reagents

### 1. Immunoassay Buffer C

Add 100  $\mu$ l Immunoassay Buffer C (1X) to background wells. Add 50  $\mu$ l Immunoassay Buffer C (1X) to 100% initial activity wells. *NOTE: If the unknown inhibitor is in a solvent, use the same solvent concentration diluted in Immunoassay Buffer C (1X) for the 100% initial activity wells.*

### 2. Inhibitor/Control

Add 50  $\mu$ l of unknown inhibitor or diluted SARS-CoV-2 Inhibitor Control per well. Each inhibitor should be assayed in duplicate (triplicate recommended). If inhibitors in different solvents are to be assayed at the same time, separate sets of 100% initial activity wells should be run for each solvent. *NOTE: To determine an  $IC_{50}$  value for an inhibitor, multiple concentrations of the inhibitor should be tested in the assay.*

### 3. ACE2 Inhibitor Screening Reagent

Add 50  $\mu$ l ACE2 Inhibitor Screening Reagent to each well except the blank wells.

### 4. Spike Inhibitor Screening Reagent

Add 50  $\mu$ l Spike Inhibitor Screening Reagent to each well except the blank and background wells.

## First Incubation

Cover the plate with a 96-Well Cover Sheet (Item No. 400012) and incubate for 60 minutes at room temperature on an orbital shaker.

## Addition of the Anti-His-HRP Conjugate

### 1. Wash plate

Empty the wells and rinse five times with ~300  $\mu$ l Wash Buffer (1X). Each well should be completely filled with Wash Buffer (1X) during each wash. Invert the plate between wash steps to empty the fluid from the wells. After the last wash, gently tap the inverted plate on absorbent paper to remove the residual Wash Buffer (1X).

### 2. Anti-His-HRP Conjugate

Add 150  $\mu$ l of Anti-His-HRP Conjugate (1X) to each well except the blank wells.

## Second Incubation

Cover the plate with a 96-Well Cover Sheet and incubate for 30 minutes at room temperature on an orbital shaker.

## Development of the Plate

1. Empty the wells and rinse five times with ~300  $\mu$ l Wash Buffer (1X).
2. Add 175  $\mu$ l TMB Substrate Solution (Item No. 400074) to each well.
3. Cover the plate with the 96-Well Cover Sheet. Incubate the plate for 15 to 30 minutes at room temperature on an orbital shaker. Allow the wells to turn medium-to-dark blue before stopping the reaction. *NOTE: This reaction can be monitored by reading absorbance at 650 nm prior to stopping the reaction. An optical density (OD) of 0.8-1.0 at 650 nm ( $OD_{650}$ ) yields an  $OD_{450}$  of approximately 2.0-2.5. Do not overdevelop.*
4. Remove the plate cover being careful to keep TMB Substrate Solution from splashing on the cover. *NOTE: Any loss of TMB Substrate Solution will affect the absorbance readings.*
5. **DO NOT WASH THE PLATE.** Add 75  $\mu$ l of HRP Stop Solution (Item No. 10011355) to each well of the plate. Blue wells should turn yellow and colorless wells should remain colorless. *NOTE: The stop solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.*

## Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Read the plate at a wavelength of 450 nm.

Result	Interpretation
No/Low Signal	Inhibitor binds ACE2 and interferes
	Inhibitor binds spike-RBD and interferes
High Signal	Inhibitor does not bind
	Inhibitor binds ACE2 and does not interfere
	Inhibitor binds spike-RBD and does not interfere

Table 1. Interpretation of results

## ANALYSIS

### Calculations

*NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.*

1. Determine the average absorbance (AA) of each sample.
2. Subtract the AA of the background wells from the AA of the 100% initial activity and inhibitor wells. These are the corrected values.
3. Determine the percent inhibition or percent activity for each inhibitor using one of the following equations:

$$\% \text{ inhibition} = \left[ \frac{(\text{corrected 100\% initial activity} - \text{corrected inhibitor activity})}{\text{corrected 100\% initial activity}} \right] \times 100$$

$$\% \text{ activity} = \left[ \frac{(\text{corrected inhibitor activity})}{\text{corrected 100\% initial activity}} \right] \times 100$$

4. Graph the percent inhibition or percent activity as a function of inhibitor concentration to determine the  $IC_{50}$  value (the concentration at which there is 50% inhibition) of the inhibitor. Inhibition of the SARS-CoV-2 spike RBD-ACE2 interaction by the SARS-CoV-2 Inhibitor Control is shown in figure 3 (see page 21).

## Performance Characteristics

### Z' Factor:

Z' factor is a term used to describe the robustness of an assay, which is calculated using the equation below.<sup>16</sup>

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|}$$

Where  $\sigma$ : Standard deviation  
 $\mu$ : Mean  
c+: Positive control  
c-: Negative control

The theoretical upper limit for the Z' factor is 1.0. A robust assay has a Z' factor >0.5. The Z' factor for Cayman's SARS-CoV-2 Spike-ACE2 Interaction Inhibitor Screening Assay Kit was determined to be 0.86.

### Sample Data:

The data shown here is an example of the data typically produced with this kit; however, your results will not be identical to these. Do not use the data below to directly compare to your samples. Your results could differ substantially.

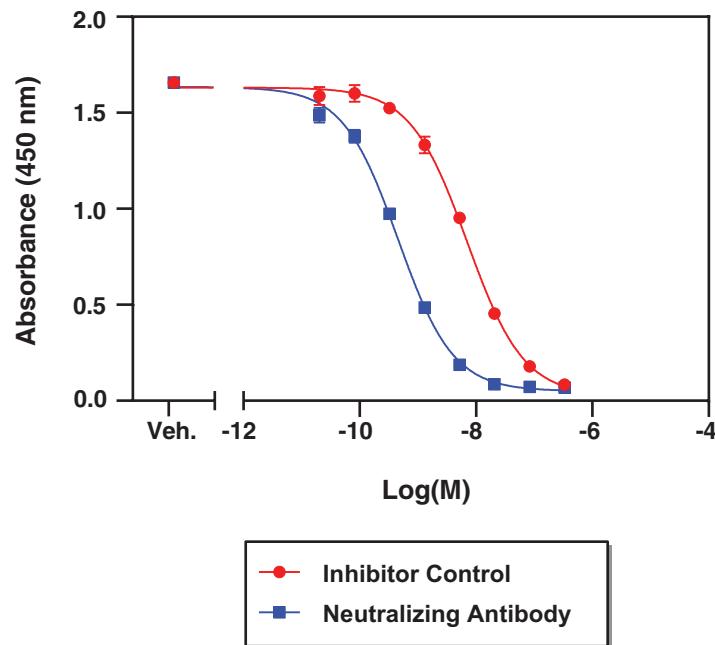
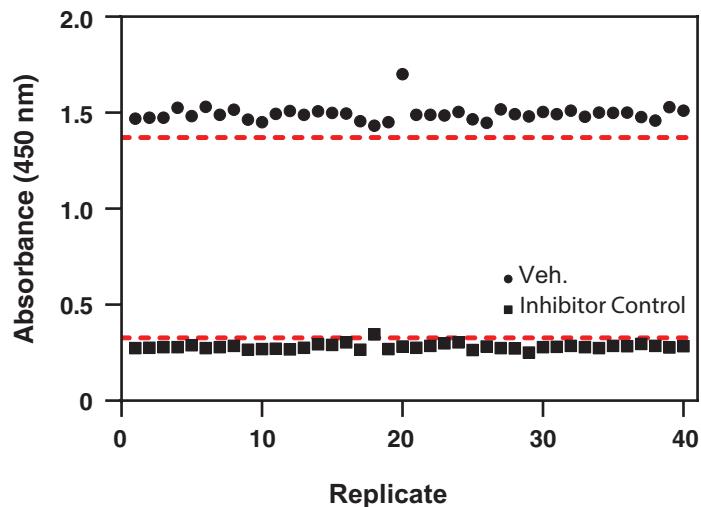


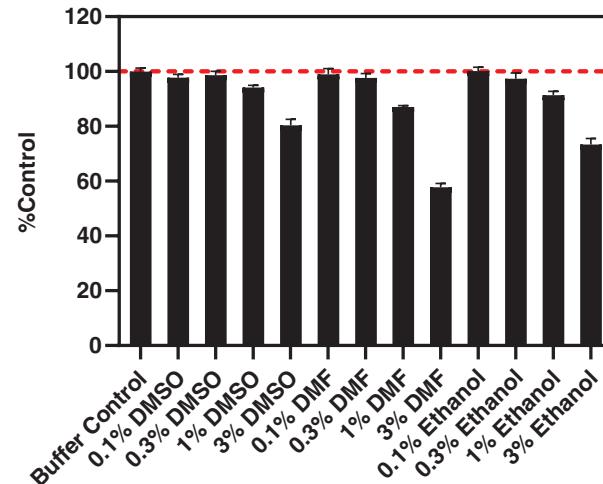
Figure 3. Inhibition of recombinant SARS-CoV-2 RBD by the SARS-CoV-2 Inhibitor Control and neutralizing antibody. Data are plotted as the mean of triplicate measurements  $\pm$  the standard deviation.



**Figure 4. Typical Z' data for the SARS-CoV-2 Spike-ACE2 Interaction Inhibitor Screening Assay Kit.** Data are shown from 40 replicates each for vehicle control (Veh.) and the SARS-CoV-2 Inhibitor Control prepared as described in the kit booklet. The calculated Z' factor for this experiment was 0.86. The red lines correspond to three standard deviations from the mean for each control value.

#### Effects of Solvents:

A titration of organic solvents showed the signal decreases slightly with increasing solvent concentration; therefore the proper vehicle control should be included in the assay.



**Figure 5. The effect of solvent on the readout of SARS-CoV-2 Spike-ACE2 Interaction Inhibitor Screening Assay Kit activity.** The data are shown as the mean  $\pm$  standard deviation for triplicate reactions containing the indicated concentration of solvents.

### Precision:

Intra-assay precision was determined by analyzing 8 measurements of the background and 40 measurements of vehicle and 0.25 mM SARS-CoV-2 Inhibitor Control on the same day. The intra-assay coefficients of variance were 29.1, 2.3, and 16.9%, respectively. The intra-assay coefficient of variation for the IC<sub>50</sub> value of 9 inhibition curves performed on the same day was 5.0%.

The inter-assay coefficient of variation for the IC<sub>50</sub> value of 3 inhibition curves performed on three different days was 9.1%.

## RESOURCES

### Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No absorbance detected above background in the inhibitor wells	A. Not all reagents added to the well(s) B. Inhibitor concentration is too high and completely inhibited the spike RBD-ACE2 interaction	A. Make sure to add all of the reagents to the well(s) B. Reduce the inhibitor concentration and re-assay
No inhibition seen with compound	A. The compound concentration is not high enough B. The compound is not an inhibitor of the SARS-CoV-2 spike-ACE2 interaction	Increase the compound concentration and re-assay

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### Warranty and Limitation of Remedy

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