SARS-CoV-2 Spike S1 RBD-ACE2 Binding Cellular Imaging Assay Kit

Item No. 701970

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening the kit, store individual components as stated below.

Item Number	Item	Quantity/Size	Storage
701972	Cell-Based Assay Buffer B (10X)	1 vial/25 ml	4°C
701914	Rabbit Fc-tagged S1-RBD Protein	5 vials/120 µl	-80°C
701916	Positive Control Neutralizing Antibody	1 vial/50 µl	-80°C
701917	BSA (10%)	1 vial/15 ml	-20°C
701973	Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate	1 vial/60 µl	-20°C
701971	ACE2 Reverse Transfection Black/Clear Plate	1 plate	-20°C
400023	Foil Plate Cover	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.

The Rabbit Fc-tagged Spike S1 RBD Protein, Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate, and Positive Control Neutralizing Antibody should be thawed on ice shortly before use. It is recommended to avoid freeze-thaw cycles of these materials.

If You Have Problems

Technical Service Contact Information

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

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 fluoresence microscope or imaging fluorescence plate reader capable of imaging at excitation/emission (ex/em) wavelengths of 562/576 and 350/470 nm, which correspond to TRITC and DAPI filters, respectively
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of pure water; glass-distilled, ultrapure, or HPLC-grade water is acceptable *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. HEK293T/17 cells and complete culture medium (DMEM + 10% FBS with 1X penicillin/streptomycin)
5. Cell incubator at 37°C with 5% CO_2 and >80% relative humidity (RH)
6. Microcentrifuge tubes or a polypropylene 96-well plate
7. DAPI and paraformaldehyde (PFA) (optional)

NOTE: The components in each kit lot have been quality assured and warranted in this specific combination only; please do not mix them with components from other lots.

INTRODUCTION

Background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped positive-stranded RNA virus, a member of the *Betacoronavirus* genus, and the causative agent of COVID-19.[1-5] The SARS-CoV-2 genome contains approximately 30 kilobases encoding four structural proteins: surface glycoprotein, envelope, membrane, and nucleocapsid.[1,2] The surface glycoprotein, also known as the spike glycoprotein, is located on the outer envelope of the virion.[1] It is composed of an S1 and S2 subunit divided by a furin S-cleavage site not found in other SARS-CoVs.[6,7] 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.[8-12] 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 but is downregulated by SARS-CoV-2 binding.[13,14] ACE2 acts as a negative regulator of signaling through angiotensin II by converting angiotensin II to the vasodilatory and anti-inflammatory peptide angiotensin 1-7.[14] 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-CoV-2 spike glycoprotein reduce viral entry *in vitro*.[8,15] SARS-CoV-2 infection results in the production of neutralizing antibodies, which bind to the SARS-CoV-2 spike S1 RBD preventing further viral entry and infection, starting approximately 4-10 days after symptom onset.[16,17]

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 S1 RBD-ACE2 Binding Cellular Imaging Assay Kit provides a sensitive and unique imaging platform for identifying novel modulators of SARS-CoV-2 spike S1 RBD and ACE2 binding. This assay uses Cayman's proprietary reverse transfection platform to express human ACE2 on the surface of HEK293T/17 cells for binding by a recombinant rabbit Fc-tagged SARS-CoV-2 spike S1 RBD protein as a model of SARS-CoV-2 cellular infection.[18] Binding of the spike S1 RBD to ACE2 is detected with a DyLight™ 550-conjugated anti-rabbit Fc antibody by fluorescence imaging. Inhibition of spike S1 RBD binding to ACE2 results in decreased fluorescence. A positive control neutralizing antibody is included to inhibit the binding between the SARS-CoV-2 spike S1 RBD and ACE2.

A schematic of this process is shown in Figure 1, on page 9.

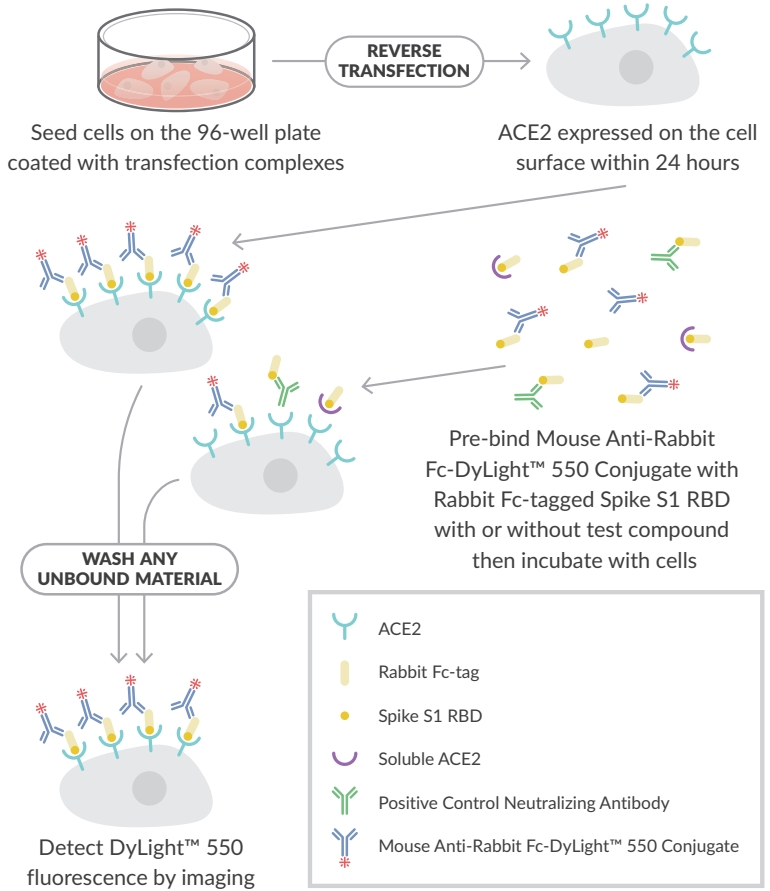


Figure 1. Schematic of the SARS-CoV-2 Spike S1 RBD-ACE2 Binding Cellular Imaging Assay Kit

PRE-ASSAY PREPARATION

Buffer Preparation

The diluted blocking and assay buffers may be stored at 4°C for up to one week.

1. **Cell Blocking Buffer (1X)**

	Volume	Final Concentration
Cell-Based Assay Buffer B (10X) (Item No. 701972)	3 ml	1X
BSA (10%) (Item No. 701917)	15 ml	5%
Pure water	12 ml	
Total Volume	**30 ml**	

2. **Cell-Based Assay Buffer B (1X)**

	Volume	Final Concentration
Cell-Based Assay Buffer B (10X) (Item No. 701972)	20 ml	1X
Pure water	180 ml	
Total Volume	**200 ml**	

Sample Preparation

All samples (including culture medium, plasma, protein, or small molecules) should be diluted in Cell Blocking Buffer (1X) at 10X the final assay concentration. Culture medium and plasma can be used directly at 10X the final assay concentration without dilution, if needed. Compounds dissolved in DMSO must be diluted at least 20-fold in Cell Blocking Buffer (1X) to bring the final concentration of DMSO below 0.5%.

Reagent Preparation

1. **4% PFA (optional)**

   Dilute 2 ml of 16% PFA with 0.8 ml of Cell-Based Assay Buffer B (10X) and 5.2 ml of pure water.

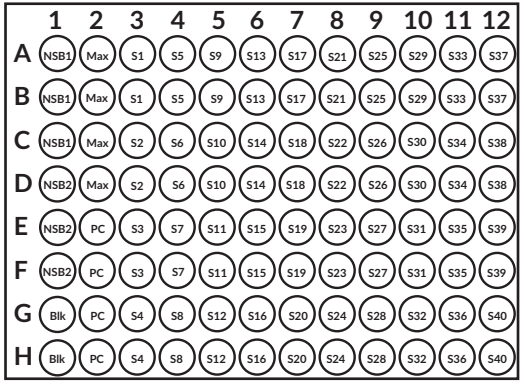
2. **DAPI (1X) (optional)**

   Dissolve DAPI (Item No. 14285) in DMSO at 1 mg/ml to make a stock solution, then dilute with Cell-Based Assay Buffer B (1X) to a final concentration of 1 µg/ml.

Plate Set Up

The 96-well plate included with this kit is coated with reverse transfection complexes. The unopened package should be equilibrated to room temperature before opening inside a biosafety cabinet to seed cells. Column 1 of the plate contains a negative control transfection complex that does not contain ACE2 DNA, while columns 2 to 12 contain the ACE2 transfection complex. Column 1 is intended for blank (Blk) wells and controls for non-specific binding (NSB) by the Rabbit Fc-tagged Spike S1 RBD Protein plus Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate mixture (NSB1; spike S1 RBD-DyLightTM 550 conjugate mixture) and by the Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate alone (NSB2; DyLightTM-Conjugate).

There is no specific pattern for using the wells on the plate. However, it is necessary for column 1 to contain a minimum of two Blk, two NSB1, and two NSB2 wells. In addition, it is necessary to have a minimum of three wells for 100% Rabbit Fc-tagged Spike S1 RBD Protein binding (Max) in any column from columns 2 to 12. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* It is suggested that each modulator, including the Positive Control Neutralizing Antibody (PC), be assayed at least in duplicate. For statistical purposes, we recommend assaying modulators in triplicate. It is suggested that the contents of each well be recorded on the template sheet provided on page 29. A typical layout is shown in Figure 2, on page 13. The arrangement of the Blk and NSB wells in column 1 may be changed and the location and type of wells in columns 2 to 12 varied as necessary for each particular experiment.



NSB1 = Non-Specific Binding Wells for the Spike S1 RBD-DyLight™ 550 Conjugate Mixture
NSB2 = Non-Specific Binding Wells for the DyLight™ 550 Conjugate
Blk = Blank Wells
Max = 100% Rabbit Fc-tagged Spike S1 RBD Protein Binding Wells
PC = Positive Control Neutralizing Antibody Wells
S1-S40 = Sample Wells

Figure 2. Sample plate format

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.

General Information

- The final volume of the assay is 50 µl in all the wells.
- All reagents should be prepared as described above with the Rabbit Fc-tagged Spike S1 RBD Protein, Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate, and Positive Control Neutralizing Antibody thawed on ice before beginning the assay.
- It is recommended to assay the samples in duplicate or triplicate, but it is the user's discretion to do so.
- The assay is performed at room temperature without shaking.

Performing the Assay

Addition of Cells to the Reverse Transfection Plate

NOTE: Before starting the assay, pre-warm a sufficient volume of culture medium and ensure the number of actively growing cells will be available at the time of the assay.

1. Allow the ACE2 Reverse Transfection Black/Clear Plate (Item No. 701971) to equilibrate to room temperature within the sealed bag.
2. After the plate has reached room temperature and before opening the bag, clean the bag with 70% alcohol and place the plate inside a biosafety cabinet.
3. Seed HEK293T/17 cells at a density of 20,000-40,000 cells/well in 200 µl of complete culture medium (DMEM with 10% FBS and 1X penicillin/streptomycin). Adjust seeding density, as needed, to achieve 80-90% confluency after 20-30 hours of incubation.
4. Allow the cells to equilibrate to an even distribution for 30 minutes.
5. Place the plate in a CO_2 incubator at 37°C and incubate for 20-30 hours.

Incubation with Cell Blocking Buffer (1X)

1. After 20-30 hours of incubation, remove culture medium by inverting the plate over a waste bucket. Blot with absorbent paper.
2. Carefully remove residual media from the wells using a multichannel pipettor by placing the pipette tip halfway down the side of the wells.
3. Add 200 µl of Cell Blocking Buffer (1X) to each well.
4. Cover the plate with the lid and incubate for 30-45 minutes at room temperature.

Preincubation of Rabbit Fc-tagged Spike S1 RBD Protein and Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate

1. Add 540 µl of Cell Blocking Buffer (1X) to the vial containing Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate (Item No. 701973) to prepare a Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate working solution. Mix by pipetting up and down gently.
2. Prepare a mixture of Rabbit Fc-tagged Spike S1 RBD Protein (Item No. 701914) and Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate (spike S1 RBD-DyLight™ 550 conjugate mixture) in a polypropylene tube according to the volumes listed in Table 1, below:
3. Incubate the spike S1 RBD-DyLight™ 550 conjugate mixture at room temperature for 15 minutes.

Component	Volume
Cell Blocking Buffer (1X)	3,850 µl
Rabbit Fc-tagged Spike S1 RBD Protein	550 µl
Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate Working Solution	550 µl
Total Volume	**4,950 µl**

Table 1. Spike S1 RBD-DyLight™ 550 conjugate mixture preparation

Preincubation of Sample and Control Mixtures

1. Combine the samples and controls in microcentrifuge tubes or a polypropylene 96-well plate as shown in Table 2, below. If a different number of replicates is used, adjust the volumes accordingly.
2. Incubate for 15 minutes at room temperature.

	Spike S1 RBD + Ab-DyLight™ 550 NSB Wells (3 wells)	Ab-DyLight™ 550 NSB Wells (3 wells) (optional)	Blk Wells (2 wells)	Max Signal Wells (4 wells)	Positive Control Neutralizing Antibody Wells (4 wells)	Sample Wells (duplicate wells)
Cell Blocking Buffer (1X)	16.5 µl	148.5 µl	110 µl	22 µl		
Spike S1 RBD-DyLight™ 550-Conjugate Mixture	148.5 µl			198 µl	198 µl	99 µl
Sample						11 µl
Positive Control Neutralizing Antibody					22 µl	
Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate Working Solution		16.5 µl				
Total Volume	**165 µl**	**165 µl**	**110 µl**	**220 µl**	**220 µl**	**110 µl**

Table 2. Preincubation volumes for samples and controls

Incubation of Sample and Control Mixtures on Cell

1. Empty the wells of the ACE2 Reverse Transfection Black/Clear Plate by inversion and gently blot on a paper towel to remove any residual Cell Blocking Buffer (1X).
2. Transfer 50 µl of the preincubated sample and control mixtures to the corresponding wells in the ACE2 Reverse Transfection Black/Clear Plate. *NOTE: Dispense the sample mixtures near the bottom of the well without disturbing the cell layer.*
3. Cover with the Foil Plate Cover (Item No. 400023) and incubate for one hour at room temperature.
4. Carefully pipette 200 µl of Cell-Based Assay Buffer B (1X) to each well with a multichannel pipettor. *NOTE: Dispense toward the wall slowly.*
5. Gently invert the plate to empty the wells.
6. Repeat washing with 200 µl of Cell-Based Assay Buffer B (1X) per well two more times.
7. Proceed to the Cell Fixation and Counterstaining (Optional) section, on page 19, or carefully remove residual liquid from the wells, pipet 50 µl of Cell-Based Assay Buffer B (1X), into each well and proceed to the Plate Development section, on page 19.

Cell Fixation and Counterstaining (Optional)

1. Fix the cells by adding 50 µl of 4% PFA to each well.
2. Cover the plate with the Foil Plate Cover and incubate at room temperature for 20 minutes.
3. Carefully pipet 200 µl of Cell-Based Assay Buffer B (1X) into each well with a multichannel pipettor.
4. Gently invert the plate to empty the wells.
5. Repeat washing with 200 µl of Cell-Based Assay Buffer B (1X) per well two more times.
6. Carefully pipet 50 µl of DAPI (1X) into each well with a multichannel pipettor.
7. Cover with the Foil Plate Cover and incubate at room temperature for 20 minutes.
8. Carefully pipet 200 µl of Cell-Based Assay Buffer B (1X) into each well with a multichannel pipettor.
9. Gently invert the plate to empty the wells.
10. Repeat washing with 200 µl of Cell-Based Assay Buffer B (1X) per well two more times. Carefully remove residual liquid from the wells.
11. Pipet 50 µl of Cell-Based Assay Buffer B (1X) into each well.

Plate Development

1. Acquire images from all wells using an imaging fluorescence plate reader or fluorescence microscope capable of imaging at excitation/emission (ex/em) wavelengths of 562/576 and 350/470 nm (optional).
2. Quantify the fluorescence signal according to the instrument instructions.

ANALYSIS

Calculations

Percentage of Inhibition

The average relative fluorescence unit (RFU) from the NSB1 wells in column 1 should be subtracted from the sample wells in columns 2-12 to yield the net RFU. The average net RFU values of the Max wells are considered 100% binding. The percentage of binding by the samples is calculated using the equation:

$$\% \text{ binding} = \left[\frac{(\text{sample net RFU})}{(\text{average Max net RFU})} \right] \times 100$$

The percentage of inhibition by the samples is calculated using the equation:

$$\% \text{ inhibition} = \left[\frac{1 - (\text{sample net RFU})}{\text{average Max net RFU}} \right] \times 100$$

Plotting the Dose-Response Curve of Test Compounds

Use the percentage of inhibition values calculated above from each well to plot against the final concentrations of test compound in the well. A non-linear regression with a four-parameter variable slope is recommended for the calculation of IC_{50} values.

Performance Characteristics

Z´ Factor:

Z´ factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[19]

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

Where σ: Standard deviation
μ: Mean
c+: Max wells
c-: Neutralizing antibody

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 S1 RBD-ACE2 Binding Cellular Imaging Assay Kit was determined to be 0.6.

Sample Data:

The data presented here is an example of 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 may vary with different cell lines, cell passages, and culture conditions.

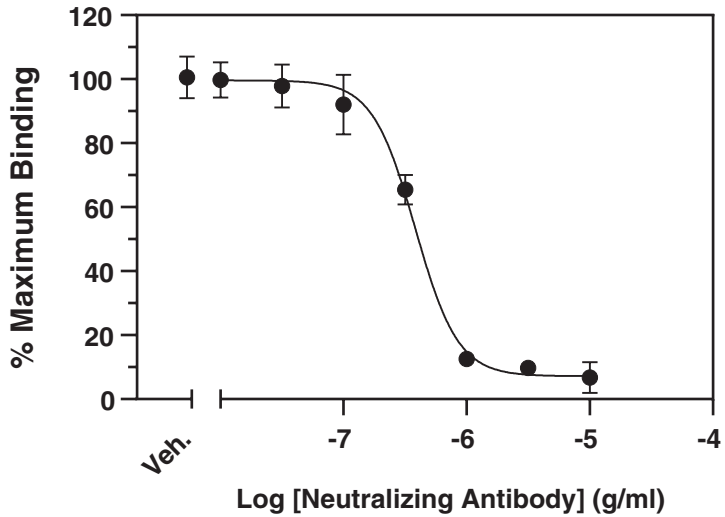


Figure 3. Inhibition of the binding between the SARS-CoV-2 spike S1 RBD and ACE2 by the Positive Control Neutralizing Antibody

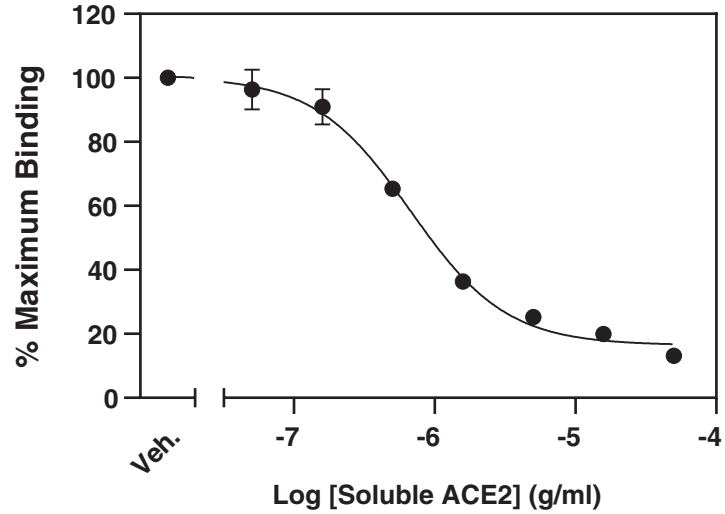


Figure 4. Inhibition of the binding between the SARS-CoV-2 spike S1 RBD and ACE2 by a competitor control soluble ACE2 *NOTE: Soluble ACE2 is not provided in the kit.*

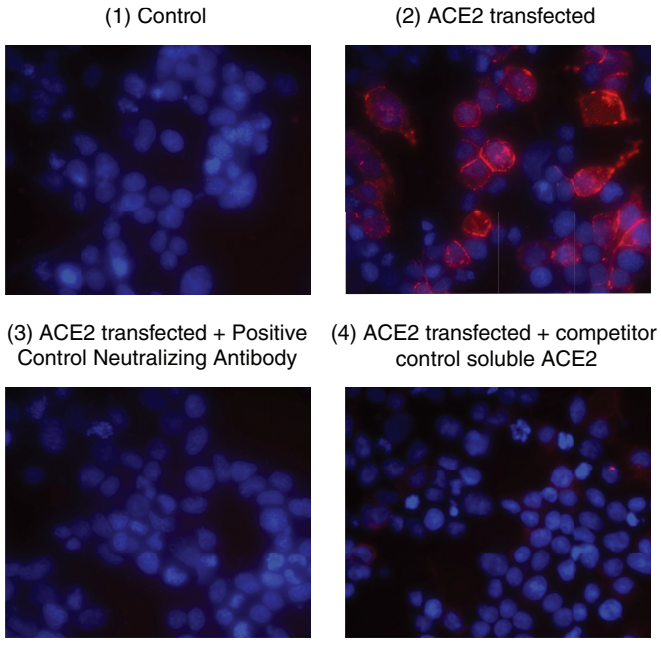


Figure 5. Inhibition of Spike S1 RBD-ACE2 binding by the Positive Control Neutralizing Antibody and a competitor control soluble ACE2. Twenty-four hours after seeding HEK293T/17 cells on reverse transfection complexes containing ACE2 DNA (2-4) or control DNA (1), the cells were incubated with a spike S1 RBD-DyLight™ 550 conjugate mixture alone (1 & 2) or with the addition of the Positive Control Neutralizing Antibody (3) or competitor control soluble ACE2 (4) for one hour at room temperature. Cell nuclei were counterstained with DAPI in blue. The presence of the Positive Control Neutralizing Antibody or competitor control soluble ACE2 significantly inhibited binding of the Spike S1 RBD with ACE2 on the cell surface and abolished the spike S1 RBD-DyLight™ 550 immunostaining in red.

Precision:

Intra-assay precision was determined by analyzing 60 measurements of the maximum binding wells (Max) on the same day. The intra-assay coefficient of variation was 9.8%. The intra-assay coefficient of variation for the IC_{50} value of four inhibition curves performed on the same day was 13.9%.

Inter-assay precision was determined by analyzing inhibition with the Positive Control Neutralizing Antibody in separate assays on four different days. The inter-assay coefficient of variance for the IC_{50} value was 27%.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Uneven cell distribution B. Cell displacement/floating off C. Poor pipetting/pipetting error D. Bubble(s) in assay wells	A. Make sure cells are in homogenous suspension at plating and allow 30-45 minutes for the cells to evenly distribute before placing into the incubator. B. Plate was handled too roughly during inversion and washing. Gently tap the bottom of the plate to remove residual liquid during emptying. C. Check the pipettor volume. D. Carefully tap the side of the plate or use an autopipettor to remove bubbles.
Sample signal is too high	A. Cell density was too high B. Incorrect dilution of the Rabbit Fc-tagged Spike S1 RBD protein C. Incorrect dilution of the Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate D. Improper/inadequate washing of wells	A. Reduce cell plating density. B. Check Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate dilution and use the amount suggested. C. Check Rabbit Fc-tagged Spike S1 RBD protein dilutions and use the amount suggested. D. Follow the protocol for washing the wells using the correct number of times and volume.
Sample signal is too low	A. Cell density is too low B. Incorrect dilution of the Rabbit Fc-tagged Spike S1 RBD C. Incorrect dilution of the Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate D. Pipetting error E. Splashing of sample F. Residual wash buffer carryover	A. Increase the cell seeding density. B. Check the Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate dilution and use the amount suggested. C. Check the Rabbit Fc-tagged Spike S1 RBD protein dilutions and use the amount suggested. D. Check the pipettor volume. E. Dispense pipettor contents carefully. F. Make sure the wells are empty of residual liquid.
High background (NSB)	A. Mouse Anti-Rabbit Fc-DyLight™ 550 Conjugate concentration is too high B. Insufficient blocking C. Cell lysis	A. Check the BSA concentration in the blocking buffer and the volume of blocking buffer dispensed and use the concentration and volume suggested. B. Handle the plate gently during all pipetting, washing, and incubation steps.

References

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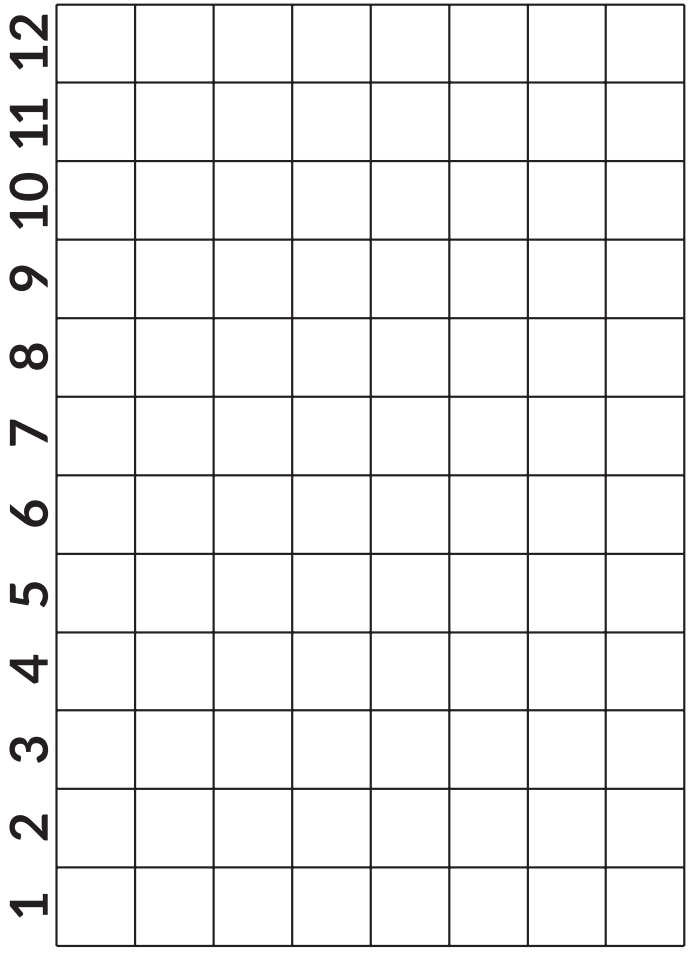

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.