RayBio® COVID-19 Spike-AXL Binding Assay Kit I

For screening COVID-19 drugs and antibodies targeting the Spike-AXL protein interaction

Catalog Numbers: CoV-AXLS1-1 (1 plate kit)
CoV-AXLS1-2 (2 plate kit)
CoV-AXLS1-5 (5 plate kit)

User Manual
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RayBiotech
Empowering your proteomics

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RayBio COVID-19 Spike-AXL Binding Assay Kit I Protocol

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I. INTRODUCTION

The coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (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. Viral entry is also mediated by the interaction between the S-protein with the human Neuropilin-1 (NRP1) and tyrosine-protein kinase (AXL) receptors. NRP1 binds to the S-protein's CendR (C-end rule) motif while AXL binds to the S-protein's N terminal domain (NTD), which are at the C- and N-terminus of the S protein's subunit 1 (S1) domain, respectively. Due to the pivotal role of Spike in COVID-19 infection, the Spike-receptor complexes are targets of COVID-19 therapies and vaccines.

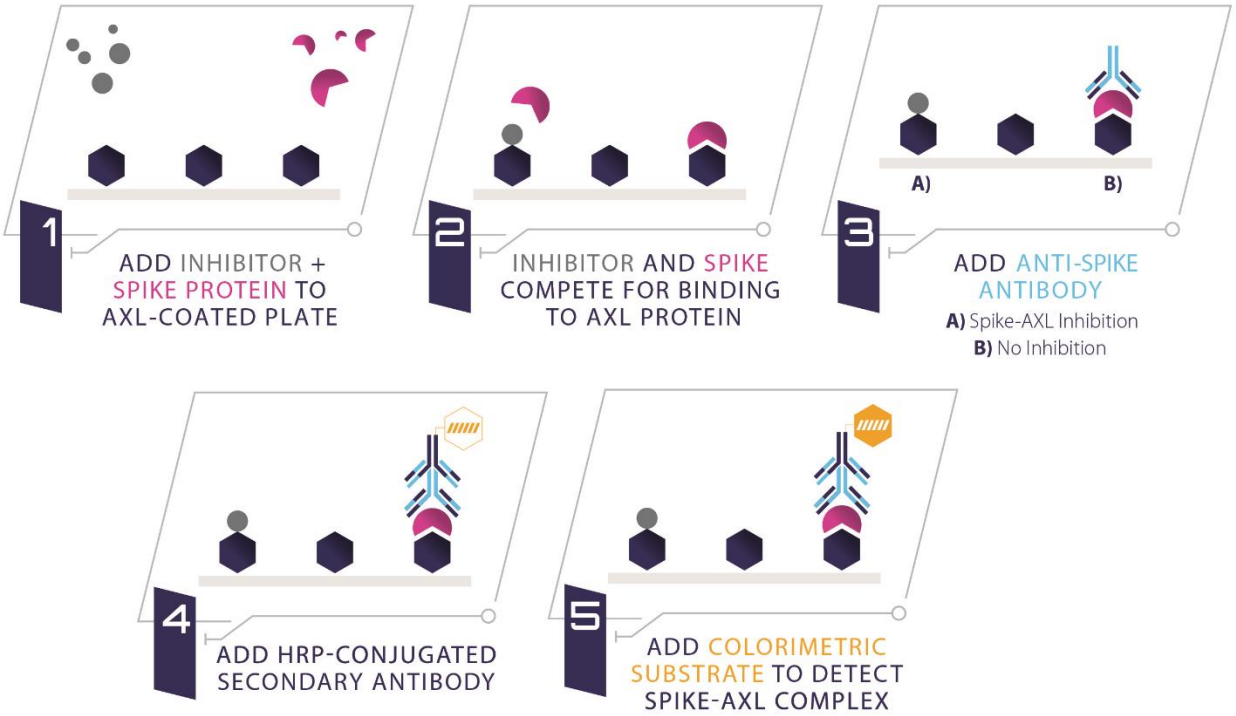
The RayBio® COVID-19 Spike-AXL Binding Assay Kit I is a rapid, simple, and sensitive method to characterize the binding affinity of the S-AXL complex in the presence of potential inhibitors within 4 hours. Various inhibitor types can be tested, including small molecules, peptides, antibodies, or patient sera, for neutralizing activity against SARS-CoV-2. Using this approach, inhibitors can be screened in a high throughput fashion, accelerating the development of therapeutic drugs and vaccines to treat and prevent COVID-19.

The RayBio® COVID-19 Spike-AXL Binding Assay Kit I contains a 96-well plate coated with recombinant AXL. The researcher's potential inhibitor is then added to the wells in the presence of recombinant Spike S1 protein. Unbound S1 is removed with a wash step, and a mouse anti-S1 IgG detection antibody is added, binding to the S-AXL complex. After washing, an HRP-conjugated anti-mouse secondary IgG is then applied to the wells in the presence of 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The HRP reacts with the TMB solution, producing a blue color that is proportional to the amount of bound S1. 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.

References

Wang et al. AXL is a candidate receptor for SARS-CoV-2 that promotes infection of pulmonary and bronchial epithelial cells. Cell Research. (2021): 1-15.

Note: *RayBiotech also offers binding assay kits to screen inhibitors of the Spike-ACE2 interaction (cat no. CoV-SACE2, CoV-ACE2S2) and Spike-NRP1 interaction (cat no CoV-NRP1S1).*



A schematic showing how the RayBio® Spike-AXL Binding Assay Kit I can measure the inhibition of the Spike S1 subunit and AXL 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
AXL-coated Microplate (Item A)	96 wells (12 strips x 8 wells) coated with recombinant human AXL extracellular domain	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
Spike S1 Protein (Item F)	2 vials of purified recombinant SARS-CoV-2 Spike S1 Protein (1 vial is enough to assay half of the microplate)	5 days at 4°C
Detection Antibody S1 (Item C-1)	2 vials of mouse anti-Spike S1 IgG antibody (1 vial is enough to assay half of the microplate)	5 days at 4°C
HRP-Conjugated Anti-Mouse IgG (Item D-2)	15 µl of 1000x concentrated HRP-Conjugated Anti-Mouse 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), Spike S1 Protein (Item F), Detection Antibody S1 (Item C-1), and HRP-Conjugated Anti-Mouse 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 Concentrate** (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 25 ml of 20x Wash Buffer Concentrate into 475 ml of 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 **Spike S1 Protein** (Item F) before use. Add **100 µl** of 1x Assay Diluent into the **S1 Protein** vial to prepare a "50x S1 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 S1 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 S1** (Item C-1) before use. 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-Mouse IgG** (Item D-2) before use. HRP-Conjugated Anti-Mouse IgG should be diluted 1000-fold with 1x Assay Diluent to yield a "1x HRP-conjugated IgG" solution. Each reaction requires 100 µl 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-AXL complex, it is highly recommended to perform a titration curve. This approach will help the researcher to empirically determine the dose-responsive range of the 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 Spike-AXL binding to different extents, there is no recommended starting dilution. Rather, it is encouraged that the researcher determines a dilution series based on their knowledge of the Test Reagent.

Preparation of Reactions. Since the Spike S1 protein putatively competes with the Test Reagent for binding to AXL, it is critical that the Spike S1 be present at 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 Spike S1 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 Spike S1 Protein Concentrate (prepared on page 6 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 researcher's chosen dilutions.

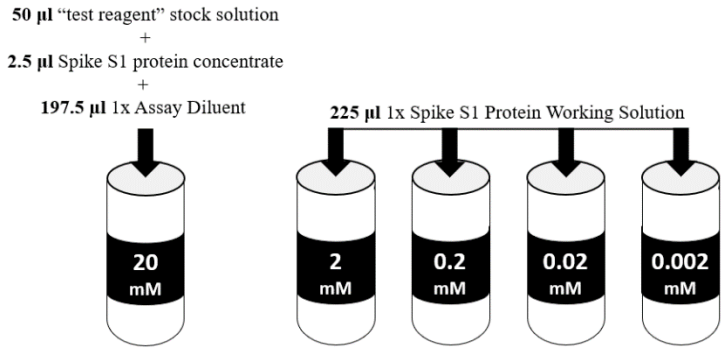
SERIAL DILUTION EXAMPLE

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.

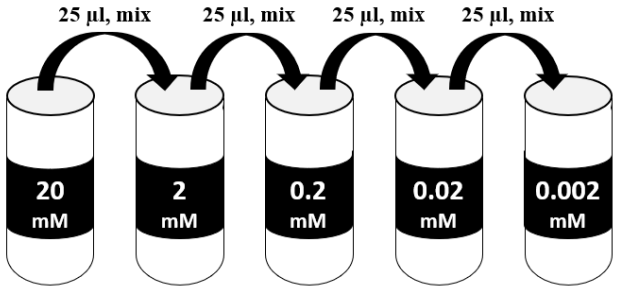
1. *Label 5 serial dilution tubes as follows: 20 mM, 2 mM, 0.2 mM, 0.02 mM, and 0.002 mM.*
2. *Make the first reaction mix:*
   a. *In the tube labeled "20 mM", prepare the first reaction mix by mixing the following components:*
      - *50 µl of the Test Reagent stock solution*
      - *2.5 µl of Spike S1 Protein Concentrate (prepared on page 6 in Part V, 4)*
      - *197.5 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-AXL 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 Spike S1 Protein Concentrate prepared in Part V step 4 (page 6) should be diluted 100-fold with Assay Diluent to make 1200 µl of Spike S1 Protein Working Solution.*
   b. *Pipette 225 µl of the 1x Spike S1 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 Spike S1 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 Reagents 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** into an appropriate well.

   Note: For data reliability, it is recommended that all Test Reagent 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** (see Section V, 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 HRP-conjugated IgG** (see Section V, 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 Reagents as instructed.

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

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

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

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 data to the positive control, 0 mM sample (no Test Reagent).

   Note: *A Test Reagent's absorbance will decrease if the Spike-AXL 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 / OD of positive control)] x 100

   Note: *This calculation uses OD's after subtraction of vehicle control.*

X. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
Signal with Test Reagent is too high	Test Reagent concentration is too low	Increase Test Reagent concentration
	Test Reagent does not inhibit the Spike interaction	Use a different Test Reagent.
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 "no Test Reagent sample" signal	Improper storage of kit	Upon receipt, the kit should be stored at 4°C
	Inaccurate amount of Stop Solution added. Plate not read immediately after adding Stop Solution.	Stop Solution should be added to each well. The OD should be read immediately after adding the Stop Solution.
	Improper dilution of protein, primary or secondary antibody	Ensure correct dilution

This product is for research use only.