VIRONGY BIOSCIENCES INC.

Rapid Cell-Based SARS-CoV-2 Neutralization Assay with HA-CoV-2 Pseudovirus

Firefly Luciferase Reporter and READY TO USE CELLS

Purpose: Neutralization Assay utilizing the HA-CoV-2 pseudovirus for titering antibody containing serum using multiple dilutions and 18hr incubation on a 96 well plate.

Equipment, Supplies and Reagents

Item	**Source, Catalog #, Lot #**
Samples	
Human antibody (AB) serum	Patients or purchased AB solutions
Equipment Not Included	
Sterile Reagent Reservoir	Fisher #03-449-255
Olympus 96-Well PCR Plate, Non-Skirted Ultra Thin Wall, Natural, 25 Plates/Unit	Genesee Scientific # 24-300
96-Well Tissue Culture Plate, Greiner Bio-One (With Lid, µClear® White Flat Round, Chimney)	VWR #82050-758
Luciferase Reader: GloMax® Discover Microplate Reader	Promega #GM3000
Reagents Included	
HEK293T(ACE2+TMPRSS2)#21 cells READY TO USE FROM THE CRYOTUBE TUBE	Virongy Biosciences Inc. # RCSNAK-01
VBI Infection Medium	Virongy Biosciences Inc. # RCSNAK-01/ RCSNAK-02
HA-CoV-2(Luc) pseudovirus	Virongy Biosciences Inc. # RCSNAK-01/RCSNAK-02
Standard SARS-CoV-2 Neutralizing Antibody	Virongy Biosciences Inc. # RCSNAK-01/RCSNAK-02
10X Cell Lysis Buffer	Virongy Biosciences Inc. # RCSNAK-01/RCSNAK-02
D-luciferin Solution	Virongy Biosciences Inc. # RCSNAK-01/RCSNAK-02
Firefly Luciferase Buffer Solution	Virongy Biosciences Inc. # RCSNAK-01/RCSNAK-02

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Neutralization Assay Procedure:

*All cell culture work should be performed in a laminar airflow Class II biosafety cabinet and strict sterile procedures must be used.

1. Prepare the Plate Maps:

Mixing Plate (96-well PCR Plate):

Recommended Mixing Plate Map						
	Dilution 1 1:5	Dilution 2 1:25	Dilution 3 1:125	Dilution 4 1:625	Dilution 5 1:3125	Dilution 6 1:15625
Sample 1	10µL AB +40µL Medium	10µL Dilution 1 +40µL Medium	10µL Dilution 2 +40µL Medium	10µL Dilution 3 +40µL Medium	10µL Dilution 4 +40µL Medium	10µL Dilution 5 +40µL Medium
Sample 2	10µL AB+40µL Medium	10µL Dilution 1 +40µL Medium	10µL Dilution 2 +40µL Medium	10µL Dilution 3 +40µL Medium	10µL Dilution 4 +40µL Medium	10µL Dilution 5 +40µL Medium
Sample 3	10µL AB+40µL Medium	10µL Dilution 1 +40µL Medium	10µL Dilution 2 +40µL Medium	10µL Dilution 3 +40µL Medium	10µL Dilution 4 +40µL Medium	10µL Dilution 5 +40µL Medium
Sample 4	10µL AB+40µL Medium	10µL Dilution 1 +40µL Medium	10µL Dilution 2 +40µL Medium	10µL Dilution 3 +40µL Medium	10µL Dilution 4 +40µL Medium	10µL Dilution 5 +40µL Medium
Sample 5	10µL AB+40µL Medium	10µL Dilution 1 +40µL Medium	10µL Dilution 2 +40µL Medium	10µL Dilution 3 +40µL Medium	10µL Dilution 4 +40µL Medium	10µL Dilution 5 +40µL Medium
Sample 6	10µL AB+40µL Medium	10µL Dilution 1 +40µL Medium	10µL Dilution 2 +40µL Medium	10µL Dilution 3 +40µL Medium	10µL Dilution 4 +40µL Medium	10µL Dilution 5 +40µL Medium
Sample 7	10µL AB+40µL Medium	10µL Dilution 1 +40µL Medium	10µL Dilution 2 +40µL Medium	10µL Dilution 3 +40µL Medium	10µL Dilution 4 +40µL Medium	10µL Dilution 5 +40µL Medium
Standard Neutralizing Antibody	10µL AB+40µL Medium	10µL Dilution 1 +40µL Medium	10µL Dilution 2 +40µL Medium	10µL Dilution 3 +40µL Medium	10µL Dilution 4 +40µL Medium	10µL Dilution 5 +40µL Medium

Infection Plate (96-well White Chimney TC Treated Plate):

Recommended Infection Plate Map													
	1	2	3	4	5	6		7	8	9	10	11	12
Sample 1	1:20	1:100	1:500	1:2500	1:12500	1:62500	Sample 1	1:20	1:100	1:500	1:2500	1:12500	1:62500
Sample 2	1:20	1:100	1:500	1:2500	1:12500	1:62500	Sample 2	1:20	1:100	1:500	1:2500	1:12500	1:62500
Sample 3	1:20	1:100	1:500	1:2500	1:12500	1:62500	Sample 3	1:20	1:100	1:500	1:2500	1:12500	1:62500
Sample 4	1:20	1:100	1:500	1:2500	1:12500	1:62500	Sample 4	1:20	1:100	1:500	1:2500	1:12500	1:62500
Sample 5	1:20	1:100	1:500	1:2500	1:12500	1:62500	Sample 5	1:20	1:100	1:500	1:2500	1:12500	1:62500
Sample 6	1:20	1:100	1:500	1:2500	1:12500	1:62500	Sample 6	1:20	1:100	1:500	1:2500	1:12500	1:62500
Sample 7	1:20	1:100	1:500	1:2500	1:12500	1:62500	Sample 7	1:20	1:100	1:500	1:2500	1:12500	1:62500
Standard Neutralizing Antibody	1:20	1:100	1:500	1:2500	1:12500	1:62500	Controls	IC	IC	IC	PBC	PBC	PBC

Infections: 15µL AB + 45µL HaCoV2(Luc) Particles + 15µL cells
IC= Infection Control (15µL Medium + 45µL HaCoV2(Luc) Particles + 15µL cells)
PBC= Particle Background Control (30µL Medium + 45µL HaCoV2(Luc) Particles)

2. Antibody Treatment Preparation in a 96-Well PCR Plate:

The volumes are doubled in the mixing plate for each sample to distribute on the **Infection Plate in duplicate. In a 96-well PCR plate **(Mixing Plate)**, prepare the antibody(AB) serum dilutions according to the Mixing Plate map .

1. Place **40µL** of VBI Infection Medium in each row of the **Mixing Plate**.
2. Perform a serial dilution with each of the serum sample and control standard antibody according to the table below: Add **10 µL** of each AB/serum sample to the first column and mix well. Transfer **10 µL** from column 1 to column 2 and mix well using a higher volume setting on the pipette, pipette-mix the AB/serum and medium thoroughly before transferring to the next

column. Continue to dilute until the 6th dilution is complete. **Note:** Be sure to exchange pipette tips between well transfers and ensure that antibody/serum and medium are mixed thoroughly to produce accurate results.

Well #	Antibody Serial Dilutions
1A (1:5) AB	10 µL of AB/Serum +40µL VBI Infection Medium
2A (1:25) AB	10 µL of well #1 + 40µL VBI Infection Medium
3A (1:125) AB	10 µL of well #2 + 40µL VBI Infection Medium
4A (1:625) AB	10 µL of well #3 + 40µL VBI Infection Medium
5A (1:3125) AB	10 µL of well #4 + 40µL VBI Infection Medium
6A (1:15625) AB	10 µL of well #5 + 40µL VBI Infection Medium

3. In the **Infection Plate add 45 µL** of HA-CoV-2(Luc) particles to each infection well following the Infection Plate map.
4. Transfer **15 µL** of the AB/serum dilutions from the **Mixing Plate** to the **Infection Plate** with the HACoV2 particles and mix well. Repeat this step to prepare duplicate wells according to the Infection Plate map.
5. Set 3 wells as the **Infection Control** wells by adding **15µL** of VBI Infection Medium to the particles and mix well
6. Set 3 wells as the **Particle Background Control** wells by adding **15µL** of the VBI Infection Medium to the particles and mix well. **Note: These wells will not receive cells in the next step.**
7. Incubate the HA-CoV-2(Luc) particles and AB mixture for 30 mins at 37℃. **Prepare the cells while the particle and antibody incubate.**

Final Dilutions Antibody/Serum + Virus Complex	
A	1 : 20
B	1 : 100
C	1 : 500
D	1 : 2500
E	1 : 12500

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F	1 : 62500

1. **Prepare the HEK293T(ACE2+TMPRSS2) READY TO USE CELLS**
    1. Quickly thaw the HEK293T(ACE2+TMPRSS2)#21 READY TO USE cells in a 37°C water bath. **Note:** water bath can quickly become contaminated, its recommended to wrap the tube with parafilm.
    2. Once thawed immediately transfer the tubes contents to 5 mL of **prewarmed VBI Infection Medium.**
    3. Centrifuge the cell for 5 minutes at 1200rpm and discard the supernatant. Resuspend the cells in **2mL** of prewarmed VBI Infection Medium**.**
    4. Transfer **15µL** of the resuspend cells into each infection well of the **Infection Plate**. Each well should receive around $2.5x10^4$ cells in 15 µL of VBI Infection Medium. **Note: For the Particle Background Control wells add 15µL of VBI Infection Medium.**
    5. Incubate the Infection Plate overnight at 37°C for 16-18 hours. **Note:** This incubation can be done in the GloMax plate reader and can be set to automatically add the lysis buffer when completed with the injector add on.

2. **Luciferase Procedure**

1. Following the incubation of the **Infection Plate** add **7.5 µL** of the **10X Cell Lysis Buffer** directly to each well and mix by orbital shaking for 2 mins. Allow cells to lyse for at least 5 minutes.
2. In a dark location, away from direct light, prepare the Firefly Luciferase Assay Solution by mixing the D-Luciferin Solution with the Firefly Luciferase Buffer Solution in a 1:50 ratio. For a full 96 well plate prepare **5.5 mL** of the luciferase substrate solution by mixing **110 µL of the D-Luciferin Solution** with **5.390 mL of the Substrate Buffer**. **Note:** Use the Firefly Luciferase Assay Solution within 30 minutes of preparation. Do not reuse the Firefly Luciferase Assay Solution.
3. Add **50 µL of the Firefly Luciferase Assay Solution** to the cell lysates. **Note:** For more accurate reading the Firefly Luciferase Assay Solution should be injected into each well by a programable injector so the timing of the assay can be precisely controlled.
4. The plate should be run within 10-15 minutes of adding the samples to the plate. (Tip: Run the plate reader in a dark room to reduce any background signal.) **Note:** More lysate can be added to generate a stronger luciferase signal. Calculate the results by subtracting the background signal determined from the average of the “Control No Infection” wells.