Virongy Detailed Protocol:

Rapid Cell-Based Neutralization Assay with the Hybrid Alpha Pseudovirus (Ha-PV)

The Hybrid-Alpha pseudovirus allows for rapid and robust (high signal-to-noise ratio) expression of reporter genes in target cells (*Hetrick et al., 2022*). Ha-PV is assembled with all structural proteins from the virus of interest as well as a reporter genome derived from an alphavirus-based vector. Ha-PV serves as a platform for quick, easy, and reliable quantification of neutralizing antibodies, viral variants, and antiviral drugs (*Dabbagh et al., 2021*; *He et al., 2021*). A video protocol for this assay can be found at the following link: https://www.youtube.com/watch?v=FeRkEAOJNbk&t=3s

I. Equipment, Reagents, and Storage Conditions

Reagents & Materials Included	Storage Conditions:
1. Ready-to-Use Target Cells	1. Short term: -80 ºC freezer; Long term: Liquid N Tank
2. Ha-PV(Luc) Pseudovirus Particles	2. -80 ºC freezer
3. 10 x Cell Lysis Buffer	3. -80 ºC freezer
4. D-Luciferin Solution	4. -80 ºC freezer
5. Firefly Luciferase Buffer Solution	5. -80 ºC freezer
6. VBI Infection Media	6. -20 ºC freezer
7. Optional: Optional Standard Neutralizing Antibody (NAB) Control	7. -80 ºC freezer
8. 96-well plate (Genesee Scientific, Cat#: 25-109)	8. Room temperature
Equipment Not Included	**Recommended (Vendor, Cat No)**
1. Sterile Reagent Reservoir	1. Fisher, Cat# 03-449-255
2. 96-Well Plate/Microcentrifuge Tubes (for antibody mixing)	2. Genesee Scientific, Cat# 24-300
3. Microplate Luciferase Reader w/ Orbital Shaker	3. Promega, Cat# GM3000
4. 37ºC Water Bath	4. PRECISION, Cat# 51221048
5. 37ºC Incubator w/ or w/o CO2	5. Forma Scientific, Cat# 95926-12567
6. Biosafety Cabinet	6. ESCO, Cat# AC2-559-NS G4

II. Before You Begin

- This experiment will be completed in **two parts over two days**.
- It is recommended that the first part of the experiment is done **later in the day** to allow for the proper **incubation period of 16-18 hours.** You should allot approximately **1 hour** for Part 1of the experiment and **1-2 hours** Part 2 of the experiment.

III. Procedure – Day 1

1) Example Antibody Treatment Preparation in a 96-Well Plate:

1. **Before beginning, heat the VBI Infection Media in a 37ºC water bath until warm throughout.**

   Allow Ha-PV(Luc) pseudovirus particles and optional Standard Neutralizing Antibody (NAB) to thaw on ice before use.

2. **In a biosafety cabinet, collect the needed materials:**
   a. 1 microcentrifuge tube rack
   b. 6 microcentrifuge tubes or an antibody mixing plate (alternatively, you can dilute the antibody in the 96-well plate)
   c. 1 96-well, white, chimney, flat, clear-bottom plate
   d. VBI Infection Media
   e. On ice:
      a. Ready-to-Use Target Cells
      b. Ha-PV(Luc) Pseudovirus Particles
      c. Optional Standard NAB
3. **Label the microcentrifuge tubes numbers 1-6.**

   Each number corresponds to a dilution. Tube 1 will contain the 1:5 dilution, Tube 2 will contain the 1:25 dilution, and so on.
4. **Pipette 40µL of warmed VBI Infection Media into each (6) microcentrifuge tube.**
5. **Serial Dilution: Add 10µL of optional Standard Neutralizing Antibody (or sample serum/drug) to Tube 1. Mix well by flicking the tube 8-10 times and briefly spin.**
6. **Transfer 10 µL from Tube 1 to Tube 2 and mix well by flicking Tube 2 8-10 times and briefly spin.**

   Be sure to change pipette tips between transfers and ensure that the antibody and medium are mixed thoroughly.
7. **Continue to dilute using the transfer method described above (10 µL each time and flicking and spinning to mix) until the 6th dilution is complete. Repeat steps 2-6 for as many samples as desired (maximum of 7 samples for duplicate runs in 1 96-well plate).**

   Refer to the dilution table below for further clarification:

Tube	Antibody Serial Dilutions
1 (1:5) AB	10 µL of Antibody + 40 µL Medium
2 (1:25) AB	10 µL of Tube 1 + 40 µL Medium
3 (1:125) AB	10 µL of Tube 2 + 40 µL Medium
4 (1:625) AB	10 µL of Tube 3 + 40 µL Medium
5 (1:3125) AB	10 µL of Tube 4 + 40 µL Medium
6 (1:15625) AB	10 µL of Tube 5 + 40 µL Medium

2) **Infection Preparation in a 96-Well Plate:**

Infection of target cells with Ha-PV(Luc) pseudovirus particles will be done in duplicate. Refer to the table below for a visual representation of the infection preparation procedure.

1. **Ha-PV(Luc) Pseudovirus Particle: In the 96-well plate, add 45 µL of Ha-PV(Luc) Pseudovirus Particle into each infection well of the infection plate (every well except the Cells Only (CO) wells (G10-G12)).**

   Do not add Ha-PV(Luc) in the CO wells (wells G10-G12).

2. **VBI Infection Media:**
   a. **In the Infection Control (IC) wells (G7-G9), add 15 µL of VBI Infection Media.**
   b. **In the Cells Only (CO) wells (G10-G12), add 60 µL of VBI Infection Media.**

3. **Optional Standard NAB Serial Dilution: Add:**
   a. **15 µL of Tube 1** (1:5 dilution tube) to well **G7**
   b. **15 µL of Tube 2** (1:25 dilution tube) to well **G8**
   c. **15 µL of Tube 3** (1:125 dilution tube) to well **G9**
   d. **15 µL of Tube 4** (1:625 dilution tube) to well **G10**
   e. **15 µL of Tube 5** (1:3125 dilution tube) to well **G11**
   f. **15 µL of Tube 6** (1:15625 dilution tube) to well **G12**

4. **Follow the same procedure as step 3 for each sample (7 possible samples) adding sample antibody/drug in place of Virongy's optional Standard NAB. Repeat in duplicate for each sample.**

		Sample 1	Sample 1	Sample 2	Sample 2	Sample 3	Sample 3	Sample 4	Sample 4	Sample 5	Sample 5	Sample 6	Sample 6
		1	2	3	4	5	6	7	8	9	10	11	12
	Row A	NAB: 1:5	NAB: 1:5	NAB: 1:5	NAB: 1:5	NAB: 1:5	NAB: 1:5	NAB: 1:5	NAB: 1:5	NAB: 1:5	NAB: 1:5	NAB: 1:5	NAB: 1:5
	Row B	NAB: 1:25	NAB: 1:25	NAB: 1:25	NAB: 1:25	NAB: 1:25	NAB: 1:25	NAB: 1:25	NAB: 1:25	NAB: 1:25	NAB: 1:25	NAB: 1:25	NAB: 1:25
	Row C	NAB: 1:125	NAB: 1:125	NAB: 1:125	NAB: 1:125	NAB: 1:125	NAB: 1:125	NAB: 1:125	NAB: 1:125	NAB: 1:125	NAB: 1:125	NAB: 1:125	NAB: 1:125
	Row D	NAB: 1:625	NAB: 1:625	NAB: 1:625	NAB: 1:625	NAB: 1:625	NAB: 1:625	NAB: 1:625	NAB: 1:625	NAB: 1:625	NAB: 1:625	NAB: 1:625	NAB: 1:625
	Row E	NAB: 1:3125	NAB: 1:3125	NAB: 1:3125	NAB: 1:3125	NAB: 1:3125	NAB: 1:3125	NAB: 1:3125	NAB: 1:3125	NAB: 1:3125	NAB: 1:3125	NAB: 1:3125	NAB: 1:3125
	Row F	NAB: 1:15625	NAB: 1:15625	NAB: 1:15625	NAB: 1:15625	NAB: 1:15625	NAB: 1:15625	NAB: 1:15625	NAB: 1:15625	NAB: 1:15625	NAB: 1:15625	NAB: 1:15625	NAB: 1:15625
Sample 7	Row G	NAB: 1:5	NAB: 1:25	NAB: 1:125	NAB: 1:625	NAB: 1:3125	NAB: 1:15625	IC	IC	IC	CO	CO	CO
Sample 7	Row H	NAB: 1:5	NAB: 1:25	NAB: 1:125	NAB: 1:625	NAB: 1:3125	NAB: 1:15625	Standard NAB 1:5	Standard NAB 1:25	Standard NAB 1:125	Standard NAB 1:625	Standard NAB 1:3125	Standard NAB 1:15625

Infection Plate Map

NAB= Optional Standard Neutralizing Antibody Serial Dilution:
45 µL Ha-PV(Luc) Particles + 15 µL Standard NAB + 15 µL Cells
IC=Infection Control:
45 µL Ha-PV(Luc) Particles + 15 µL Cells + 15 µL Medium
Cells Only:
15µL Cells + 60 µL Medium

5. **Cover the plate and incubate for 15-30 minutes at room temperature while preparing the target cells.**

3) **Target Cell Preparation:**

Warm VBI Infection Media to 37°C. The Ready-to-Use Target Cells must also be thawed in a 37°C water bath before being added to the VBI Infection Media.

1. **Add 5 mL of warmed VBI Infection Media to a 10 mL tube.**
2. **After thawing the Ready-to-Use Target Cells in a 37°C water bath, briefly spin down and immediately transfer to the 10 mL tube with the VBI Infection Media. Pipette gently to mix.**
3. **Centrifuge the cells for 5 minutes at 1,200 rpm (140 xg for a 87mm radius centrifuge) and discard supernatant.**
4. **Resuspend the cells by adding 1.6 mL of pre-warmed VBI Infection Media to the 10 mL tube. Pipette gently to mix.**

4) **Infection Procedure:**

1. **Aliquot 15 µL of the resuspended Ready-To-Use Target Cells into each well of the infection plate.**

   Each well should receive around 5.00 x 10^4 cells in 15 µL (anywhere from 2.5 to 5.0 x 10^4 is sufficient for the assay). Use of a multichannel pipette and sterile reservoir is recommended to ensure consistency between wells.

2. **Cover and gently mix the cells by orbital shaking at 1 rotation/second for 60 seconds.**

3. **Cover and note the time of infection. Place the plate in a 37°C incubator for 16-18 hours (overnight).**

Start Time of Incubation Period: _____

IV. Procedure – Day 2

5) **Luciferase Preparation:**

1. **Thaw on ice 10 x Cell Lysis Buffer, Firefly Luciferase Buffer Solution (FLBS), and D-Luciferin Solution. These reagents are light sensitive, proceed with the following steps in a reduced light environment.**

End Time of Incubation Period (16-18 hours): _____

2. **Following the overnight incubation of the infection, add 7.5 µL of the 10 x Cell Lysis Buffer directly to each well.**

3. **Cover and mix by orbital shaking for 2 mins.**

   Allow cells to lyse for at least 5 minutes at room temperature.

4. **In a dark location, prepare the Firefly Luciferase Assay Solution (FLAS) by mixing the D-Luciferin Solution with the FLBS in a 1:50 ratio.**

   For a full 96-well plate, prepare 3 mL of the FLAS by mixing 60 µL of the D-Luciferin Solution with 2940 µL of the FLBS in the provided bottle. Invert 6-8 times. **Note:** Use the FLAS within 30 minutes of preparation. Do not reuse the FLAS. See the following table for the needed volumes of the above reagents for a full 96-well plate:

Table:

Number of Wells	Firefly Luciferase Buffer Solution	D-Luciferin Solution	Total Volume of Firefly Luciferase Assay Solution
Full 96-Well Plate	**2940 µL**	**60 µL**	**3 mL (3,000 µL)**

Note: Please refer to the supplemental protocol for instructions and examples on how to calculate the needed volumes for the FLAS if you are looking to run fewer samples that require less wells and reagents.

6) **Add 25 µL of FLAS to the cell lysates. Mix the plate by orbital shaking for 1 min.**
   For a more accurate luciferase reading, we recommend using a programable injector, when possible, to add the FLAS, so the timing of the assay can be precisely controlled.

7) **Luciferase Reading:**
   1. **Analyze the plate using a luminometer.**
      Luminometer set-up: Read the luciferase signal using your luminometer's manufacturer-recommended range (between 550-570nm) with a read time of 0.3 seconds/well. 560nm is preferred as that is the peak emission wavelength. The plate should be analyzed within 10-15 minutes of adding the FLAS and should be read with the lid off.

      Tip: Run the plate reader in a dark room to reduce any background signal; more FLAS can be added to generate a longer and stronger luciferase signal. Calculate the results by subtracting the background signal determined from the average of the control Cells Only wells.

8) **Data Analysis:**
   1. **Use your preferred data analysis software (Microsoft Prism, Microsoft Excel, etc.) to analyze data. Please refer to the supplemental protocol for more detailed information on analyzing data and example results.**

A video protocol can be found on YouTube at the following link:
https://www.youtube.com/watch?v=FeRkEAOJNbk&t=3s

If you have any questions about the procedures, please feel free to reach out to info@virongy.com or call us at +1 (703) 257-5500.