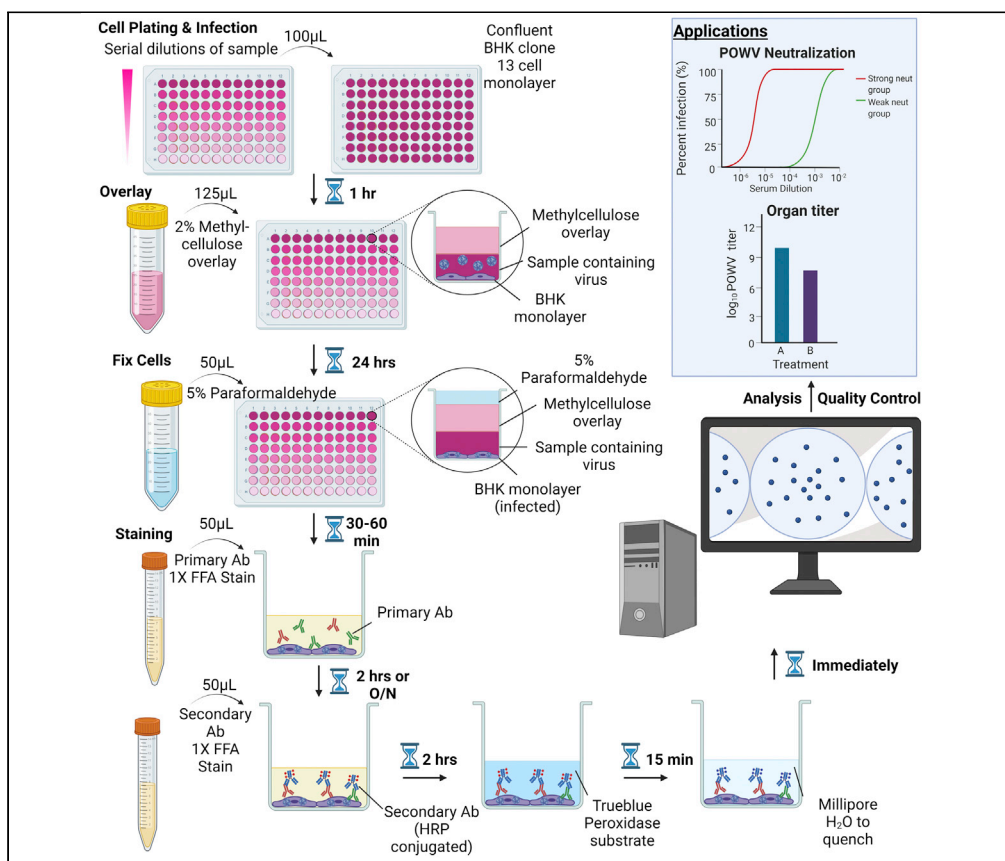


## Protocol

# Titration and neutralizing antibody quantification by focus forming assay for Powassan virus



The development of high-throughput assays measuring Powassan virus (POWV) lineage I and II represents an important step in virological and immunological studies. By adapting focus-forming assays previously optimized for West Nile virus and Zika virus, this protocol is able to determine viral load, evaluate antivirals, and measure neutralizing antibodies. Although limited by its requirement of a detection antibody, this protocol includes a rapid and high-throughput assay for measuring viral titer. By utilizing a baby hamster kidney cell line and a 96-well plate format, this protocol allows for more sensitivity in the detection of POWV lineage I.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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### Highlights

Powassan virus focus forming assay for evaluation of antivirals

Antibody focus reduction neutralization assay (FRNT) for Powassan virus

Steps describing Powassan virus lineage I and II titration

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## Protocol

## Titration and neutralizing antibody quantification by focus forming assay for Powassan virus

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<https://doi.org/10.1016/j.xpro.2022.101473>

## SUMMARY

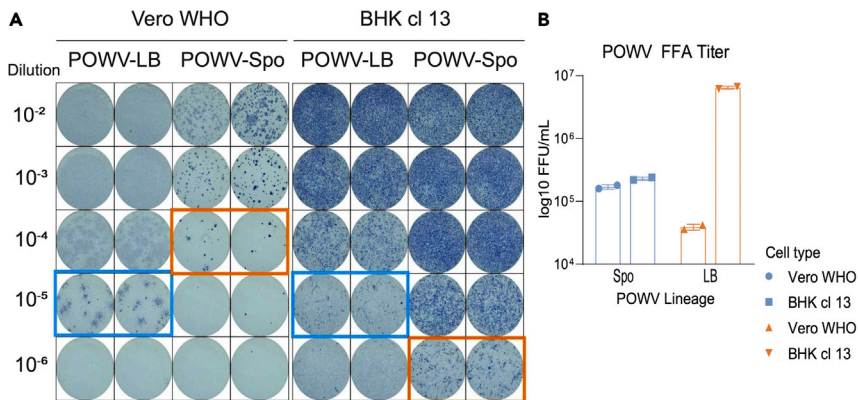
The development of high-throughput assays measuring Powassan virus (POWV) lineage I and II represents an important step in virological and immunological studies. By adapting focus-forming assays previously optimized for West Nile virus and Zika virus, this protocol is able to determine viral load, evaluate antivirals, and measure neutralizing antibodies. Although limited by its requirement of a detection antibody, this protocol includes a rapid and high-throughput assay for measuring viral titer. By utilizing a baby hamster kidney cell line and a 96-well plate format, this protocol allows for more sensitivity in the detection of POWV lineage I.

For complete details on the use and execution of this protocol, please refer to Stone et al. (2022).

## BEFORE YOU BEGIN

1. Establish baby hamster kidney cells (BHK). It is well-established in literature describing POWV virus stock generation that BHK cells are a preferable choice for propagating virus, over the well-characterized African green monkey kidney Vero cell line (referred to as Vero or Vero WHO) (Leonova et al., 2009; Abdelwahab et al., 1964; Anderson and Armstrong, 2012). Although both cell lines are known to be type I interferon-deficient (Osada et al., 2014; Macpherson and Stoker, 1962) in our hands we have found that titration of identical serial dilutions of the POWV-LB lineage on the two cell types yields drastically different titers, with Vero WHO cells showing ~2 logs less virus than BHK-21 cells (Figure 1). For this reason, our assays are optimized with the use of BHK-21 clone 13 cells.
2. Determine desired application. This protocol describes the specific steps for titration of POWV by focus forming assay in a 96-well plate format. However, we have also used this protocol in 12-, and 24-well plate formats for titration of other flaviviruses (Brien et al., 2008, 2019; Hassert et al., 2018, 2019, 2021). This protocol can also be adapted for antiviral compound screening, determining organ titer, and neutralizing antibody quantification.
3. Determine dilution of virus stock for assay (if needed). For antiviral compound screening and neutralizing antibody quantifications, the researcher should determine the dilution of virus stock/sample that will yield a count of ~70–100 foci per well before proceeding to the next steps. For quantification of neutralizing antibody, serial dilutions of serum samples should be made in growth media (Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal bovine serum (FBS and 1% HEPES, see materials and equipment) and incubated with the appropriate dilution of





**Figure 1. Baby hamster kidney cells should be used in Powassan virus titration**

(A) A representative focus forming assay showing the results of plating identical ten-fold serial dilutions of Powassan virus Spooner lineage (Spo, blue) or Powassan virus LB lineage (LB, orange) on Vero WHO cells (left) or baby hamster kidney 21 clone 13 cells from the ATCC (right). Boxed in blue and orange are the serial dilutions that could be used to determine the viral titer.

(B) Quantification of POWV infectious viral titer images presented in (A). Data are reported as the mean foci for the two replicates.

virus (yielding 70–100 foci per well) for 1 h 37°C, 5% CO<sub>2</sub> to allow for immune complex formation. Mixture should then be applied to cell monolayer to allow for virus adsorption and handled identically to the focus forming assay (FFA) described below.

- Determine method of spot counting. This protocol utilizes an ImmunoSpot S6 FluoroCore M2 analyzer, and outlines the parameters that produce the most rapid and accurate foci counts.

### Institutional permissions

This protocol requires biosafety level 3 containment facilities. Standard operating procedures, protocols and disinfectants approved by the Institutional Biosafety Committee (IBC) or equivalent biosafety regulatory bodies should be used, and all work is performed inside Biosafety Cabinets (BSC) unless otherwise noted.

### Culture cell lines

⌚ Timing: 4–10 days

The maintenance of a healthy cell culture is essential to achieve accurate and reproducible titration assays, drug screens, and neutralizing antibody quantifications.

- Baby hamster kidney American Type Cell Culture clone 13 (referred to as BHK-21 clone-13 or BHKs) should be passaged 2–3 times in 5% DMEM (see [materials and equipment](#)) upon initial thawing from liquid N<sub>2</sub> prior to plating for focus forming assays. Cells may be carried in T150 flasks and split 1:20 every 2–3 days. Cells should be carried in 20 mL of 5% DMEM to 90%–95% confluency and washed two times with 10 mL Ca<sup>2+</sup> Mg<sup>2+</sup> free phosphate buffered saline (PBS) and lifted via incubation with 4 mL trypsin. Following trypsinization, 6 mL of 5% DMEM should be added to halt the trypsin reaction, resulting in a single cell suspension of ~10 mL. To the same T150 flask, 1 mL of this single cell suspension may be added, along with 19 mL of fresh 5% DMEM and returned to the incubator.

### Prepare reagents

⌚ Timing: 6 h

6. Prepare 2% methylcellulose, 5× FFA Staining buffer, and 1× FFA Wash buffer (see tables for recipe):

### Cell plating and titration by focus forming assay

⌚ Timing: Active time: 2–3 h, With incubations: 48–72 h

7. The day before the assay, plate BHK-21 clone-13 (ATCC CCL-10) cells in a tissue culture treated 96-well plate in growth media overnight.
  - a. Before beginning the assay, cells should reach ~90% confluency; BHKs will not form an even monolayer with complete cell-to-cell contact as seen with Vero cells.
8. Prepare 10-fold serial dilutions of the sample of interest in duplicate or triplicate.
9. Remove media from 96-well flat bottom plate containing BHKs. Add 100 µL per well of serially diluted samples in growth media to BHK cell monolayer.
10. Incubate for 1 h to allow for virus adsorption.
11. During incubation, prepare methylcellulose overlay and warm to room temperature.
12. Add methylcellulose overlay to minimize lateral spread of viral infection.
13. Incubate 24 h.
14. Following incubation, fix cells in freshly made 5% paraformaldehyde (PFA) in PBS for 30–60 min.
15. Wash cells twice with 1× PBS and once with 1× FFA Wash buffer. Incubate 5–10 min at room temperature to permeabilize cells.
16. Remove plates from BSL-3 containment and proceed to immunostaining.

### Immunostaining

⌚ Timing: Active time: 2–3 h, With incubations: 8–24 h

17. Prepare and add primary antibody (Monoclonal Anti-Langat Virus Envelope Glycoprotein) in 1× FFA Staining buffer.
18. Incubate at room temperature for 1.5–2 h or overnight at 4°C.
19. Wash three times with 1× FFA Wash buffer.
20. Prepare and add secondary antibody (goat anti-mouse conjugated to HRP) in 1× FFA Staining buffer.
21. Incubate at room temperature for 2–3 h.
22. Wash three times with 1× FFA Wash buffer.
23. Add Trueblue substrate and incubate 15–20 min or until spots are fully defined with minimal background.
24. Quench the reaction by removing substrate and rinsing with Millipore water.
25. Tap on a paper towel to remove water and image.

### Imaging (optional)

⌚ Timing: Active time: 15–60 min, with computer/software run time: 30–120 min

26. A CTL Immunospot analyzer can be used for high-throughput quantification of foci using CTL Immunospot 'Scan', 'Count' and 'Quality Control' functions. Researchers who do not have access to an Elispot may elect to manually count wells under a dissecting microscope, however this will reduce the high-throughput nature of the assay.

⚠ **CRITICAL:** Prior to fixation step, all agents should be handled in appropriate biological containment.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-Mouse IgG (whole molecule)–Peroxidase antibody produced in goat, 1:5,000	Sigma-Aldrich	Cat# A8924, RRID: AB_258426
Monoclonal Anti-Langat Virus Envelope Glycoprotein (E), Clone 10F6, 3 :10,000	BEI Resources	NR-40316
<b>Bacterial and virus strains</b>		
POWV strain LB	Ebel laboratory	<a href="#">Mandl et al., 1993</a>
POWV-DTV strain Spooner	Ebel laboratory	<a href="#">Ebel et al., 1999</a>
<b>Experimental models: Cell lines</b>		
BHK-21 clone 13	ATCC	ATCC Cat# CCL-10, RRID: CVCL_1915
<b>Other</b>		
Dulbecco's Modified Eagle's Media	Sigma-Aldrich	Cat# D5796-500ML
1 M HEPES solution	Sigma-Aldrich	Cat# H3537-100ML
Fetal bovine serum	Sigma-Aldrich	Cat# F0926
Methylcellulose	Sigma-Aldrich	Cat# M0512-250G
20% Electron microscopy grade paraformaldehyde	EMS	Cat# 15713-S
Saponin	Sigma-Aldrich	Cat# 47036
KPL TrueBlue Substrate	SeraCare	Cat# 5510-0052
ImmunoSpot® S6 FluoroCore M2 Analyzer	ImmunoSpot®	N/A

## MATERIALS AND EQUIPMENT

### Growth Media (5% DMEM)

Reagent	Final concentration	Amount
Dulbecco's Modified Eagle's Media	n/a	500 mL
1 M HEPES solution	1%	5 mL
Fetal Bovine Serum	5%	25 mL
<b>Total</b>	<b>n/a</b>	<b>530 mL</b>

Store at 4°C away from light. Handle aseptically.

### 2% Methylcellulose

Reagent	Final concentration	Amount
Methylcellulose	2%	10 grams
Millipore H <sub>2</sub> O	n/a	500 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

Store at 4°C. Handle aseptically.

### 5% Paraformaldehyde (PFA)

Reagent	Final concentration	Amount
20% EM Grade PFA	5%	2.5 mL
1× PBS	n/a	7.5 mL
<b>Total</b>	<b>n/a</b>	<b>10 mL</b>

Store at 4°C. Prepare fresh on day of fix.

### 1 × FFA Wash Buffer

Reagent	Final concentration	Amount
10× PBS	1×	1 L
Millipore H <sub>2</sub> O	n/a	9 L
Triton X-100	0.05%	5 mL
<b>Total</b>	<b>n/a</b>	<b>10 L</b>

Store at room temperature.

### 5 × FFA Staining Buffer

Reagent	Final concentration	Amount
10× PBS	5×	250 mL
Millipore H <sub>2</sub> O	n/a	250 mL
Saponin	1 mg/mL	2.5 grams
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

Store at 4°C. Handle aseptically.

### Primary Antibody in 1 × FFA Staining Buffer

Reagent	Final concentration	Amount
5× FFA Staining Buffer	1×	2 mL
Millipore H <sub>2</sub> O	n/a	8 mL
Primary Antibody ( $\alpha$ -LGTV, clone 10F6)	3:10,000	3 $\mu$ L
<b>Total</b>	<b>n/a</b>	<b>10 mL</b>

Store at 4°C. Prepare fresh on day of immunostaining.

### Secondary Antibody in 1 × FFA Staining Buffer

Reagent	Final concentration	Amount
5× FFA Staining Buffer	1×	2 mL
Millipore H <sub>2</sub> O	n/a	8 mL
Secondary Antibody (Goat $\alpha$ -mouse conjugated HRP IgG)	1:5,000	2 $\mu$ L
<b>Total</b>	<b>n/a</b>	<b>10 mL</b>

Store at 4°C. Prepare fresh on day of immunostaining.

**Storage:** Growth media, 2% methylcellulose and 5× FFA Staining buffer can be made in advance and should be stored at 4°C and handled aseptically. All other solutions should be prepared on the day of the assay and stored at 4°C until use.

**△ CRITICAL:** Paraformaldehyde is a respiratory hazard and should be handled and disposed of with appropriate precautions and PPE in place.

**Alternatives:** Polyclonal sera can be used in place of The LGTV mAb, clone 10F6 (NR-40316) for the primary (detection) antibody. FFA Staining buffer can be used as an alternative to FFA Wash buffer for all wash steps. However, due to increased likelihood of contamination associated with the use of Saponin not seen with Triton X-100 it is advised to use FFA Wash buffer for the steps indicated. FFA Wash buffer is not an alternative for FFA Staining buffer.

## STEP-BY-STEP METHOD DETAILS

### Prepare reagents

⌚ Timing: 6 h

Advance preparation of 2% methylcellulose, 5× FFA Staining buffer, and 1× FFA Wash buffer will expedite the assay and ensure visualization of individually discernable foci.

1. Prepare 2% methylcellulose:
  - a. Autoclave the following prior to preparation:
    - i. 1 L glass bottle, 10 g methylcellulose, and a large stir bar.
    - ii. 1 L glass bottle with 500 mL of Millipore water.
  - b. In a tissue culture hood, add the 500 mL hot autoclaved Millipore water to bottle containing methylcellulose and a large stir bar. If the autoclaved water has cooled, reheat in the microwave until the bottle is hot to the touch, but water should not boil.
  - c. Partially cap bottle and stir on a hotplate set between medium/high until methylcellulose is in solution (1–4 h).
  - d. In a tissue culture hood, aliquot cooled 2% methylcellulose into 50 mL conical tubes. Pipette slowly, as the mixture will be viscous. Store at 4°C until further use in the assay, which will require bringing the methylcellulose to room temperature.
2. Prepare 5× FFA Staining buffer according to tables found in [materials and equipment](#):
  - a. To 250 mL of 10× PBS, add:
    - i. 250 mL of Millipore water.
    - ii. 2.5 g of Saponin.
  - b. Stir until dissolved, then sterile filter and store at 4°C until the day of the assay.
3. Prepare 1× FFA Wash buffer (1× PBS, 0.05% Triton X-100) according to table found in [materials and equipment](#):
  - a. To 9 L of Millipore water, add:
    - i. 1 L 10× PBS.
    - ii. 5 mL Triton X-100.
4. Prepare media for carrying BHK-ATCC clone-13 cells (hereafter referred to as growth media):
  - a. To 500 mL 5% DMEM, add:
    - i. 5 mL HEPES solution.
    - ii. 25 mL Fetal Bovine Serum (FBS).
    - iii. Store at 4°C until further use.

### Cell plating and titration by focus forming assay

⌚ **Timing:** Active time: 2–3 h, With incubations: 48–72 h

Quantification of Powassan viral titer by focus forming assay relies on the addition of sample with an unknown quantity of virus to a monolayer of permissive cells (BHKs). This step outlines the plating conditions and titration schema that, in our hands, produces the most reproducible conditions for determining POWV titer. Researchers are encouraged to determine the appropriate cell density and culture conditions for their own cell lines before titrating precious samples. Additionally, this protocol details titration using a 10-fold dilution series, researcher may find it useful to increase or decrease the fold dilution series based on the need for sensitivity for the titration series. This step also outlines the process of adding sample to the cell monolayer, overlaying to minimize lateral spread of infection, and the process of fixing the cells for removal from biological containment.

5. The day before the assay: to a tissue culture treated 96-well plate, add  $2.5 \times 10^4$  cells per well of BHK-21 ATCC clone-13 cells in 100  $\mu$ L of growth media per well. Incubate overnight at 37°C, 5% CO<sub>2</sub>.
  - a. Before starting the assay, examine cells under a light microscope to ensure you have proper cell confluence the day of the assay (~90%); BHKs will not form an even monolayer with complete cell-to-cell contact as seen with Vero cells.
6. In a 96-well round bottom plate, prepare 10-fold serial dilutions of the sample containing virus by adding 20  $\mu$ L of sample to 180  $\mu$ L of growth media. Sample dilutions should be performed in duplicate or triplicate, and at least one column should include a 'mock' infected PBS control.

7. Remove media from 96-well flat bottom plate containing BHKs. Add 100  $\mu\text{L}$  per well of serially diluted samples in growth media to the 96-well flat bottom plate containing BHKs.
8. Incubate at 37°C, 5%  $\text{CO}_2$  for 1 h without shaking to allow for virus adsorption.
9. During incubation, prepare methylcellulose:
  - a. Mix one part 2% methylcellulose (see [prepare reagents](#)) with one part growth media. Place on a rocker at room temperature with gentle agitation to mix methylcellulose and media.
10. Add 125  $\mu\text{L}$  per well of diluted methylcellulose in growth media to the flat bottom plate containing BHKs and + serially diluted samples. This step will minimize lateral spread of viral infection.
11. Incubate 24 h without shaking at 37°C, 5%  $\text{CO}_2$ .
12. Following incubation, fix the 96-well flat bottom plates as follows:
  - a. Prepare 5% paraformaldehyde (PFA) by diluting 20% electron microscopy grade PFA four-fold in 1 $\times$  PBS.
  - b. Fix cells with 5% paraformaldehyde by adding 50  $\mu\text{L}$  per well and incubate at room temperature for 30–60 min.
  - c. Wash cells gently three times with 150  $\mu\text{L}$  per well of 1 $\times$  PBS.
  - d. Add 150  $\mu\text{L}$  per well of 1 $\times$  FFA Wash buffer and let sit for 5–10 min at room temperature.
  - e. Remove plates from BSL-3 containment and proceed to immunostaining.

### Immunostaining

⌚ Timing: Active time: 2–3 h, With incubations: 8–24 h

After cells have been fixed, individual foci must be visualized following an immunostaining protocol that relies on the detection of viral antigen in the cells. This step outlines the immunostaining protocol that, in our hands, allows the researcher to visualize foci with minimal background.

13. Prepare 1 $\times$  FFA Staining buffer by diluting 5 $\times$  FFA Staining buffer five-fold in Millipore water. 5 $\times$  FFA Staining buffer should be handled aseptically.
14. Remove Wash buffer and add primary antibody (e.g., Monoclonal Anti-Langat Virus Envelope Glycoprotein (E), Clone 10F6) that has been diluted 3:10,000 in 1 $\times$  FFA Staining buffer (50  $\mu\text{L}$  per well).
15. Incubate at room temperature for 1.5–2 h with gentle agitation on an orbital shaker, or overnight at 4°C without shaking.
16. Wash three times with 1 $\times$  FFA Wash buffer (150  $\mu\text{L}$  per well).
17. Remove Wash buffer and add secondary antibody (goat anti-mouse conjugated to HRP) that has been diluted 1:5,000 in 1 $\times$  FFA Staining buffer (50  $\mu\text{L}$  per well).
18. Incubate at room temperature for 2–3 h.
19. Wash three times with 1 $\times$  FFA Wash buffer (150  $\mu\text{L}$  per well).
20. Remove Wash buffer; add Trueblue substrate (50  $\mu\text{L}$  per well) and incubate 15–20 min without shaking, in the dark, or until spots are fully defined with minimal background.
21. Flick off substrate and wash twice with 200  $\mu\text{L}$ /well of cool Millipore water to quench the reaction. The reaction should not be quenched with hot (>37°C) water.
22. Tap on a paper towel to remove water and count or image within 15–30 min of quenching for best counting results.

### Imaging (optional)

⌚ Timing: Active time: 15–60 min, With computer/software run time: 30–120 min

After immunostaining, individual foci should be visible wells. While POWV titer can be calculated manually by counting under a dissecting microscope, we have found that the high-throughput nature of the 96-well plate format is more suitable for automated counting. For this purpose, we utilize



an ImmunoSpot S6 FluoroCore M2 analyzer, hereafter referred to as a CTL Elispot machine. The steps below outline the parameters on a CTL Immunospot analyzer that, in our hands, produce the most rapid and accurate foci counts.

23. Turn on both the computer and CTL machine.
24. Open the CTL Switchboard and select the 'Scan' function.
25. Select 'Full plate scan'.
26. Under 'Dashboard', ensure the correct filepath and plate setting.
  - a. The '96 well BD Falcon' setting can be used for most FFAs.
27. Load and name the plate.
28. Start the scan, confirm that the CTL machine can correctly identify the A1, A12, and H1 wells, and allow the images to be collected.
29. After the scan has completed, return to the CTL Switchboard.
30. Select the 'Count' function.
31. Select 'SmartCount'.
32. Find the file folder containing the plate that was just scanned/imaged and load the plate(s) into the autcount queue.
33. Select a few wells with clear, distinct foci. Adjust the parameters of the spot count, giving special consideration to the appropriate gate size, background balance, and spot separation to yield an accurate count.
  - a. It may be necessary to adjust parameters using a manually counted well or series of wells.
34. Once the appropriate parameters are selected, start the autcount.
35. After the autcount has completed, return to the CTL Switchboard.
36. Select the 'Quality Control' Function.
37. Find the file folder containing the plate that was just counted and load the plate(s) into the quality queue.
  - a. Quality control functions of the CTL Elispot should be used to manually correct aberrant counts, remove false spots/hits, and, if necessary, re-count wells with different parameters.
  - b. Parameters should be determined/adjusted in the same manner as the original autcount.
38. Titration should be based on wells with individually discernable foci.

## EXPECTED OUTCOMES

Once a sample has been sufficiently diluted, individual foci should be visible and countable in the well. For determination of viral titer, the researcher should utilize the dilution that yields a suitable number (between 20–120) of distinct foci.

## QUANTIFICATION AND STATISTICAL ANALYSIS

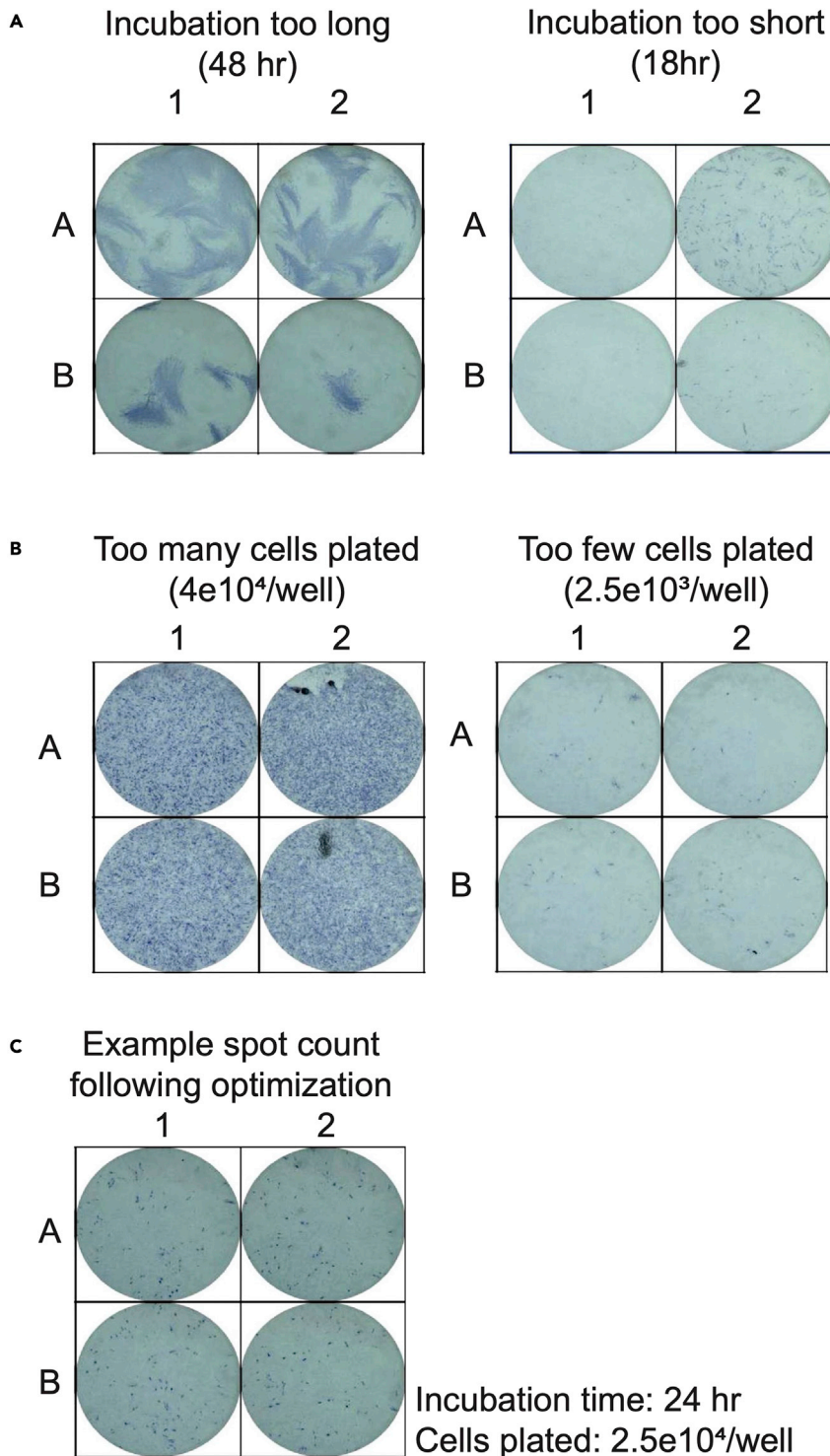
For quantification of neutralizing antibodies at a given serum dilution, use the following formula:

$$\left( \frac{(\# \text{ of foci rep 1} + \# \text{ of foci rep 2})}{2} \div \# \text{ of foci in PBS control} \right) \times 100 = \% \text{ POWV inhibition}$$

Alternatively, the reduction in foci can also be subtracted from 100 to give the '% POWV infection'. Serum serial dilutions should then be log transformed. In GraphPad Prism (v9), Python, R, etc., perform a nonlinear regression (curve fit) for a 'log(agonist) vs. response, variable slope (four parameters) analysis, with constraints set to top equal to 100 and bottom equal to 0. This analysis can also be used to find the EC<sub>50</sub> and EC<sub>90</sub> values, or the reciprocal dilution of serum required to obtain 50% or 90% neutralization, respectively.

## LIMITATIONS

All focus forming assays rely on antibody binding for the detection of viral antigen within infected cells. The assay is therefore highly reliant on the quality and specificity of the antibody for the viral antigen being detected. Researchers should take care in selecting the primary antibody being



**Figure 2. Common Powassan virus focus forming assay issues**

Representative images of focus forming assays performed with lineage 1 Powassan virus LB in duplicate depicting some common issues.

(A) These images show the results of incubation times that are too long (48 h, left) or too short (18 h, right). For incubations that are too long, foci will run together and will not be individually discernable. For incubations that are too short, foci will appear too small for detection, or will not appear at all.

**Figure 2. Continued**

(B) These images show the results of plating cell densities that are too high ( $4 \times 10^4$  cells per well, left) or too low ( $2.5 \times 10^3$  cells per well, right). For plating an excess of cells, background staining or ‘stacking’ of cells vertically (i.e., not on the cell monolayer) is possible. An excess of cells also increases the likelihood of washes damaging the cell monolayer (well A2). For plating too few cells, gaps in the cell monolayer may be visible, and viral titer will likely appear lower than expected.

(C) Representative image showing a focus forming assay with a cell dilution of  $2.5 \times 10^4$  cells and 24 h incubation, suitable for viral titer determination, as well as compound screening or neutralizing antibody quantification, resulting in distinct and individually discernible foci.

used. For those using polyclonal serum it is recommended that different dilutions of serum and incubation times and conditions are tested to minimize non-specific binding, which can greatly increase the background and make counting difficult.

## TROUBLESHOOTING

### Problem 1

Cell plating.

In the [cell plating and titration by focus forming assay](#) section step one the addition of an excess of cells, or too few cells, to the 96-well plate before the addition of POWV can result in highly variable or inaccurate titration. Examples of focus forming assays which were conducted on over-confluent or under-confluent plates are shown in [Figure 2](#).

### Potential solution

Researchers are encouraged to determine the appropriate cell density and culture conditions for their own cell lines before titrating precious or limited samples.

### Problem 2

Organ toxicity.

When titrating by focus forming assay in the [cell plating and titration by focus forming assay](#) step, it is possible for organ homogenate to be cytotoxic to the cell monolayer. This may alter the sensitivity of the assay, decreasing the limit of detection, and causing inaccuracy in viral titer determination. This is often the case with organs such as lungs, salivary glands, and livers.

### Potential solution

Organ toxicity can be overcome by diluting the organ homogenate (i.e., rather than applying 20  $\mu$ L of organ homogenate diluted in 180  $\mu$ L of media (final dilution 1:100), consider diluting 100-fold, then adding 20  $\mu$ L of the 100-fold dilution to 180  $\mu$ L of media (final dilution 1:1,000). It is, however, possible that in instances of low viral titer the dilution required to mitigate organ toxicity may exceed the sensitivity of the assay, thus preventing accurate recording of infectious virus.

### Problem 3

Incubation times.

In the [cell plating and titration by focus forming assay](#) section step seven, the twenty-four-hour incubation step following the methylcellulose overlay is based on the amount of time needed for the POWV lineages to replicate to sufficient levels for foci to be detected. This timing is highly variable among different viruses or even among different strains of the same virus. Examples of focus forming assays which were conducted using excessively long or short incubation times are shown in [Figure 2](#).

### Potential solution

Researchers are encouraged to test many incubation times prior to the fixation step according to the cell type, strain, and doubling time of their own cell lines before titrating limited samples. Generally, 24–72 h is sufficient for POWV focus forming assays for either lineage I or lineage II.

### Problem 4

Removal of monolayer.

In the [cell plating and titration by focus forming assay](#) section and the Immunostaining steps eight, two, four, five, seven and eight, vigorous pipetting or washing can remove the monolayer, leading to lost data or inaccurate reporting of titer results.

### Potential solution

Care must be taken when liquid is added to the plates containing the monolayer so as not to scratch the monolayer with the pipet tip or agitate the monolayer too harshly. This will prevent dislodging the cells.

### Problem 5

Fibers and hairs.

In the immunostaining and imaging sections, fibers and hairs present during the washing of the assay plates can remain associated with the monolayer and be carried through to the imaging step. The presence of hairs and fibers can cause significant errors if using an automated counting program.

### Potential solution

Refrain from using absorbent materials which contain fibers that can be released into the assay and maintain a clean laboratory environment.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Amelia K. Pinto ([amelia.pinto@health.slu.edu](mailto:amelia.pinto@health.slu.edu)).

### Materials availability

There are restrictions to the availability of the primary detection antibody (polyclonal mouse sera) due to limited supply. We will provide the serum to labs that request it, while our supply lasts. However, as these sera were generated in our lab, and we cannot guarantee that there will be no differences or batch-to-batch variation in different lots of POWV serum. We therefore recommend that researchers seeking a primary detection antibody utilize the cross-reactive Langkat virus (LGTV) antibody (clone 10F6, Cat#: NR-40316) available from BEI Resources.

### Data and code availability

This study did not generate or analyze neither datasets nor code.

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## AUTHOR CONTRIBUTIONS

Experimental conceptualization: all authors; data curation: E.T.S. and A.K.P., formal analysis: E.T.S.; funding acquisition: A.K.P. and J.D.B.; investigation: A.K.P. and E.T.S.; methodology: A.K.P., J.D.B., and E.T.S.; project administration, A.K.P.; resources: all authors; software, A.K.P. and J.D.B.; supervision: A.K.P.; validation: A.K.P.; visualization: A.K.P. and E.T.S.; writing – original draft: A.K.P. and E.T.S.; writing – review & editing, all authors, who reviewed and approved the content and submission of the final manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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