Chapter 11

ELISA and Neutralization Methods to Measure Anti-West Nile Virus Antibody Responses

Brian M. Friedrich and David W.C. Beasley

Abstract

Measurements of humoral immune responses to West Nile virus (WNV) infection in mouse or other animal models are valuable components of basic laboratory investigations to assess immunogenicity of candidate vaccines or to evaluate seroconversion following challenge with WNV. Here, we outline the steps for screening or titrating of total antibodies by indirect enzyme linked immunosorbent assay (ELISA) as well as assessment of neutralizing antibody titers by immunofocus detection.

Key words West Nile virus, Humoral immunity, Antibody titration, ELISA, Neutralization, Immunofocus, FRNT

1 Introduction

Measurements of humoral immune responses to West Nile virus (WNV) infection in mouse or other animal models are valuable components of basic laboratory investigations to assess immunogenicity of candidate vaccines or to evaluate seroconversion following challenge with virulent or attenuated WNV variants/strains. This chapter provides a representative method for screening or titration of IgG or total antibodies by indirect enzyme linked immunosorbent assay (ELISA) that can be performed using in-house or commercially available recombinant protein antigens representative of envelope (E) or non-structural protein 1 (NS1), two major targets of the humoral immune response in natural WNV infections. This method is also adaptable to assessment of antibody responses in other species via the use of secondary antibodies with alternative specificity. Although several commercial immunoassays are available for testing of human serum samples for anti-WNV IgM and/or IgG antibodies, these methods can also be appropriate for experimental evaluation of field-collected human or animal serum samples.

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In addition, a method for assessment of neutralizing antibody titers is described. This method employs an immunofocus detection approach, rather than traditional detection of virus plaques, which makes it more amenable to performance in 12- or 24-well tissue culture plates, thereby reducing quantities of serum samples required for assay performance.

2 Materials

Prepare all solutions using distilled, deionized water. Prepare and store all reagents at room temperature (unless indicated otherwise). Each of the reagents described below is for example and could be readily substituted with alternative commercially available equivalents with minimal assay optimization.

2.1 Indirect ELISA Materials

1. Coating buffer: borate saline pH 9.0. Add about 900 ml of water to a glass beaker or container. Weigh 7.0 g NaCl and transfer to beaker. Weigh 3.1 g H_3BO_3 and transfer to beaker. Add 24 ml 1.0 N NaOH to beaker. Mix until all solid chemicals have gone into solution then adjust pH to 9.0. Make up to 1 l final total volume with water.
2. Phosphate buffered saline (PBS): Add one phosphate buffered saline tablet (e.g., Fisher, catalog# BP2944-100 or Sigma, catalog# P4417-100TAB) to 200 ml of water and mix to dissolve. For preparation of large quantities of PBS or washing buffer (see below), a 5× PBS stock solution (e.g., 25 tablets in 1 l of water) can be prepared and subsequently diluted 1:5 in appropriate volumes of water.
3. Blocking buffer: 3% milk or 3% bovine serum albumin (BSA) in PBS. Add about 75 ml of PBS to a glass beaker or container. Weigh 3 g dried milk (or BSA) and transfer to beaker and mix to dissolve (BSA can sometimes take several minutes to dissolve into solution). Add water to make final volume 100 ml. Mix and store at 4 °C. Sufficient quantities of blocking buffer should be prepared for a single use or for expected use over 3–5 days (*see* **Note 1**).
4. Washing buffer: PBS containing 0.5% Tween 20 (PBST). Add 0.5% v/v of Tween 20 to PBS and mix to dissolve (e.g., 5 ml Tween 20 to 995 ml PBS).
5. MaxiSorp round-bottom 96-well plates (Thermo Scientific Nunc, catalog# 449824).
6. Adhesive PCR plate foil or other adhesive plate cover.
7. 3,3′,5,5′-tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (e.g., Sigma, catalog# T0440).

Table 1
WNV antibodies

Antibody	Source/catalog number	Protein specificity	References
7H2	VRL Cat#81-002 (*see* **Note 2**)	E, domain III	[1]
5H10	VRL Cat#81-003	E, domain III	[1]
3A3	VRL Cat#81-004	E, domain III	[1]
5C5	VRL Cat#81-005	E, domain III	[1]
3D9	VRL Cat#81-006	E, domain II (fusion loop)	[1, 2]
3.1112G	Millipore Cat#MAB8152	NS1	[3]

8. Stop solution: 3 M HCl. A 3 M working solution should be prepared by appropriate dilution of a commercial hydrochloric acid stock.
9. Plate reader. (Needs to be able to read dual absorbances at 450 and 595 nm.)
10. Positive control antibodies: A range of anti-WNV E/NS1 monoclonal antibodies and polyclonal antisera are available from commercial sources or reference laboratories and can be employed as positive controls. Some examples are provided in Table 1 above.
11. HRP-labeled Secondary Antibody. Anti-Mouse IgG (whole molecule)—Peroxidase conjugated antibody produced in goat (e.g., Sigma, catalog# A4416)—or whichever species your primary antibodies are derived from (*see* **Note 3**).
12. Antigens for coating plates: Various methods have been reported in the literature for expression and purification of WNV and other flavivirus E (e.g., [4, 5]), EIII (e.g., [1, 6]), and NS1 (e.g., [4, 7]) recombinant proteins using bacterial, mammalian or insect cell expression systems. For studies in our laboratory we use an in-house prepared WNV EIII antigen; however, similar EIII antigens (e.g., SinoBiological Inc., catalog # 40345-V08Y-100) as well as 80% E (e.g., ProSpec Bio., catalog# WNV-001) and NS1 (e.g., Immune Tech, catalog# IT-006-0053p) antigens can be obtained from commercial suppliers.

2.2 Focus Reduction Neutralization Test (FRNT) Components

1. Vero growth media: MEM + 8% FBS + 1% L-glut + 1% NEAA + 1% pen/strep. To one bottle of MEM (e.g., Corning Cellgro, catalog# 10-010-CV), add 40 ml FBS (e.g., Hyclone, catalog# SH30071), 5 ml L-glutamine (e.g., Gibco, catalog# 25030), 5 ml MEM nonessential amino acid solution 100× (e.g., Sigma, catalog# M7145), and 5 ml penicillin–streptomycin (e.g., Corning Cellgro, catalog# 30-001-CI). FBS should

be heat inactivated by incubating at 56 °C for 30 min prior to use in media preparation. A bottle of FBS can be heat inactivated, aliquoted into appropriate volumes in sterile tubes and stored at −20 °C prior to use.

2. Maintenance media: MEM + 2 % FBS + 1 % L-glut + 1 % NEAA + 1 % pen/strep. To one bottle of MEM add 10 ml FBS, 5 ml L-glutamine, and 5 ml of penicillin–streptomycin.
3. 2×MEM: 2× MEM + 4 % FBS + 0.5 % phenol red + 2 % L-glut + 2 % pen/strep. To one bottle of 2×-MEM (e.g., Quality Biological 115-073-101) add 20 ml FBS, 2.5 ml phenol red Solution (e.g., Sigma, catalog# P0290), 10 ml L-glutamine, and 10 ml penicillin–streptomycin.
4. 2 % agar: Add 6 g agar (e.g., Sigma, catalog# A1296-1KG) to 300 ml water. Autoclave to dissolve and sterilize.
5. Overlay media: Make a 1:1 mixture of 2× MEM and 2 % agar (*see* **Note 4**).
6. PBS: Add 1 phosphate buffered saline tablet (e.g., Fisher, catalog# BP2944-100 or Sigma, catalog# P4417-100TAB) to 200 ml of water and mix to dissolve.
7. Vero cells (e.g., ATCC, Vero (catalog# CCL-81), Vero 76 (catalog# CRL-1587), or Vero E6 (catalog# CRL-1586)): Grow cells in culture flasks using culture media (above). A single confluent T-150 flask (e.g., Corning, catalog# 430823) generally yields sufficient cells to seed approximately five 12-well plates.
8. 12-well plates (e.g., Corning Costar, catalog# 3513, or any cell culture treated 12-well plate).
9. 96-well plate(s) (e.g., Corning Costar, catalog# 3788, or any untreated 96-well plate).
10. West Nile virus. When preparing the virus, prepare sufficient quantities of aliquots to allow repeated performances of the FRNT assay without having to reoptimize with a new virus prep each time. Please refer to Chapter 3 for methods on propagation and titration of WNV strains.
11. Serum or samples for testing (*see* **Note 5**).
12. 10 % buffered formalin (e.g., Protocol, catalog# 245-684).
13. 70 % ethanol, stored at −20 °C.
14. Primary detecting antibody (e.g., mouse anti-flavivirus MAb 4G2 [EMD Millipore, catalog# MAB10216]).
15. HRP-labeled secondary antibody. Anti-mouse IgG (whole molecule)—Peroxidase antibody produced in goat (e.g., Sigma, catalog# A4416)—or whichever species your primary antibodies are derived from (*see* **Note 3**).

16. PBS I-Block Buffer: Add 0.5% I-Block (e.g., Applied Biosystems, catalog# T2015) to PBS.
17. Precipitating substrate solution (e.g., KPL True Blue [KPL, catalog# 50-78-02] or Vector VIP [Vector Laboratories, Cat# SK-4600]).
18. Rocking platform.

3 Methods

3.1 Indirect ELISA

Carry out all procedures at room temperature unless otherwise specified. Plates should not be allowed to dry out during incubation steps—generally this should not be a problem for 60 min or shorter incubations at room temperature, but it is best to keep the plates covered while incubating.

3.1.1 Coating Plates with Antigen

1. For each ELISA plate, prepare two tubes. The first tube will have borate saline only (background control wells): measure 2700 µl borate saline into a tube. The second tube will contain antigen diluted into borate saline: EIII, 80%E or NS1 Antigen will be diluted into borate saline to a final volume of 2700 µl. For EIII, measure about 5400–6750 ng (corresponding to 100–125 ng/well) and dilute volume to 2700 µl. (*See* **Note 6** for other antigens.)
2. Pipette 50 µl per well of diluted antigen to the desired number of wells in one or more MaxiSorp ELISA plates. If appropriate, also dispense 50 µl per well of borate saline to an equal number of wells (*see* **Note 7** for plate set up).
3. Cover plate (e.g., with adhesive foil/film or a plastic lid) and incubate plate overnight in a refrigerator at approximately 4 °C.

3.1.2 Indirect ELISA

1. After incubating overnight, discard the contents of all wells, and wash once with washing buffer. Washing can be performed by hand using a squirt bottle filled with wash buffer. Fill each well with washing buffer and then invert the plate and shake over a sink to empty. Residual wash buffer can be removed from the wells by tapping the inverted plate on some paper toweling.
2. Block the ELISA plates by adding 60–75 µl/well of blocking buffer (*see* **Note 1**).
3. Cover plates with adhesive foil and incubate for 60 min to allow blocking. (Additional incubation at this step is ok.)
4. During the blocking incubation, prepare dilutions of the serum/antibody samples in wash buffer. For example, individual samples may be screened at 1:100 and 1:500 dilutions in

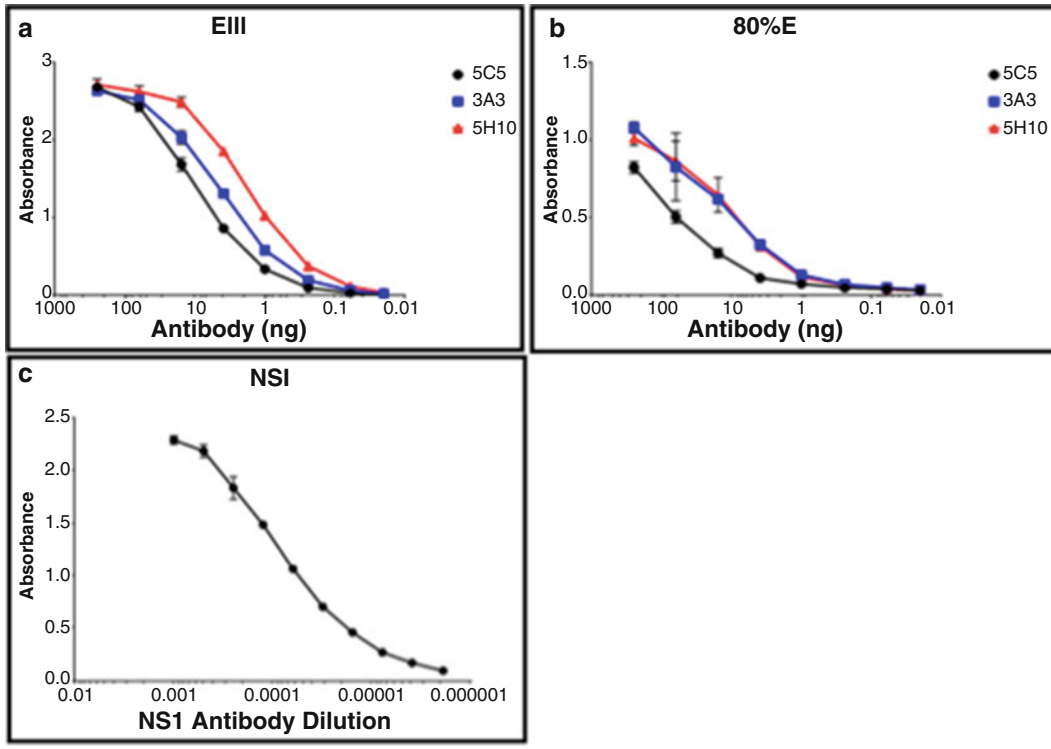

Fig. 1 Representative titrations of positive control monoclonal antibodies. EIII-specific monoclonal antibodies (VRL) were titrated (serial fourfold dilutions) against (**a**) EIII or (**b**) 80 % E antigens. (**c**) Titration of NS1-specific monoclonal antibody 3.112G (Millipore) (serial fourfold dilutions) against NS1 antigen ELISA. Note that the anti-NS1 antibody used was not provided by the manufacturer at a defined concentration/quantity

duplicate against antigen coated and uncoated wells. In that case, each sample will be used in four wells (duplicates for both antigen-coated and background controls), so a minimum of 200 µl (4 wells × 50 µl/well) is needed for each sample. A positive control antibody/antiserum can be included with each plate/batch, such as an anti-WNV-EIII (e.g., 7H2, 5C5, 5H10), anti-WNV-E (e.g., 3D9), or anti-WNV-NS1 (3.1112G) at a 1:1000 dilution (Fig. 1). Store diluted samples at 4 °C until ready. A negative control can be included with each plate/batch that contains "wash buffer only."

5. After blocking, wash two times with wash buffer (by completely filling wells and then discarding into the sink), then ensure any remaining wash buffer is removed from wells by tapping inverted plates on paper towels.
6. Add 50 µl/well volumes of diluted primary antibodies/sera and controls to appropriate wells. If using "borate saline only" wells/plates for background controls then dispense antibodies/sera into those as well.
7. Cover plate/s with adhesive foil and incubate for 45–60 min at room temperature.

8. Make dilution of secondary antibody: HRP-labeled anti-mouse IgG antibody at a 1:5000 dilution in PBST. (For other secondary antibodies, *see* **Note 3**.) For each plate, dilute 1 μl antibody in 5 ml PBST.
9. Wash plate/s three times with wash buffer as described above, and then ensure any remaining wash buffer is removed from wells by tapping inverted plates on paper towels.
10. Dispense 50 μl of diluted secondary HRP-labeled antibody to each well. This can be done using a multichannel pipette and trough or with a multidosing pipette.
11. Cover plate/s with adhesive foil and incubate for 45–60 min.
12. Discard secondary antibody solution from each well and wash plate(s) three times with wash buffer, and then was an additional two times with PBS alone; then remove any remaining buffer volume from wells by tapping inverted plate(s) on paper towels.
13. Add 50 μl of TMB substrate to each well using a multichannel pipette, taking care not to allow tips to touch the plate.
14. Incubate for 10 min (or until color develops sufficiently in positive control wells while remaining low in negative control wells), and then stop reaction by adding 50 μl of 3 M HCl to each well.
15. Absorbance values can then be read on a plate reader; use dual wavelength—450 and 595 nm reference values. When interpreting the data, subtract the average of the duplicate borate saline wells from the average of the duplicate antigen-coated wells of the same samples. That will give you the actual absorbance signal for the assay and subtract out any nonspecific binding or background. Cutoffs for "positive" and "negative" samples should be established for the particular types of sample(s) being tested. A good rule of thumb for a simple cutoff is a value three standard deviations above the mean absorbance of negative control wells. However, alternative cutoffs for positive and negative samples may be determined to be more appropriate depending upon the types of samples being tested or intended use of the data (positive/negative determination vs. quantitative or semi-quantitative comparison). If samples are being titrated via serial dilution, the titer typically represents the inverse of the last positive dilution (Fig. 2).

3.2 FRNT

Important: All cell culture work should be done in a clean biosafety cabinet (BSC) in BSL2 conditions. All work involving use of live WNV must be done in a BSC in a BSL3 facility. Serum samples should be heat inactivated at 56 °C for 30 min prior to use in this assay.

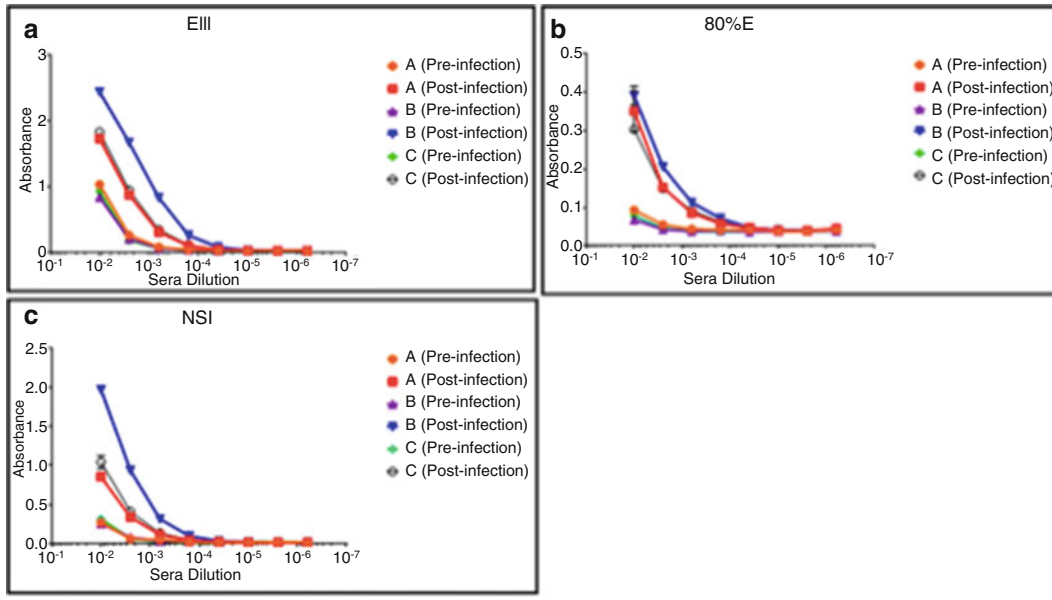

Fig. 2 Representative data from (**a**) EIII, (**b**) 80 % E, and (**c**) NS1 ELISAs to assess antibody levels in pre- and post-WNV-challenge samples from mice previously immunized with two doses of a commercial WNV veterinary vaccine. Serum samples were serially fourfold diluted from 1:100 to 1:1,638,400. The average background absorbance value (borate saline only wells) was 0.026 with a standard deviation of 0/013. Therefore the positive/negative cutoff of this particular assay would be 0.065

3.2.1 Seeding of Plates with Vero Cells

1. Seed 1 ml per well of a cell mixture containing approximately 1.2×10^5 cells/ml (Vero cells) in 12 well tissue culture plates. In the method described here, 18 wells (1.5 plates) are required per serum sample tested in duplicate, although a smaller or larger number of dilutions may be performed depending on the need to identify neutralization endpoint titers. (The recommended batch size is 15–20 total plates (10–13 samples) to minimize handling times during each of the processing steps.)
2. Plates should normally be used 1 day after seeding and should ideally be slightly less than 100 % confluent at time of infection.

3.2.2 Dilutions and Infections for FRNT

The specific range of dilutions to be tested can be varied based on available sample volumes or requirement to identify particular endpoint titer(s). In the following steps, samples are tested at doubling dilutions between 1/20 and 1/2560. Serum dilutions are prepared between 1/10 and 1/1280 in maintenance media and then mixed 1:1 with an equal volume of diluted virus to give the final dilutions in the neutralization test.

1. In a 96-well plate, pipette 108 μl of maintenance media into wells in column 1 (two rows for each sample to be tested in duplicate). (This volume would be modified for alternative starting dilutions.)

2. Pipette 60 µl of maintenance media into wells in columns 2–9.
3. Add 12 µl of serum sample(s) to wells of column 1 and mix by pipetting (this is a 1:10 dilution). Each serum sample will be diluted in two rows.
4. Using a multichannel pipette, perform serial doubling dilutions across the plate, using a transfer volume of 60 µl. Mix each dilution by pipetting and discard tips between each transfer. Discard the final transfer volume after column 8, leaving column 9 as culture medium only. The final volume in each well is 60 µl. Take particular care when using a multichannel pipette to ensure by visual inspection that all tips contain correct volumes during each transfer.
5. Prepare virus dilution. The amount of virus needed should be determined beforehand where the final assay gives approximately 40 plaques per well (*see* **Note 8**). For each serum sample tested, a minimum of 1080 µl diluted virus (9 dilutions in duplicate [18] × 60 µl/well) is required. Each virus stock tube should be thawed at room temperature and mixed by pipetting prior to use to ensure even distribution of the virus.
6. To each well of the 96-well plate(s) used for serum dilutions add 60 µl of diluted virus and mix by pipetting. A multichannel pipette can be used. Virus prep can be placed into a sterile trough, and clean tips are required for each addition. Incubate at room temperature for 60 min.
7. Following the 60 min incubation, discard supernatants from wells of 12 well plates (five or six at a time is generally ok), rinse wells with sterile PBS (~1 ml/well) and discard.
8. Pipette 100 µl of virus–serum mixture from wells of 96-well plate(s) into appropriate wells of 12-well plates (using a clean tip for each addition), rock the plate to ensure that the virus–serum mixture is distributed over the entire monolayer, then replace the lid and incubate for 30–35 min at room temperature with periodic rocking to allow virus binding.
9. Following incubation, overlay each well with 2 ml overlay media and incubate at 37 °C/5 % CO_2 for 2 days.

3.2.3 Immunostaining

1. After 2 days, fix plates by completely filling wells with buffered formalin and incubating for 60–70 min at room temperature. Ensure that formalin contacts all interior surfaces of the wells and plate lid.
2. After incubation, discard formalin from wells, remove agar plugs with a spatula and add ~1 ml of fresh buffered formalin to each well. Incubate for an additional 10–15 min at room temperature.

3. After this second incubation, plates, still containing formalin, can then be surface decontaminated with appropriate virucidal reagent (e.g., CaviCide), placed in sealed secondary containment and transferred to BSL2 for further processing.
4. Discard the buffered formalin into appropriate waste container and then wash each well two times with PBS, using approximately 2 ml per well each time (*see* **Note 9**).
5. Discard PBS and add ~0.5 ml/well ice cold 70% ethanol and incubate for 30 min at −20 °C.
6. Prepare sufficient volume of a dilution of the primary antibody (e.g., 1:2500 anti-flavivirus MAb 4G2 diluted 1:2500 in I-Block PBS buffer) to dispense 150 μl/well in the assay plates.
7. After incubation, wash plates two times with PBS as described above.
8. Discard PBS, add 150 μl/well of the primary antibody solution, replace the lid, and incubate for 80–90 min on a rocking platform at room temperature. Rocking speed should be low and sufficient to allow gentle movement of the antibody solution over the monolayer.
9. Wash cells two times with PBS (as described above).
10. Discard PBS and add 200 μl/well of the secondary antibody (e.g., 1:5000 dilution of HRP-labeled anti-mouse IgG in I-Block). Incubate 60–70 min on rocking platform.
11. Wash cells two times with PBS (as described above).
12. Stain plates with 200 μl/well precipitating substrate solution for 10–15 min on rocking platform.
13. Discard staining solution and rinse cells with dH_2O (~2 ml) when foci become clearly visible in virus-only control wells and then air dry each plate (e.g., with lid off in a fume hood).

3.2.4 Analysis and Interpretation

Count foci in each well and record on data sheets. Foci counts in all virus-only control wells for each batch are to be averaged to determine an endpoint cutoff. For example, FRNT50 titers for each sample tested can be determined by identifying the well for each containing the highest dilution of serum with a foci count <50% of the average virus control well value (Fig. 3). Titers for individual sample replicates should be within twofold. Use of alternative endpoint cutoffs (e.g., 80 or 90% neutralization) may be considered and can be calculated from the same data.

4 Notes

1. High nonspecific binding of some sera from immunized/infected mice to plates blocked with PBS containing BSA has been observed in some of our experiments, perhaps due to

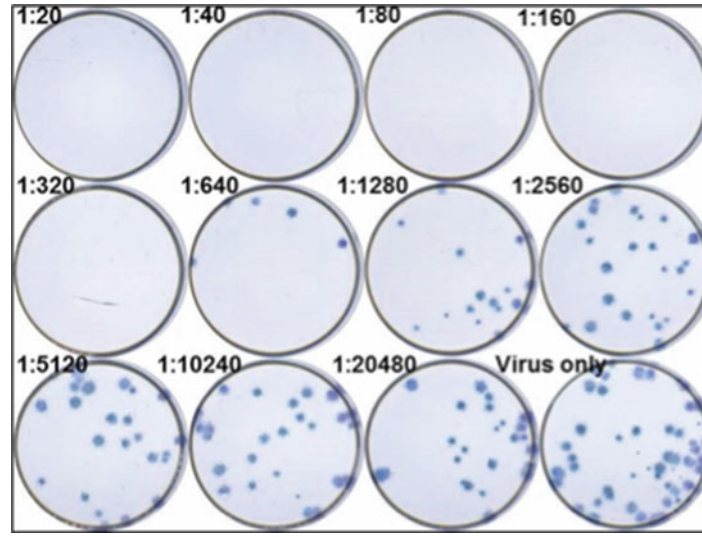

Fig. 3 Representative FRNT assay plate. The 50 % focus reduction neutralization titer (FRNT50) is the inverse of the final dilution with a foci count <50 % of the mean virus only control well counts for a batch. For this particular plate, the FRNT50 would be 1280

residual FBS in virus preparations or in some commercial WNV veterinary vaccine preparations. PBS containing 3 % milk may be a preferable blocking reagent for most applications.

2. VRL was formerly known as Bioreliance.
3. Some examples of secondary antibodies for alternative species: HRP-labeled anti-human IgG antibody (e.g., Sigma, catalog# A6029-1ML), HRP-labeled anti-rabbit IgG antibody (e.g., Sigma, catalog# A6154-1ML), HRP-labeled anti-monkey IgG antibody (e.g., Rockland, catalog# 617-103-012).
4. Overlay media—2× MEM/agar. The 2 % agar solution should be carefully heated in a microwave oven to return it to liquid form and then held in a 52–56 °C water bath for >20 min prior to use. 2× MEM should be removed from 4 °C storage and held in a 37 °C water bath for >20 min prior to use. After mixing equal quantities of the agar and 2× MEM to prepare overlay media, it will generally remain liquid at room temperature for at least 15 min which is sufficient time to overlay a typical batch of plates.
5. It is highly recommended to include a positive control neutralizing antibody to monitor performance from batch-to-batch. This could be a monoclonal antibody or polyclonal antiserum several of which are commercially available or can

be sourced from reference collections such as the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) or ATCC/BEI.

6. Appropriate dilutions of antigen for coating should be determined by block-titration of antigen and a known positive control antiserum/MAb, i.e., groups of wells are coated with serial dilutions of the antigen, and then detected using serial dilutions of the antiserum. Optimal dilutions of both antigen and control antiserum can be determined in this manner. In our hands, the optimal antigen coating concentrations are:
   (a) 80% E (ProSpec, catalog # wnv-001-b): 300–500 ng/well.
   (b) NS1 (Immune Technology Corp, catalog # IT-006-0053P): 200–300 ng/well.
   (c) EIII: 100–125 ng/well.
7. Plate coating and assay setup are flexible and may involve screening of sera at a single dilution, multiple dilutions, or performance of serial titrations to assess endpoint titers. Coated and uncoated wells may be on the same plate or different plates, depending on how many samples are being processed and dependent on the user. It is recommended to test sera at least in duplicate, although additional replicates may be appropriate. Also, for previously untested polyclonal sera, it is very beneficial to test against coated and uncoated (borate saline only) wells to identify any nonspecific binding. For example: if one half of the plate is coated in antigen and the other half with borate saline only, each plate can test 24 samples in duplicate at a single dilution or 12 samples in duplicate at two dilutions.
8. Virus dilutions: Prior to FRNT assay, the virus dilution must be determined that will give you approximately 40–50 foci per well in the final assay.
9. If immunostaining will occur at a later time, plates can be left with PBS in wells and stored at 4 °C for up to 2 days.

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References

1. Beasley DWC, Barrett ADT (2002) Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. J Virol 76:13097–13100
2. Zhang S, Li L, Woodson SE et al (2006) A mutation in the envelope protein fusion loop attenuates mouse neuroinvasiveness of the NY99 strain of West Nile virus. Virology 353: 35–40
3. Hall RA, Broom AK, Hartnett AC et al (1995) Immunodominant epitopes on the NS1 protein of MVE and KUN viruses serve as targets for a blocking ELISA to detect virus-specific antibodies in sentinel animal serum. J Virol Methods 51:201–210
4. Lieberman MM, Clements DE, Ogata S et al (2007) Preparation and immunogenic properties of a recombinant West Nile subunit vaccine. Vaccine 25:414–423
5. Beasley DWC, Holbrook MR, Travassos da Rosa APA et al (2004) Use of a recombinant envelope protein subunit antigen for specific serological diagnosis of West Nile virus infection. J Clin Microbiol 42:2759–2765
6. Bhardwaj S, Holbrook M, Shope RE et al (2001) Biophysical characterization and vector-specific antagonist activity of domain III of the tick-borne flavivirus envelope protein. J Virol 75:4002–4007
7. Chung KM, Nybakken GE, Thompson BS et al (2006) Antibodies against West Nile virus nonstructural protein NS1 prevent lethal infection through Fc γ receptor-dependent and -independent mechanisms. J Virol 80:1340–1351