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Technical report:
Technical Protocol for Tick-borne Encephalitis Virus Neutralization Test

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Executive Summary

This technical protocol provides a comprehensive, laboratory-ready workflow for **detecting and quantifying neutralizing antibodies against tick-borne encephalitis virus (TBEV)** using a **virus neutralization test (VNT) coupled with an in-cell ELISA (ic-ELISA) readout**. The method is designed to assess functional antibody responses in serum or plasma samples and enables determination of **neutralization efficacy (% of neutralization)** as well as calculation of the **50 % neutralizing antibody titer (NT_{50})**.

Tick-borne encephalitis virus (TBEV) is one of the most important arthropod-borne viral pathogens in Europe, capable of causing clinical manifestations ranging from mild febrile illness to severe neurological disease. Protective immunity against TBEV is primarily mediated by neutralizing antibodies, making their accurate measurement essential for evaluating vaccine-induced immunity, previous exposure, and immune responses in clinical and epidemiological studies. Due to the extensive serological cross-reactivity among flaviviruses, VNT is an essential tool for the differential diagnosis of flaviviral infections, enabling distinction between TBEV-specific antibodies and cross-reactive flavivirus antibodies.

The protocol is based on incubating TBEV with serially diluted test samples, followed by infection of susceptible cell cultures. After the incubation period, viral replication is quantified by **in-cell ELISA (ic-ELISA).** The assay measures the optical density (OD) signal generated by infected cells, which directly reflects the extent of viral infection. Neutralizing activity is determined by comparing OD values obtained in the presence and absence of test sera, enabling calculation of the **percentage of virus neutralization** for each sample dilution. Subsequently, dose-response analysis is used to determine the **NT_{50} value**, defined as the reciprocal serum dilution that achieves 50 % neutralization of viral infectivity.

This document describes the required reagents, equipment, and step-by-step laboratory procedures necessary for standardized assay implementation. The workflow has been optimized to provide a sensitive, reproducible, and scalable approach for assessing TBEV-specific neutralizing antibodies, while reducing the subjectivity associated with traditional cytopathic effect-based readout.

Overall, this protocol provides a standardized, reproducible method suitable for **confirming routine diagnostic test results, conducting vaccine studies, performing seroepidemiological investigations, and supporting research applications** involving TBEV. Using ic-ELISA as the readout platform facilitates harmonized data generation and quantitative comparison of neutralizing antibody responses across laboratories and studies.

Tick-borne encephalitis virus neutralization test protocol

V.1 (2026-08-04)

1 Introduction

Tick-borne encephalitis (TBE) is the most significant tick-borne viral infection in Europe and parts of Asia. In Central Europe, it is a seasonal disease, with most cases occurring between April and November. It is caused by tick-borne encephalitis virus (TBEV), which is most commonly transmitted by the bite of an infected tick, although other routes are possible, such as consumption of unpasteurised dairy products, transmission via solid organ transplantation, and laboratory infection. Infection may result in a wide spectrum of clinical manifestations, ranging from mild febrile illness to severe neurological disease, including meningitis, meningoencephalitis, and meningoencephalomyelitis. Protective immunity against TBEV is largely mediated by virus-specific neutralizing antibodies directed against the viral envelope (E) protein, which prevent viral attachment, entry, and replication in susceptible host cells. The detection and quantification of neutralizing antibodies are essential for evaluating immune responses following natural infection or vaccination, assessing population immunity, and supporting epidemiological and vaccine efficacy studies. Unlike binding antibody assays, neutralization tests measure the functional ability of antibodies to inhibit viral infectivity and are therefore considered the gold standard for assessing protective humoral immunity against TBEV.

2 Intended use

This protocol describes all laboratory procedures, reagents and equipment required for the detection and quantification of TBEV-specific neutralizing antibodies using VNT with ic-ELISA endpoint detection. The assay is intended for the analysis of serum or plasma samples from humans and enables the measurement of functional antibody responses against TBEV following natural infection or vaccination.

3 Materials and reagents

The reagents and suppliers listed in this SOP were used for protocol optimization with the TBEV Ljubljana strain and are not exclusive. Reagents from other suppliers can be used as substitutes, but re-optimization may be necessary since these reagents were not tested in the assay. The protocol is applicable to other flaviviruses, but the optimal virus concentration for the assay must be established for each virus strain.

3.1 Suitable samples

Human serum or plasma samples.

3.2 Reagents

Component	Supplier	Cat. Number
TBEV strain Ljubljana	UL, EVA-AISBL	Ref-SKU: 007V-02198
Vero CCL-81 cell line (ATCC CCL-81)	ATCC	CCl-81
DMEM (DMEM + GlutaMax-I)	GIBCO	21885-025
Fetal bovine serum	Sigma	F7524
Trypsin (0.25% Trypsin-EDTA)	GIBCO	25200-056
Trypan Blue 0.4%	Invitrogen	T10282
Formaldehyde	Sigma	1.04003.1000
MilliQ autoclavable water	ChemLab	CL02.2101.5000
1x PBS, pH 7.2	- NaCl (Supelco, 1.06404.1000), - Na2HPO4*2H2O (Sigma, 30435) - KH2PO4 (Sigma, 60220), - MilliQ water (Biotech)	
Tween 20	Sigma	8.2218.0050
Triton-x-100	Sigma	T878-50mL
Nonfat milk powder	Pomurske mlekarne	3831040010028
Primary antibody Mouse Anti-Flavivirus Group Antigen Antibody, clone D1-4G2-4-15	Sigma Aldrich	MAB10216-I-100UG
Secondary antibody goat anti-mouse IgG H&L (HRP)	Thermoscientific	AB205719
TMB Chromogen Solution	Life technologies	002023
HCl	Honeywell	30721-1L

3.3 Equipment

Component	Supplier	Cat. Number
5 mL sterile plastic pipets	SARSTEDT	86.1253.001
10 mL sterile plastic pipets	SARSTEDT	86.1254.001
25 mL sterile plastic pipets	SARSTEDT	86.1685.001
50 mL falcon tubes	TPP	91050
96-well tissue culture plates	TPP	92096
96-well dilution plate	Greiner	780271
Cell counting chamber slides	Invitrogen	C10283
Reagent reservoir	Costar Corning	RA-9425518
8-channel automatic pipette, 1200 µl	Sartorius	41684244
12-channel automatic pipette, 300 µl	Sartorius	41485299
Countess 3 FL Automated Cell Counter	ThermoFisher Scientific	2188A22072599
Absorbance reader GloMax Explorer GM3500	Promega	9720100455
CO_2 incubator MCO-170AICUVH-PE	PHCBI	210960269
ThermoMixer	Eppendorf	5382DN005540

3.4 Solutions

Solution	Preparation
10% DMEM	50 mL FBS add to 450 mL DMEM; store at 4°C for up to 4 to 6 weeks
1% DMEM	5 mL 10% DMEM add to 45 mL DMEM; prepare before use
Fixation buffer (8% Formaldehyde)	215.6 mL 37 % Formaldehyde (Sigma, 1.04003.1000) dilute with 784.4 mL ultra-pure water; store at 4°C for up to 3 months
Permeabilization buffer	Prepare 1x PBS and add Triton-X-100 at a 0.01% concentration (v/v) (example: for a 96-well plate, mix 12 mL PBS with 120 µL Triton X-100); prepare directly before use. Due to its high viscosity, pipette carefully and mix thoroughly until fully dissolved.
PBS-T (1X PBS, 0.1% Tween-20)	Prepare 1x PBS and add Tween-20 at a 0.1% concentration (v/v) (example: 1 mL Tween-20 ad to a 1 L PBS); store at 4°C for up to 4 to 6 weeks
Blocking buffer (5% skimmed milk)	Add 5% of nonfat dry milk to the desired volume of PBS-T (example: 5g of dry milk to a 100 mL of PBS-T); prepare before use
Primary antibody working solution	Dilution 1:1000 (example: 10 µL primary antibody ad to 9990 µL Blocking buffer); prepare directly before use
Secondary antibody working solution	Dilution 1:2000 (example: 5 µL secondary antibody ad to 9995 µL Blocking buffer); prepare directly before use
1N HCl	8.3 mL 37% hydrochloric acid (Honeywell, 30721) add to 100 mL ultra-pure water; store at room temperature for up to 3 months

4 Protocol

4.1 Preparation of the cell cultures (day 0)

For VNT, the cell monolayer in the wells should be almost completely confluent, with the cells evenly distributed across the entire surface. Thus, the VERO (CCL-81) cells should be prepared 1 day before the assay. For a 96-well plate, prepare the cells at a concentration of 3×10^5 cells/mL and add 100 µL per well.

1. Trypsinization: for a T25 flask, add 1mL of trypsin and incubate at 37 °C and 5% CO_2 until the cells detach from the bottom (~ 2 min). Add 4 mL of 10 % DMEM and resuspend the cells.
2. Measure the concentration of the cell suspension and dilute it accordingly: for a 96-well plate, you need 10 mL of cell suspension at a concentration of 3×10^5 cells/mL.
3. Dispense the prepared cell suspension into a 96-well plate at a volume of 100 µL/well. After seeding, tap the plate on all four sides to distribute the cells evenly across each well.
4. Incubate the plates over night at 37 °C and 5 % CO_2. Before inoculation confirm that the cell monolayer is 80-90% confluent. To obtain more reliable results, do not inoculate the outer wells (use only the 10 inner wells in 6 rows, but the outer wells should not be empty).

4.2 Virus neutralization test inoculation (day 1)

1. Prepare serum samples:
   a. Heat-inactivate 50 µL of serum sample **for 30 minutes at 56°C.**
   b. **Prepare a serial dilution of the investigated serum samples using 1% DMEM** dilution inoculated in triplicate (Table 1). The dilution range included in the assay should be determined according to the experimental design. Including more dilutions in the test improves the reliability of the NT_{50} calculation. For an efficient NT_{50} calculation, the percentage of neutralization in the final serum dilution tested should be low or negative.

Table 1: Example of two-fold serum samples serial dilution in 1 % DMEM. The volumes shown below are calculated for inoculating samples in triplicate (3x50 µL plus access volume).

Dilution (1 : x)	10	20	40	80	160	320	640	1280	2560	5120	10240
DMEM + 1% FBS	180 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
SERUM	20 µl										
Transfer	Transfer 100 µl	Transfer 100 µl	Transfer 100 µl	Transfer 100 µl	Transfer 100 µl	Transfer 100 µl	Transfer 100 µl	Transfer 100 µl	Transfer 100 µl	Transfer 100 µl	Discard 100 µl

2. Dilute virus stock to working concentration:
   a. Required final concentration of virus in well is **2.2×10^2 $TCID_{50}$/µL** (based on desired virus concentration (5.5×10^3 $TCID_{50}$/well) and the final volume of the virus in the well 25 µL)
   b. Prepare virus working batch by calculating:
      - Calculate the <u>volume of virus required</u>: for each sample dilution you need 100 µL of virus, plus an additional 100 µL of virus for the positive control (PC) for each plate.
      - Calculate the virus <u>working batch concentration</u>: 2.2×10^2 $TCID_{50}$ x volume of required virus
      - Calculate <u>virus stock dilution (in µL):</u> working batch conc / stock virus concentration ($TCID_{50}$/mL) × 1000

<u>Example of calculation:</u>

- Stock virus concentration: 5.6×10^6 $TCID_{50}$/mL
- Volume of virus required: 14 sample dilutions (each 100 µL) + PC (100 µL) = 1500 µL
- Working batch virus concentration needed: 2.2×10^2 $TCID_{50}$/µL × 1500 µL = 3.3×10^5 $TCID_{50}$
- Dilution of stock virus: 3.3×10^5 $TCID_{50}$ / 5.6×10^6 $TCID_{50}$/mL = 0.058 mL ~ which would mean: 58 µL of stock virus + 942 µL DMEM

3. Prepare virus and serum mixture:
   a. Mix virus in working concentration with diluted sample at a 1:1 ratio: add **100 µL of virus suspension** to **100 µL of sample dilution**.
   b. Prepare the **negative control (NC)**: 200 µL of 1% DMEM (inoculated in triplicate).
   c. Prepare the **positive control (PC)**: 100 µL of 1% DMEM + 100 µL of virus suspension (inoculated in triplicate).
   d. Seal the plate with adhesive film and incubate for **1 hour at 37°C with 5% CO_2.**

4. Prepare the cell culture plates for inoculation:
   a. During incubation, prepare the plates containing the pre-seeded cells. Confirm that the cell monolayer is 80-90% confluent. Appropriate cell density is critical for obtaining a reliable OD signal.
   b. Leave the edge wells unused. Remove 50 µL of growth medium from the inner wells that will be inoculated. Label the plate according to the working protocol.

5. Inoculate the cells:
   a. After incubation, carefully remove the sealing film and inoculate prepared cell culture plates with virus/sample mixture and controls in **triplicate**, adding **50 µL** per well.
   b. Incubate the plates for **3 days** at 37°C with 5% CO_2.

4.3 VNT detection with ic-ELISA readout (day 3)

1. Add **100 µL/well of fixation buffer** (final concentration of 4% formaldehyde/well) and incubate for 30 min at room temperature.
2. Wash the cells with 1x PBS, **2×250 µL/well**.
3. Permeabilize the cells by **adding 100 µL/well of permeabilization buffer** and incubate for 10 minutes at room temperature.
4. Remove the permeabilization buffer, **add 100 µL/well of blocking buffer** and incubate for 1 hour at room temperature.
5. Remove the blocking buffer and add 100 µL/well of **primary antibody working solution** and incubate for 2 hours at room temperature or overnight at 4°C.
6. Wash the cells with PBS-T, **4 × 250 µL/well**.
7. Add 100 µL/well of the **secondary antibody working solution** and incubate for 1 hour at room temperature.
8. Wash the cells with PBS-T, **4 × 250 µL/well**.
9. Add 100 µL/well of **TMB Chromogen Solution** and incubate in the dark for 15 min.
10. Add 100 µL/well **1N HCl** to stop the reaction and measure absorbance at 450 nm.

4.4 Calculation % of NT

1. Export the raw data into Excel.
2. Calculate the **mean OD** for each serum sample dilution, PC and NC.
3. Calculate **neto OD** (also for PC): mean OD_{sample} – mean OD_{NC}

4. Calculate **% of infection**: (neto OD_{sample} / neto OD_{PC}) × 100
5. Calculate **% of neutralization**: 100 - % of infection
6. Calculate NT_{50}:
    a. Prepare xy table: x = log sample dilution and y = % of neutralization for each serum dilution
    b. Analyze: Nonlinear Regression → log(agonist) vs. response (define constrain: F = constant equal to 50, Bottom = constant equal to 0 and Top = constant equal to 100)
    c. As a result, NT_{50} is calculated

5 Annexes

TBEV NT_interactive protocol.xlsx – An Excel template for calculation reagent volumes and workflow design.

6 Contact

For further information please contact eurl-ph-vbv.imi@mf.uni-lj.si

7 Suggested citation:

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