ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) DESCRIPTION

Methods

Antigen coated plates were provided by the VRL Reagents Lab or purchased commercially. ELISA was performed as per Standard Operating Procedures. Test sera were diluted 1:50 using 5% Skim Milk, and 100 µl of diluted test sera was added to the appropriate wells. The plates were incubated at 37°C ± 2°C for 1 hour. Plates were washed 3-5 times with wash buffer. Bound antibodies were detected by a secondary antibody linked to Horse Radish Peroxidase (HRP). The enzymatic activity of bound HRP was detected by addition of 100 µl/well ABTS HRP substrate.

Assay results were read spectrophotometrically at 405nm using a Molecular Devices VMax plate reader. Raw data was collected and analyzed using LIS software (Ronware version 3.15).

Test Materials

The reagents used in the ELISA were prepared in the VRL Reagents Lab according to Standard Operating Procedure. Samples were tested against negative control antigen at the same time they were tested for the presence of antigen specific antibodies in the serum. As internal positive controls, serum known to contain antibodies against the selected antigens was used. As the negative controls, serum known to contain no antibodies against the selected antigens was used. Positive and negative control sera are used at minimum sensitivity to ensure the reliability of the assay.

Antigens: The antigens used in the assay were derived from virus inoculated into appropriate cell lines and grown to an appropriate Cytopathic Effect (CPE). At that time the infected cells were harvested, washed and the antigen extracted. The antigen was coated onto 96 well plates according to Standard Operating Procedures.

Plates: Batches of plates were made by the coating the odd column wells of 96 well plates with antigen and the even column wells with control antigen at the appropriate dilution. Once the plates were coated they were stored at -80°C (+20°C/-10°C) until used for sample analysis. The plate coating was performed according to Standard Operating Procedures.

Conjugate: Conjugate was used at the dilution suggested by the vendor.

Reagents: PBS-Tween, 5% Skim Milk and Washing Buffer

Critical Equipment: Molecular Devices VMax Plate Reader

Methodology for Serological Testing

The ELISA methodology consists of an indirect ELISA for the detection of antibodies against specific viruses and bacteria. Virus specific antigen is coated on 96 well ELISA plates, followed by addition of the sample. Plates are washed to remove the non-binding sample components and incubated with species specific anti IgG antibodies. Binding to the antigen in the plate is detected by development of color in the presence of HRP substrate. The ELISA assay is used as a screening assay and is designed to have a low cut-off value to ensure that low level positives are not missed. The cut-off was defined by testing positive and negative samples, plotting the OD values in a frequency distribution pattern and identifying the lowest OD obtained from the low level positive samples. To differentiate between background and a positive reactivity, a confirmatory test is run in all samples with a net OD value above the cut-off value. Positive reactivity is confirmed by Immunofluorescence Assay (IFA) or Western Blot (WB). The IFA test use infected to cells to detect antibodies in the serum sample. A positive sample will show the characteristic pattern associated with virus infection.

The quality of the ELISA results is controlled by the use of internal assay controls that are included in every test.

- The negative control serum is maintained and tested at the same dilution as the samples. Tests are considered invalid if the negative control value exceeds the cut-off value.
- Positive control sera are used at a dilution that gives a 1.0 OD value. These dilutions are determined by block titrations of antigen and conjugate against serum dilutions. These sera are maintained under the same conditions as the submitted samples. The test is considered invalid if the positive control serum fails to achieve a value of at least 0.8 OD units.

Evaluation of ELISA Test Results

The amount of cleaved substrate was measured using a Molecular Devices VMax plate reader as an optical density (OD) measurement. The net amount of antibody binding to the antigen is obtained by subtracting the OD value of the sample reactivity in the control antigen containing wells (even columns) from the sample reactivity in the antigen containing wells (odd columns). A positive specimen is defined as one whose net OD value is higher than or equal to a predetermined cut off value, in this case 0.17. The cut off value depends on the agent tested.

Antigen OD – Control Antigen OD = Net OD value

Confirmation of positive reactivity in the ELISA is done by Indirect Fluorescent Antibody (IFA) tests. The Final result will be the result of the confirmatory test.

Appendix A. Additional details describing the ELISA tests performed

These email responses were received from VRL in October of 2025 in response to additional questions about the specific details of the hantavirus ELISA tests that they performed for NEON.

Hello Sara

Many of those individuals have moved on from VRL. If needed in the future myself and Dr. Moll, who is the current site director for VRL Maryland, are the best contact points. In regards to your questions your assumptions are correct.

1. What specific hantavirus was tested for in the analysis?
    - *The antigen run for this study did target specifically HTNV (strain 76–118).*
2. Which antibodies were being tested?
    - *IgG*

If there is anything further you would like to know about this study please let us know. I am unsure what other type of information would be useful.

Best,
Jason

Jason Lankasky
Laboratory Manager VRL-MD
401 Professional Dr., Suite 210
Gaithersburg, MD 20879

Good Morning Sara,

The work done for the study was done under test code *80324: Hantaan virus*, focusing on the one virus. While VRL has the capability to test for other Hantaviruses, that was not the work requested for the NEON project. While I was not a part of this decision it did prove to be a wise one from a laboratory practicality standpoint. Getting sufficient volume from the various field locations to screen for a single virus proved to be a challenge. I certainly do not envy someone trying to draw blood from a wild rodent in the field.

Best,
Jason

Jason Lankasky
Laboratory Manager VRL-MD
401 Professional Dr., Suite 210
Gaithersburg, MD 20879