

West Nile Virus Antibodies

**Enzyme ImmunoAssay (ELISA)
for the qualitative
determination of antibodies to
West Nile Virus
in human serum and plasma**

- for “in vitro” diagnostic use only-



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West Nile Virus Ab

A. INTENDED USE

Enzyme ImmunoAssay (ELISA) for the qualitative determination of antibodies to West Nile Virus in human plasma and sera. The product is intended mostly for the follow-up of West Nile Virus infection.

For "in vitro" diagnostic use only.

B. INTRODUCTION

West Nile Virus is an arbovirus and is transmitted to people through complex life cycles involving birds and mosquitoes.

Each infection is most often not transmitted from person to person.

Most people who are infected with West Nile Virus will not have any symptoms. Clinician estimate that 20% of the people who become infected will develop West Nile fever: mild symptoms, fever, headache, and body aches, occasionally with a skin rash on the trunk of the body and swollen lymph glands. 1 in 150 West Nile Virus infections (< 1%) result in meningitis or encephalitis. The case fatality rates for WNV are similar to St.Louis encephalitis virus and western equine encephalitis virus: 5-15%.

C. PRINCIPLE OF THE TEST

Microplates are coated with purified immunodominant West Nile virus specific recombinant antigens. In the 1st incubation, the solid phase is treated with diluted samples and anti WNV antibodies are captured, if present, by the antigens.

After washing out all the other components of the sample, in the 2nd incubation bound anti WNV are detected by the addition of a mix of both anti IgG antibody, labeled with peroxidase (HRP) and anti IgM antibody, labeled with peroxidase (HRP). The enzyme captured on the solid phase, acting on the substrate/chromogen mixture, generates an optical signal that is proportional to the amount of WNV antibodies present in the sample.

D. COMPONENTS

Code WNAB.CE contains sufficient reagents to perform 96 tests.

1. Microplate: MICROPLATE

12 strips x 8 microwells coated with a purified WN virus recombinant antigens. Plates are sealed into a bag with desiccant.

Allow the microplate to reach room temperature before opening; reseal unused strips in the bag with desiccant and store at 4°C.

2. Negative Control: CONTROL -

1x2.0 ml/vial. Ready to use. Pale yellow color coded.
It contains diluted human serum negative for WNV Ab, 2% casein, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.2% Tween 20, 10% Fetal Calf Serum (FCS), 0.09% Na-azide and 0.1% Kathon GC as preservatives.

3. Positive Control: CONTROL +

1x2.0 ml/vial. Ready to use. Green color coded.
It contains diluted human serum positive for WNV Ab, 2% casein, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.2% Tween 20, 10% Fetal Calf Serum (FCS), 0.09% Na-azide and 0.1% Kathon GC as preservatives..

4. Wash buffer concentrate: WASHBUF 20X

1x60ml/bottle. 20x concentrated solution. Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0 +/-0.2, 0.05% Tween 20 and 0.05% Kathon GC.

5. Enzyme conjugate : CONJ

1x16ml/vial. Ready to use and red colour coded. It contains Horseradish peroxidase conjugated polyclonal antibodies to human IgG and Horseradish peroxidase conjugated polyclonal antibodies to human IgM, 5% BSA, 10 mM Tris buffer pH 6.8 +/-0.1, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives.

6. Chromogen/Substrate: SUBS TMB

1x16ml/vial. Ready to use. It contains 50 mM citrate-phosphate buffer pH 3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methylbenzidine (or TMB) and 0.02% hydrogen peroxide (or H₂O₂).

Note: To be stored protected from light as sensitive to strong illumination.

7. Sulphuric Acid: H₂SO₄ 0.3 M

1x15ml/vial. It contains 0.3 M H₂SO₄ solution.

Attention: Irritant (H315, H319; P280, P302+P352, P332+P313, P305+P351+P338, P337+P313, P362+P363).

8. Specimen Diluent: DILSPE

2x60ml/vial. It contains 2% casein, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.2% Tween 20, 10% Fetal Calf Serum (FCS), 0.09% Na-azide and 0.1% Kathon GC as preservatives.

To be used to dilute the sample.

9. Plate sealing foils n°2

10. Package insert n°1

Important note: Only upon specific request, Dia.Pro can supply reagents for 192 tests, as reported below:

1. Microplate	n°2
2.NegativeControl	1x4.0ml/vial
3.PositiveControl	1x4.0ml/vial
4.Wash buff conc	2x60ml/bottle
5.Enz. Conjugate	2x16ml/vial
6.Chromog/Subs	2x16ml/vial
7.Sulphuric Acid	2x15ml/vial
8.SampleDiluent	4x60ml/vial
9.Plate seal foils	n° 4
10. Pack. insert	n° 1
Number of tests	192
Code	WNAB.CE.192

E. MATERIALS REQUIRED BUT NOT PROVIDED

- Calibrated Micropipettes (1000, 100 and 10ul) and disposable plastic tips.
- EIA grade water (bidistilled or deionised, charcoal treated to remove oxidizing chemicals used as disinfectants).
- Timer with 60 minute range or higher.
- Absorbent paper tissues.
- Calibrated ELISA microplate thermostatic incubator (dry or wet) set at +37°C (+/-0.5°C tolerance).
- Calibrated ELISA microwell reader with 450nm (reading) and with 620-630nm (blanking) filters.
- Calibrated ELISA microplate washer.
- Vortex or similar mixing tools.

F. WARNINGS AND PRECAUTIONS

For in vitro diagnostic use only.

- The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
- All the personnel involved in performing the assay have to wear protective laboratory clothes, talc-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained

in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.

3. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.

4. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen (TMB) from strong light and avoid vibration of the bench surface where the test is undertaken.

5. Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.

6. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.

7. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.

8. Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample.

9. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.

10. Do not use the kit after the expiration date stated on the external container and internal (vials) labels. A study conducted on an opened kit did not pointed out any relevant loss of activity up to six 6 uses of the device and up to 3 months.

11. Treat all specimens as potentially infective. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.

12. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.

13. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated before waste. Suggested procedures of inactivation are treatment with a 10% final concentration of household bleach for 16-18 hrs or heat inactivation by autoclave at 121°C for 20 min..

14. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.

15. The Sulphuric Acid is an irritant. In case of spills, wash the surface with plenty of water

16. Other waste materials generated from the use of the kit (example: tips used for samples and controls, used microplates) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

3. Haemolysed ("red") and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.

4. Sera and plasma can be stored at +2°..8°C for up to seven days after collection. For longer storage periods, samples can be stored frozen at -20°C for several months. Any frozen samples should not be frozen/thawed more than once as this may generate particles that could affect the test result.

5. If particles are present, centrifuge at 2.000 rpm for 20 min or filter using 0.2-0.8u filters to clean up the sample for testing.

H. PREPARATION OF COMPONENTS AND WARNINGS

Microplate:

Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant is not turned to dark green, indicating a defect of conservation.

In this case call Dia.Pro's customer service.

Unused strips have to be placed back into the aluminium pouch, in presence of desiccant supplied, firmly zipped and stored at +2°..8°C. When opened the first time, residual strips are stable till the indicator of humidity inside the desiccant bag turns from yellow to green.

Controls

Ready to use. Mix well on vortex before use.

Wash buffer concentrate:

The whole content of the concentrated solution has to be diluted 20x with bidistilled water and mixed gently end-over-end before use. During preparation avoid foaming as the presence of bubbles could impact on the efficiency of the washing cycles.

Note: Once diluted, the wash solution is stable for 1 week at +2..8° C.

Enzyme conjugate:

Ready to use. Mix well on vortex before use.

Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes.

If this component has to be transferred use only plastic, possibly sterile disposable containers.

Chromogen/Substrate:

Ready to use. Mix well on vortex before use.

Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes.

Do not expose to strong illumination, oxidizing agents and metallic surfaces.

If this component has to be transferred use only plastic, possibly sterile disposable container

Specimen Diluent:

Ready to use component. Mix carefully on vortex before use.

Sulphuric Acid:

Ready to use. Mix well on vortex before use.

Attention: Irritant (H315, H319; P280, P302+P352, P332+P313, P305+P351+P338, P337+P313, P362+P363).

Legenda:

Warning H statements:

H315 – Causes skin irritation.

H319 – Causes serious eye irritation.

Precautionary P statements:

P280 – Wear protective gloves/protective clothing/eye protection/face protection.

P302 + P352 – IF ON SKIN: Wash with plenty of soap and water.

P332 + P313 – If skin irritation occurs: Get medical advice/attention.

G. SPECIMEN: PREPARATION AND WARNINGS

1. Blood is drawn aseptically by venepuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis. No influence has been observed in the preparation of the sample with citrate, EDTA and heparin.

2. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. Bar code labeling and electronic reading is strongly recommended.

P305 + P351 + P338 – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

P337 + P313 – If eye irritation persists: Get medical advice/attention.

P362 + P363 – Take off contaminated clothing and wash it before reuse.

I. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. Micropipettes have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-2%. Decontamination of spills or residues of kit components should also be carried out regularly.
2. The ELISA incubator has to be set at +37°C (tolerance of +/- 0.5°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.
3. The ELISA washer is extremely important to the overall performances of the assay. The washer must be carefully validated and correctly optimised using the kit controls and reference panels, before using the kit for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 350µl/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly their number, it is recommended to run an assay with the kit controls and well characterized negative and positive reference samples, and check to match the values reported below in the section O "Internal quality Control". Regular calibration of the volumes delivered by, and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instructions of the manufacturer.
4. Incubation times have a tolerance of $\pm 5\%$.
5. The ELISA microplate reader has to be equipped with a reading filter of 450nm and with a second filter (620-630nm, strongly recommended) for blanking purposes. Its standard performances should be (a) bandwidth ≤ 10 nm; (b) absorbance range from 0 to ≥ 2.0 ; (c) linearity to ≥ 2.0 ; repeatability $\geq 1\%$. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer's instructions.
6. When using an ELISA automated work station, all critical steps (dispensation, incubation, washing, reading, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the section "Internal quality Control". The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing and for washing. This must be studied and controlled to minimize the possibility of contamination of adjacent wells. The use of ELISA automated work stations is recommended when the number of samples to be tested exceed 20-30 units per run.
7. Dia.Pro's customer service offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure compliance with the requirements described. Support is also provided for the installation of new instruments to be used with the kit.

L. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label (primary container). Do not use if expired.
2. Check that the liquid components are not contaminated by visible particles or aggregates.
3. Check that the Chromogen (TMB) is colourless or pale blue by aspirating a small volume of it with a sterile plastic pipette.
4. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box (primary container). Check that the aluminium pouch, containing the microplate, is not punctured or damaged.
5. Dilute all the content of the 20x concentrated Wash Solution as described above.
6. Allow all the other components to reach room temperature (about 1 hr) and then mix gently on vortex all liquid reagents.
7. Set the ELISA incubator at +37°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.
8. Check that the ELISA reader is turned on or ensure it will be turned on at least 20 minutes before reading.
9. If using an automated work station, turn on, check settings and be sure to use the right assay protocol.
10. Check that the micropipettes are set to the required volume.
11. Check that all the other equipment is available and ready to use.
12. In case of problems, do not proceed further with the test and advise the supervisor.

M. ASSAY PROCEDURE

The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

1. Dilute samples 1:101 into a properly defined dilution tube (example: 1000 µl Sample Diluent + 10 µl sample). Do not dilute the Controls as they are ready to use. Mix carefully all the liquid components on vortex and then proceed as described below.
2. Place the required number of microwells in the microwell holder. Leave the A1 well empty for the operation of blanking.
3. Dispense 100 µl of Negative Control and 100 µl of Positive Control in duplicate. Then dispense 100 µl of diluted samples in each properly identified well.
4. Incubate the microplate for **60 min at +37°C**.

Important note: Strips have to be sealed with the adhesive sealing foil, supplied, only when the test is carried out manually. Do not cover strips when using ELISA automatic instruments.

5. Wash the microplate with an automatic as reported previously (**section I.3**).
6. Pipette 100 µl Enzyme Conjugate into each well, except A1 blanking wells, and cover with the sealer. Check that this red coloured component has been dispensed in all the wells, except A1.

Important note: Be careful not to touch the plastic inner surface of the well with the tip filled with the Enzyme Conjugate. Contamination might occur.

7. Incubate the microplate for **60 min at +37°C**.
8. Wash microwells as in step 5.
9. Pipette 100 µl Chromogen/Substrate mixture into each well, the blank wells A1 included. Then incubate the microplate at **room temperature (18-24°C)** in the dark for **20 minutes**.

Important note: Do not expose to strong direct illumination. High background might be generated.

10. Pipette 100 µl Sulphuric Acid to stop the enzymatic reaction into all the wells using the same pipetting sequence as in step 9. Addition of acid will turn the positive control and the positive samples from blue to yellow.
11. Measure the colour intensity of the solution in each well, as described in section I.5, at 450nm filter (reading) and at 620-630nm (background subtraction, strongly recommended), blanking the instrument on A1.

Check	Requirements
Blank well	< 0.100 OD450nm value
Negative Control	< 0.200 OD450nm
Positive Control	≥ 0.500 OD450nm
Mean CV%	< 30%

If the results of the test match the requirements stated above, proceed to the next section.

If they do not, do not proceed any further and operate as follows:

Problem	Check
Blank well ≥ 0.100 OD450nm	1. that the Chromogen/Substrate solution has not got contaminated during the assay
Negative Control ≥ 0.200	1. that the washing procedure and the washer settings are as validated in the pre qualification study; 2. that the proper washing solution has been used and the washer has been primed with it before use; 3. that no mistake has been done in the assay procedure (dispensation of a positive calibrator instead of the negative one); 4. that no contamination of the negative calibrator or of their wells has occurred due spills of positive samples or the enzyme conjugate; 5. that micropipettes haven't got contaminated with positive samples or with the enzyme conjugate 6. that the washer needles are not blocked or partially obstructed.
Positive Control ≤ 0.500	1. that the procedure has been correctly executed; 2. that no mistake has been done in its distribution (ex.: dispensation of a wrong calibrator instead); 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the calibrator has occurred.

Should one of these problems have happened, after checking, report to the supervisor for further actions.

P. RESULTS

Calculate the mean OD450nm value of the Negative Control (NC) and then apply the following formula:

$$\text{CUT-OFF} = \text{NC} + 0.250$$

Q. INTERPRETATION OF RESULTS

Test results are interpreted as a ratio of the sample OD450nm value (S) and the cut-off value (Co), or S/Co, according to the following table:

S/Co	Interpretation
≤ 0.9	Negative
1.0 – 1.1	Equivocal
≥ 1.2	Positive

An example of dispensation scheme for Qualitative Analysis is reported below:

Microplate												
	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S4										
B	NC	S5										
C	NC	S6										
D	PC	S7										
E	PC	S8										
F	S1	S9										
G	S2	S10										
H	S3	S11										

Legenda: BLK = Blank NC = Negative Control

PC = Positive Control Sn = Samples

O. INTERNAL QUALITY CONTROL

A validation check is carried out on the controls any time the kit is used in order to verify whether the performances of the assay are as expected and required by the IVDD directive 98/79/EC. Control that the following data are matched:

Samples showing an OD 450nm value lower than the Cut-Off value are considered negative for anti West Nile Virus antibodies.

Samples showing an OD450nm value higher than the Cut-Off value are considered positive for anti West Nile Virus antibodies. Any patient showing an equivocal result should be retested on a second sample taken 1-2 weeks after the initial sample.

Important notes:

1. Interpretation of results should be done under the supervision of the laboratory supervisor to reduce the risk of judgment errors and misinterpretations.
2. When test results are transmitted from the laboratory to another facility, attention must be paid to avoid erroneous data transfer.
3. Diagnosis has to be done and released to the patient by a suitably qualified medical doctor.
4. When a diagnosis is released it should take into account the cross-reactions that are well known among the members of the FlaviViruses.

An example of calculation is reported below.

The following data must not be used instead or real figures obtained by the user.

Negative Control: 0.090 – 0.080 OD450nm

Mean Value: 0.085 OD450nm

Lower than 0.200 – Accepted

Positive Control: 1.000 OD450nm

Higher than 0.500 – Accepted

Cut-Off = 0.085+0.250 = 0.335

Sample 1: 0.080 OD450nm

Sample 2: 1.800 OD450nm

Sample 1 S/Co \leq 0.9 = negative

Sample 2 S/Co \geq 1.2 = positive

R. PERFORMANCE CHARACTERISTICS

Evaluation of Performances has been conducted on panels of positive and negative samples with reference to a CE marked reference kit based on WNV recombinant antigen.

1. Diagnostic Sensitivity and Specificity:

The Diagnostic **Sensitivity** was calculated on a panel of samples derived from people that had clinical signs of West Nile virus infection, certified positive for antibodies to WNV by the supplier and confirmed positive in a reference CE-marked kit based on recombinant antigens.

A value of \geq 98% was observed when referring to the reference device.

The Diagnostic **Specificity** was calculated on a panel of samples derived from people leaving in regions still almost WNV free (Italy and Europe);

A value \geq 98% was observed on the panel of European origin. Summarizing:

Sensitivity	\geq 98 %
Specificity	\geq 98 %

2. Reproducibility:

A study conducted on two samples of different WNV Ab reactivity, examined in 16 replicates in three separate runs has shown CV% values ranging 10-20% depending on the OD450nm readings.

The variability observed did not result in sample misclassification.

S. LIMITATIONS

Due to the well-known high genetic homology of West Nile Virus with the other Flaviviruses as Zika Virus and Dengue Virus, and with Chikungunya virus as well, cross-reactions were observed in those populations affected by such infections (mainly South American individuals that have been infected by Dengue virus since years and recently by Zika virus).

The assay should in principle be used only for testing individuals with clinical signs and symptoms associated with a West Nile virus infection and in compliance with CDC epidemiological criteria (e.g., history of residence in or travel to a geographic region with active WNV virus transmission at the time of travel, or other epidemiologic criteria for West Nile virus testing).

Assay results are therefore useful for the presumptive identification of antibodies to West Nile virus. Positive and equivocal results alone are not definitive for diagnosis of West Nile virus infection. Confirmation should be done according to CDC indications (neutralization assay in particular).

Frozen samples containing fibrin particles or aggregates after thawing have been observed to generate some false results. These samples should be tested only after clarification by filtration or centrifugation.

The kit is not suitable to detect the presence of only IgM in absence of IgG. For a better determination of anti WNV IgM antibodies in a recent event of infection it is recommended to use the product code WNVM.CE

Samples found positive for antibodies to WNV in kits based on native antigen, can be found negative with the present device (REF: WNAB.CE) based on WNV recombinant antigen, that is less subject to cross-reactions and therefore more specific

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All the IVD Products manufactured by the company are under the control of a certified Quality Management System in compliance with ISO 13485 rule. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

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