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Dengue Virus NS1 ELISA
Test instruction

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ORDER NO.	ANTIGEN	SUBSTRATE	FORMAT
EQ 266a-9601-1	Dengue virus NS1	Ab-coated microplate wells	96 x 01 (96)

Indications: The ELISA test kit provides semiquantitative or quantitative in vitro determination of dengue virus NS1 in serum or plasma to support the diagnosis of dengue virus infections.

Application: The serological detection of the highly specific dengue virus NS1 antigen in patients with a dengue virus infection is possible at onset of clinical symptoms, not only in primary, but also in secondary infections. Therefore, the determination of this antigen is an important instrument for the detection of acute dengue virus infections. The investigation of specific antibodies in parallel is recommended.

Principles of the test: The test kit contains microplate strips each with 8 break-off reagent wells coated with monoclonal anti-dengue virus NS1 antibodies against the serotypes 1, 2, 3, 4. In the first reaction step, diluted patient samples are incubated in the wells. If samples are positive, the dengue virus NS1 binds to specific anti-dengue NS1 antibodies. To detect the bound antigens, a second incubation is carried out using an enzyme-labelled anti-dengue virus NS1 antibody (enzyme conjugate) catalysing a colour reaction.

Contents of the test kit:

Description	Colour	Format	Symbol
1. Microplate wells coated with antibodies 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 x 8	STRIPS
2. Calibrator 1 100 RU/ml (dengue virus NS1, recombinant), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 10 RU/ml (dengue virus NS1, recombinant), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 1 RU/ml (dengue virus NS1, recombinant), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (dengue virus NS1, recombinant), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-dengue virus NS1 antibody (mouse), ready for use	blue	1 x 12 ml	CONJUGATE
8. Sample buffer ready for use	pink	1 x 50 ml	SAMPLE BUFFER
9. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
10. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
11. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
12. Test instruction	---	1 booklet	
13. Quality control certificate	---	1 protocol	
14. Protective foil	---	3 pieces	FOIL

LOT	Lot description		Storage temperature
IVD	In vitro diagnostic medical device	CE	Unopened usable until

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Modifications to the former version are marked in grey.

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Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. The reagents can be kept unopened until the specified expiration date if stored at +2°C to +8°C. After opening for the first time, they must still be stored at +2°C to +8°C and protected against contamination. The following table lists the storage life of the reagents after first opening. These storage life limits only apply if the indicated storage life is not exceeded:

Reagents	Stability
Coated wells	4 months
Calibrators	12 months
Controls	12 months
Enzyme conjugate	12 months
Sample buffer	12 months
Diluted wash buffer	4 weeks
Chromogen/substrate solution	12 months
Stop solution	12 months

The thermostat-adjusted ELISA incubator must be set at +37°C ± 1°C.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam. (Do not remove the desiccant bag).

- **Calibrators and controls:** Ready for use. The reagents must be mixed thoroughly before use.

- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.

- **Sample buffer:** Ready for use.

- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallisation occurs in the concentrated buffer, warm it to +37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionised or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.

- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light ☀. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue-coloured.

- **Stop solution:** Ready for use.

Storage and stability: The test kit has to be stored at a temperature between +2°C and +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Warning: The calibrators and controls of human origin have tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the patient samples

Samples: Human serum or EDTA or heparin plasma.

Stability: Patient samples to be investigated can generally be stored at +4°C for up to 14 days. Diluted samples should be incubated within one working day.

Sample dilution: Patient samples are diluted **1:2** in sample buffer.
For example: dilute 200 µl sample in 200 µl sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: The calibrators and controls are prediluted and ready for use, do not dilute them.

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Incubation

For **semiquantitative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1, 2 and 3** along with the positive and negative controls and patient samples.

(Partly) manual test performance

Sample incubation:
(1st step)

Transfer 100 µl of the calibrators, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. For manual processing of microplate wells, cover the finished test plate with the protective foil. When using an automated microplate processor for incubation follow the recommendations of the instrument manufacturer.
Incubate for **60 minutes** at **+37°C ± 1°C**.

Washing:

Manual: Remove the protective foil, empty the wells and subsequently wash 3 times using 300 µl of working-strength wash buffer for each wash.
Automatic: Remove the protective foil and wash the reagent wells 3 times with 450 µl of working-strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Mode").

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual <u>and</u> automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Note: Residual liquid (> 10 µl) in the reagent wells after washing can interfere with the substrate and lead to false low extinction readings.
Insufficient washing (e.g. less than 3 wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction readings.
Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

Conjugate incubation:
(2nd step)

Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-dengue virus NS1 antibody) into each of the microplate wells. For manual processing of microplate wells, cover the finished test plate with the protective foil. When using an automated microplate processor for incubation follow the recommendations of the instrument manufacturer.
Incubate for **60 minutes** at **+37°C ± 1°C**.

Washing:

Empty the wells. Wash as described above.

Substrate incubation:
(3rd step)

Pipette 100 µl of chromogen/substrate solution into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18°C to +25°C, protect from direct sunlight).

Stopping:

Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:

Photometric measurement of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution**. Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

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Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using an analysis device. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA test instruction. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I or the DSX from Dynex and this EUROIMMUN ELISA. Validation documents are available on enquiry.

Automated test performance using other fully automated, open-system analysis devices is possible. However, the combination should be validated by the user.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	C 2	P 6	P 14	P 22			C 1	P 4	P 12	P 20		
B	pos.	P 7	P 15	P 23			C 2	P 5	P 13	P 21		
C	neg.	P 8	P 16	P 24			C 3	P 6	P 14	P 22		
D	P 1	P 9	P 17				pos.	P 7	P 15	P 23		
E	P 2	P 10	P 18				neg.	P 8	P 16	P 24		
F	P 3	P 11	P 19				P 1	P 9	P 17			
G	P 4	P 12	P 20				P 2	P 10	P 18			
H	P 5	P 13	P 21				P 3	P 11	P 19			

The pipetting protocol for microplate strips 1 to 4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).

The pipetting protocol for microplate strips 7 to 10 is an example for the **quantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 3), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.

The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and minimises reagent wastage.

Both positive and negative controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction of the control or patient sample over the extinction of the calibrator 2. Calculate the ratio according to the following formula:

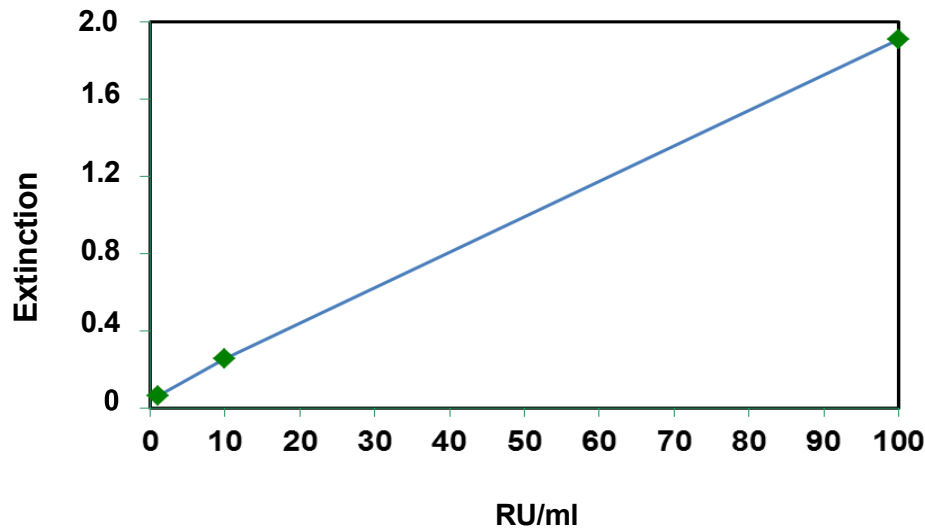$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator 2}} = \text{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <0.8:	**negative**
Ratio ≥0.8 to <1.1:	**borderline**
Ratio ≥1.1:	**positive**

In case of a borderline result, test systems for the detection of specific antibodies against dengue virus can help with the diagnosis. In some cases, also PCR diagnostics may be helpful.

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Quantitative: The standard curve from which the concentration of antigens in the patient samples can be taken is obtained by point-to-point plotting of the extinction readings measured for the 3 calibration sera against the corresponding units (linear/linear). Use "point-to-point" plotting for calculation of the standard curve by computer. The following plot is an example of a typical standard curve. Please do not use this curve for the determination of antigen concentrations in patient samples.

If the extinction for a patient sample lies above the extinction of calibrator 1 (corresponding to 100 RU/ml), the result should be reported as ">100 RU/ml". It is recommended that the sample be retested at a dilution of e.g. 1:8. The result in RU/ml read from the calibration curve for this sample must then be multiplied by factor 4.

The upper limit of the normal range of non-infected persons (**cut-off value**) recommended by EUROIMMUN is **10 relative units (RU)/ml**. EUROIMMUN recommends interpreting results as follows:

<8 RU/ml:	**negative**
≥8 to <11 RU/ml:	**borderline**
≥11 RU/ml:	**positive**

The detection of a dengue virus infection is carried out by means of PCR, NS1 antigen test or, indirectly, by the detection of specific antibodies. In the early stage of an infection, antibodies might not yet be present or only present in quantities below the limit of detection. In the case of a borderline result, a secure evaluation is not possible. If there is a clinical suspicion and a negative or borderline serological test result, we recommend clarification by means of other diagnostic methods and/or the serological investigation of a follow-up sample. A positive result indicates that there has been contact with the pathogen. In the determination of pathogen-specific IgM antibodies, polyclonal stimulation of the immune system or antibody persistence may affect the diagnostic relevance of positive findings. A titer increase and/or seroconversion in a follow-up sample taken at a later point in time may indicate an acute infection. For diagnosis, the clinical picture of the patient should always be taken into account along with the serological results. A negative finding in the serological analysis does not exclude an infection.

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Test characteristics

Calibration: As no international reference preparation exists for dengue virus NS1, the calibration is performed in relative units (RU/ml).

For every group of tests performed, the extinction readings of the calibrators and the relative units and/or ratios determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The binding activity of the antigens and the activity of the enzyme used are temperature-dependent. It is therefore recommended using a thermostat in all three incubation steps. The higher the room temperature (+18°C to +25°C) during the incubation steps, the greater will be the extinction. Corresponding variations apply also to the incubation times. However, the calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antibody: The wells of the microplates are coated with monoclonal anti-dengue NS1 antibodies (mouse). These antibodies are specific against dengue virus NS1 of serotypes 1, 2, 3, 4.

Linearity: The linearity of the Dengue Virus NS1 ELISA was determined by assaying at least 4 serial dilutions of different patient samples. The Dengue Virus NS1 ELISA is linear at least in the tested concentration range 1 RU/ml to 100 RU/ml.

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable dengue NS1 concentration. The lower detection limit of the Dengue Virus NS1 ELISA is 0.8 RU/ml.

Cross-reactivity: The quality of the antibodies used ensures a high specificity of the ELISA. Sera from patients with acute infections of different flaviviruses or vaccination against these were analysed with the Dengue Virus NS1 ELISA. All 33 samples were found to be negative.

Antibodies against	n	Dengue Virus NS1 ELISA positive
TBE virus	10	0%
Yellow fever virus	10	0%
Japanese encephalitis virus	4	0%
West Nile virus	4	0%
Zika virus	5	0%

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to a concentration of 10 mg/ml for haemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 samples. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 3 determinations performed in 10 different test runs.

Intra-assay variation, n = 20		
Sample	Mean value (RU/ml)	CV (%)
1	9	3.1
2	29	3.2
3	37	2.8

Inter-assay variation, n = 3 x 10		
Sample	Mean value (RU/ml)	CV (%)
1	12	9.6
2	34	10.6
3	43	7.9

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Sensitivity and specificity: 51 clinically characterised patient samples (INSTAND) were investigated with this EUROIMMUN Dengue Virus NS1 ELISA. The sensitivity amounted to 100%, with a specificity of 100%.

n = 51		INSTAND		
		positive	borderline	negative
EUROIMMUN Dengue Virus NS1 ELISA	positive	17	0	0
	borderline	0	0	0
	negative	0	0	34

413 precharacterised patient samples (reference method: commercially available ELISA of another manufacturer) were investigated with the EUROIMMUN Dengue Virus NS1 ELISA. The sensitivity amounted to 98.7%, with a specificity of 97.6%. Borderline results were not included in the calculation.

n = 413		ELISA of another manufacturer		
		positive	borderline	negative
EUROIMMUN Dengue Virus NS1 ELISA	positive	76	2	8
	borderline	0	0	1
	negative	1	0	325

Specificity: To investigate the specificity of the Dengue Virus NS1 ELISA, a study was performed using patient sera which were seropositive for rheumatoid factor, heterophile autoantibodies, or HAMA (mouse antibodies). Of the total of 69 samples, only one sample was positive, all other sera did not show any reaction in the Dengue Virus NS1 ELISA. Consequently, the specificity in this panel amounted to 98.6%. An overview of results can be found in the following table.

Possible influencing factors	n	EUROIMMUN Dengue Virus NS1 ELISA positive
Rheumatoid factor	49	2%
Heterophile antibodies positive	16	0%
HAMA positive	4	0%

Reference range: A panel of 150 healthy blood donors was investigated with this EUROIMMUN ELISA. 0% of the blood donors were positive for dengue virus NS1 (IgG) at a cut-off of 10 RU/ml.

Clinical significance

Dengue virus (DENV) is an arbovirus of the genus *Flavivirus* belonging to the *Flaviviridae* family. The virus is divided into four related but genetically differing serotypes (DENV 1 to 4) [1, 2]. Vectors of DENV are mosquitoes of the genus *Aedes*; primates and, most of all, humans are the reservoir hosts [3]. DENV can be transmitted via blood transfusions [4] and vertically [5]. Human-to-human transmission has not yet been observed.

The frequency of dengue fever (DF) has dramatically increased worldwide over the past decades. According to recent estimations there are 390 million dengue virus infections per year, of which 96 million show clinical manifestations [6]. DENV is endemic in more than 100 countries, especially in tropical and subtropical regions. The virus has spread in South East Asia, North and South America, the West Pacific, Africa and the eastern Mediterranean region via continuing outbreaks [1].

DENV infections can either be subclinical or cause a broad spectrum of clinical symptoms, ranging from a mild febrile disease and classic dengue fever to even lethal haemorrhagic fever (DHF) or dengue shock syndrome (DSS) [7].

After an incubation period of 4 to 7 days DF manifests suddenly with high fever, headache and retrobulbar and lumbosacral pains. These are followed by generalised muscle and joint pains that increase in intensity. Further symptoms include a macular exanthema that spreads from the trunk to the arms, legs and face, anorexia, nausea, vomiting, weakness and dizziness. The temperature curve is often biphasic. After a temporary decline of the fever, it rises again after 1 to 2 days and is accompanied by an exanthema of the entire body, excluding the face [7, 8, 9, 10].

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DHF initially shows as DF, but the high fever generally persists for more than 2 to 7 days [8, 9]. In 10 to 15% of cases clinically relevant haemorrhage can be observed; gastrointestinal and cerebral bleeding can be life-threatening. Progression to DSS is very rapid [8].

The lethality of DHF and DSS is 6 to 30%, with particularly high rates in small children. Infection confers a life-long serotype-specific immunity. The course is generally more severe in secondary infection with a different serotype [10, 11, 12].

DF should be differentiated diagnostically from other flavivirus infections, such as Zika or yellow fever, as well as chikungunya fever, leptospirosis, malaria, measles, rubella and typhoid fever [2, 7, 10, 12].

Detection of viral RNA or the virus itself, for instance, in serum or plasma is only possible during the viraemic phase in the first 4 to 5 days after onset of the disease, using RT-PCR, virus isolation or culturing [2]. The highly specific non-structural protein NS1 of DENV can be detected in the serum of patients generally from onset of clinical symptoms to day 9 in primary and secondary infections [13].

DENV-specific IgM antibodies can mostly be detected 4 to 5 days after onset of symptoms [1, 12, 13]. Seroconversion or a significant titer increase in a follow-up sample taken at least 2 to 3 weeks later also indicates an acute infection [12]. During secondary infection, the IgG antibody titer increases rapidly and remains at a high level even in the acute phase of the disease. The IgM level can be significantly lower in secondary than in primary infection and may not be detectable at all in some cases [1, 14].

Possible cross reactions with antibodies (due to infection or vaccination) against other flaviviruses, such as Zika, West Nile, Japanese encephalitis, yellow fever or TBE viruses, should be taken into consideration when interpreting results [1, 2].

There is no specific antiviral medication for the treatment of DENV infections. Beside the prevention of mosquito bites, a vaccine has been available for prophylaxis in many endemic countries since 2016. This protects from infection with all four DENV types [8, 15].

Literature

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