Dengue Virus NS1 ELISA
Test instruction

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 a semiquantitative or quantitative in vitro assay for dengue virus NS1 in serum or plasma for the diagnosis of dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS).

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 microtiter 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:

Component	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 (human), 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
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

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. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

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).
  Once the protective wrapping has been opened for the first time, the wells coated with antibodies can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.
- **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.
  The working strength diluted wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.
- **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 to +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 the agent 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.

Incubation

For **semiquantative 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 and 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 values. 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 values.
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.

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, Analyzer I-2P 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 microtiter strips 1 to 4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter 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 value of the control or patient sample over the extinction value of the calibrator 2. Calculate the ratio according to the following formula:

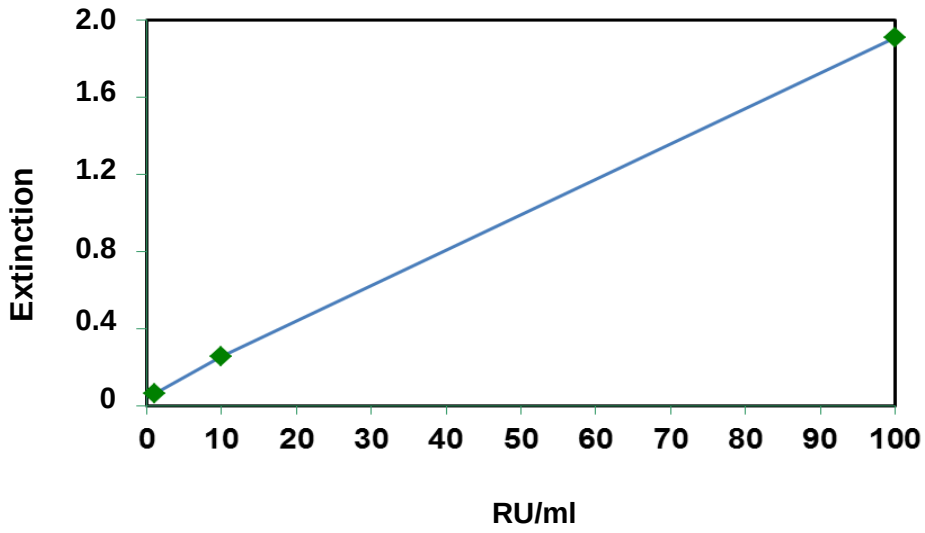
$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator 2}} = \text{Ratio}$$

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.

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 values 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 value of calibrator 1 (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 a factor of 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 time point 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.

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 values of the calibrators and the relative units and/or ratio values 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 values. 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 microtiter plates are coated with monoclonal anti-dengue NS1 antibodies (mouse). These antibodies are specific against dengue NS1 virus of serotypes 1, 2, 3, 4.

Linearity: The linearity of the Dengue Virus NS1 ELISA was determined by assaying 4 serial dilutions of different patient samples. The coefficient of determination R^2 for all sera was > 0.95. 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 1.0 RU/ml.

Cross reactivity: The quality of the antibodies used ensures the high specificity of the ELISA. Sera with potentially interfering substances like heterophile antibodies (n = 16) or human anti-mouse antibodies (n = 4) were investigated with the Dengue Virus NS1 ELISA. All 20 samples tested negative. In addition to this, patient sera which may contain proteins that are structurally similar or related to the analyte may occur, were investigated. No cross reactivities for this ELISA are known.

Patient	n	Dengue Virus NS1 ELISA positive
TBE vaccination	10	0%
Yellow fever virus vaccination	10	0%
Japanese encephalitis virus vaccination/infection	4*	0%
West Nile virus infection	4	0%
Zika virus infection	5	0%
Heterophile antibodies positive	16	0%
HAMA positive	4	0%

*(2 vaccinated, 2 acute infections)

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	9.5	3.1
2	28.8	3.2
3	36.7	2.8

Inter-assay variation, n = 3 x 10		
Sample	**Mean value (RU/ml)**	**CV (%)**
1	12.3	9.6
2	34.2	10.6
3	42.8	7.9

Sensitivity and specificity: 19 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 = 19		**INSTAND**		
		positive	borderline	negative
EUROIMMUN Dengue Virus NS1 ELISA	positive	8	0	0
	borderline	0	0	0
	negative	0	0	11

413 precharacterised patient samples (origin: Australia; 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

The specificity of the EUROIMMUN Dengue Virus NS1 ELISA was evaluated in a study performed on 49 patient sera which were seropositive for rheumatoid factors. Of the 49 samples, 48 have been tested negative with the EUROIMMUN Dengue Virus NS1 ELISA.

Possible influencing factors	**n**	**EUROIMMUN Dengue Virus NS1 ELISA positive**
Rheumatoid factor	49	2%

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 viruses (DENV) belong to the flavivirus family, along with other known members such as yellow fever virus, West Nile virus and TBE virus. Dengue fever (DF) is the most frequent and the most rapidly spreading vector-borne viral infection in humans. From 1960 to 2012 the number of cases increased 30-fold. Four different dengue serotypes exist (DENV1 to DENV4). Vectors of these viruses are mosquitoes of the Aedes species (A. aegypti, A. albopictus); reservoir hosts are primates and particularly humans.

The mosquito larvae multiply in open water reservoirs such as wells, cisterns and cloacae, as well as in small containers or waste in which rain water collects.

DENV are mainly found in Latin America, Central Africa, India, South East Asia and in some parts of the Pacific islands. The World Health Organization (WHO) defines four regions: Eastern Mediterranean Region (EMR), American Region (AMR), South-East Asia Region (SEAR) and Western Pacific Region (WPR). The fever is also regularly introduced to Europe. In Germany around 4000 people per year are infected. Thus, DF is one of the most frequent viral infections in German holidaymakers.

According to estimates of the WHO, there are around 100 million cases of DF every year. Around 95% of infected persons are children. Although most primary DENV infections are silent, in children more often than in adults, around 500,000 patients per year have a severe course, with dengue haemorrhagic fever (DHF) and/or dengue shock syndrome (DSS). The lethality rate in infants of up to one year of age is around 30%.

Following the bite of an infected mosquito and an incubation period of 2 to 10 days, the infection initially manifests with flu-like symptoms (stage I): fever that rises suddenly to 41°C, chills, severe headaches, muscle and joint pains, a noticeably low pulse and a metallic bitter taste. The fever is often biphasic. Stage II typically follows an abatement of the fever and is characterised by a second temperature phase of 4 to 5 days. This can be accompanied by measles-like exanthema and lymph node swelling. Stage III begins 5 to 6 days after this with a recovery phase which can take several weeks.

Complications occur in 0.5 to 1% of patients, leading to DHF and/or DSS. Symptoms of DHF include petechiae, black and tar-like stool, nose bleeds and plane skin bleeding. Complications such as circulatory disturbances and even shock can occur. Whereas primary infections are often mild, the risk of haemorrhagic fever significantly increases after a secondary infection with another DENV serotype. The lethality rate of DHF is between 6 to 50%, on average 12%. The cause of death is often haemor-rhagic shock or cardiovascular or multi-organ failure with acute kidney insufficiency in around 70% and/or severe hepatitis in 55% of cases.

Because of its unspecific symptoms, DF should always be differentiated from other tropical diseases such as malaria, yellow fever, West Nile virus infection, Chikungunya fever and typhus abdominalis.

The worldwide rising risk of infection with DENV has led to increased efforts to develop a specific causal treatment (e.g. using NS3 protease inhibitors). A quadrivalent vaccine will be available from 2015 for prophylaxis, to be used in affected regions alongside other measures such as protection from mosquito bites. The vaccine will be based on vaccine viruses from four DENV serotypes or on recombinant vaccine technology. Phases 1 and 2 of the clinical studies were completed in 2010.

Viral RNA or the virus itself can only be detected during the viraemic phase within the first two to seven days after the onset of the disease using RT-PCR or in vitro cultivation of the virus.

The highly-specific non-structural protein NS1 of DENV can be detected in the serum of patients generally at onset of clinical symptoms. This applies for both first and re-infections. The detection of this antigen using ELISA is an important tool for diagnosis of acute dengue infections. Antigen detection is increasingly used in dengue diagnostics in parallel to the determination of specific antibodies.

Antibodies against DENV can be detected early after the onset of symptoms using indirect immuno-fluorescence and/or ELISA. Antibodies of class IgM are detectable from the second to fourth day of illness. The IgM titer is highest approximately two weeks after the onset of symptoms. IgM antibodies remain detectable for two to three months and sometimes for up to eight months as a persisting low DENV-specific IgM titer. In cases of later infection with a different serotype, no new IgM antibodies are produced.

Since specific antibodies of class IgA are produced in parallel to IgM in around 70% of cases, the deter-mination of anti-dengue IgA in addition to IgM can help diagnosis in acute cases if a follow-up sample is not yet available. Only around 25% of patients with past infection are anti-dengue IgA positive. Around 30% of patients with IgM reactivity do not yet exhibit antibodies of class IgG. In these cases diagnosis can be supported by the additional determination of IgA.

In primary infections, DENV-specific IgG appears only after IgM antibodies, and never before the ninth day of infection. The concentration of antibodies of class IgG is highest two to three weeks after infection. Thus, a positive IgG result at the beginning of the disease can be considered as an indicator of reinfection; it most likely persists life-long. In secondary infection with a heterologic serotype of DENV types 1 to 4, a more than tenfold increase in the IgG concentration can be observed.

For a prognosis of the development of DSS and DHF the titer of specific serum antibodies is crucial. Potential cross reactions with other flaviviruses must be taken into account.

In Germany, Switzerland and Austria, a suspicion or diagnosis of DF, death from the disease, diagnosis of a haemorrhagic course and direct or indirect detection of the pathogen are notifiable.

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