研究用試薬

Anti-Dengue Virus ELISA (IgM)
Instructions for use

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 266b-9601 M	Dengue virus	IgM	Ag-coated microplate wells	96 x 01 (96)

Indication: The enzyme immunoassay (ELISA) provides semiquantitative in vitro determination of human antibodies of the immunoglobulin class IgM against dengue virus in serum or plasma to support the diagnosis of a dengue virus infection. The product is designed for use as IVD.

Principles of the test: The test kit contains microplate strips each with 8 break-off reagent wells coated with purified dengue virus antigens. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgM antibodies (also IgA and IgG) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgM (enzyme conjugate) catalysing a colour reaction.

Contents of the test kit:

Component	Colour	Format	Symbol
1. **Microplate wells coated with antigens** 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 x 8	STRIPS
2. **Calibrator** (IgM, human), ready for use	dark red	1 x 2.0 ml	CAL
3. **Positive control** (IgM, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
4. **Negative control** (IgM, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
5. **Enzyme conjugate** peroxidase-labelled anti-human IgM (goat), ready for use	red	1 x 12 ml	CONJUGATE
6. **Sample buffer** containing IgG/RF-absorbent (anti-human IgG antibody preparation obtained from goat), ready for use	green	1 x 100 ml	SAMPLE BUFFER
7. **Wash buffer** 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
8. **Chromogen/substrate solution** TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
9. **Stop solution** 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
10. **Instructions for use**	---	1 booklet	
11. **Quality control certificate**	---	1 protocol	

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

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.

Updates with respect to the previous version are marked in grey.

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 stability 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

- **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).

- **Calibrator 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. The green coloured sample buffer contains IgG/RF absorbent. Serum or plasma samples diluted with this sample buffer are only to be used for the determination of IgM antibodies.

- **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.

Warning: The calibrator 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, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

Introduction: Before the determination of specific antibodies of class IgM, antibodies of class IgG should be removed from the patient sample. This procedure must be carried out in order to prevent any rheumatoid factors of class IgM from reacting with specifically bound IgG, which would lead to false positive IgM test results, and to prevent specific IgG displacing IgM from the antigen, which would lead to false negative IgM test results.

Functional principle: The sample buffer (green coloured!) contains an anti-human IgG antibody preparation from goat. IgG from a serum sample is bound with high specificity by these antibodies and precipitated. If the sample also contains rheumatoid factors, these will be absorbed by the IgG/anti-human IgG complex.

Separation properties:
- All IgG subclasses are bound and precipitated by the anti-human IgG antibodies.
- Human serum IgG in concentrations of up to 15 mg per ml are removed (average serum IgG concentration in adults: 12 mg per ml).
- Rheumatoid factors are also removed.
- The recovery rate of the IgM fraction is almost 100%.

Performance: The **patient samples** for analysis are diluted **1:101** with green-coloured sample buffer. For example, add 10 µl sample to 1.0 ml sample buffer and mix well by vortexing. Sample pipettes are not suitable for mixing. Incubate the mixture for at least **10 minutes** at room temperature (+18°C to +25°C). Subsequently, it can be pipetted into the microplate wells according to the pipetting protocol.

Notes:
- Antibodies of the class IgG should not be analysed with this mixture.
- It is possible to check the efficacy of the IgG/RF absorbent for an individual patient sample by performing an IgG test in parallel to the IgM test using the mixture. If the IgG test is negative, the IgM result can be considered as reliable.
- The calibrator and controls are ready for use, do not dilute them.

Incubation

(Partly) manual test performance

Sample incubation: (1st step)

Transfer 100 µl of the calibrator, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:

Manual: Empty the wells and subsequently wash 3 times using 300 µl of working-strength wash buffer for each wash.
Automatic: Wash the reagent wells 3 times with 450 µl 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) remaining 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-human IgM) into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to +25°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 instructions for use. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I and the EUROIMMUN Analyzer I-2P 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	P 6	P 14	P 22								
B	pos.	P 7	P 15	P 23								
C	neg.	P 8	P 16	P 24								
D	P 1	P 9	P 17									
E	P 2	P 10	P 18									
F	P 3	P 11	P 19									
G	P 4	P 12	P 20									
H	P 5	P 13	P 21									

The above pipetting protocol is an example of the semiquantitative analysis of 24 patient samples (P 1 to P 24).

Calibrator (C), positive (pos.) and negative (neg.) control as well as the patient samples have been incubated in one well each. The reliability of the ELISA test can be improved by duplicate determinations of 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 must be assayed with each test run.

Calculation of results

The extinction value of the calibrator defines the upper limit of the reference range of non-infected persons (**cut-off**) recommended by EUROIMMUN. Values above the indicated cut-off are to be considered as positive, those below as negative.

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. Use the following formula to calculate the ratio:

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

EUROIMMUN recommends interpreting results as follows:

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

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another, EUROIMMUN recommends retesting the samples.

A negative serological result does not exclude an infection. Particularly in the early phase of infection, antibodies may not yet be present or are only present in such small quantities that they are not detectable. In the case of a borderline result, a secure evaluation is not possible. If there is a clinical suspicion and a negative or borderline 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. Significantly higher specific IgG antibody levels (increase by more than factor 2) and/or seroconversion in a follow-up sample taken after at least 7 to 10 days can indicate an acute infection. Sample and follow-up sample should be incubated in parallel in adjacent wells of the ELISA microplate within the same test run. For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Calibration: As no international reference serum exists for antibodies against dengue viruses, results are provided in the form of ratios.

For every group of tests performed, the extinction readings of the calibrator and the ratios of 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 antibodies 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 readings. Corresponding variations apply also to the incubation times. However, the calibrator is subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The antigen source is provided by dengue virus type 2 cultured in Vero cells. The microplate wells were coated with highly purified virus particles. Due to the high structural similarity of dengue virus types 1 to 4 the use of a single virus type is sufficient for the reliable detection of antibodies against all four virus types.

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 relative antibody concentration. The lower detection limit of the Anti-Dengue Virus ELISA (IgM) is ratio 0.02.

Cross-reactivity: The quality of the antigen used ensures a high specificity of the ELISA. Due to the high homologies of the target antigen within the genus of flaviviruses, cross-reactions cannot be completely ruled out. To check the cross-reactivity, sera from patients after vaccination with TBE vaccines and from patients with West Nile virus or Zika virus infections were investigated. Pronounced cross-reactivities exist mainly with anti-Zika virus IgG and anti-West Nile virus IgG antibodies. However, it should also be taken into account that double infections are possible, especially in endemic regions, or that an infection with another flavivirus may have occurred earlier. Positive findings in these cases do not result from cross reactivity of the respective antibodies.

Antibodies against	n	Positive in Anti-Dengue Virus ELISA (IgM)
TBE virus	33	21.2%
West Nile virus	53	34%
Zika virus (ZIKV)	16	6.3%

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to concentrations of 10 mg/ml haemoglobin, 20 mg/ml triglycerides and 0.4 mg/ml 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 4 determinations performed in 6 different test runs.

Intra-assay variation, n = 20		
Sample	**Mean value (ratio)**	**CV (%)**
1	1.3	4.8
2	2.1	4.1
3	2.8	5.0

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (ratio)**	**CV (%)**
1	2.0	7.5
2	2.0	8.2
3	3.0	6.2

Sensitivity and specificity:
Study I: 62 clinically pre-characterised patient samples (INSTAND) were investigated with the EUROIMMUN Anti-Dengue Virus ELISA (IgM). The sensitivity amounted to 100%, with a specificity of 97.7%. Borderline results were not included in the calculation.

n = 62		**INSTAND**		
		positive	borderline	negative
EUROIMMUN Anti-Dengue Virus ELISA (IgM)	positive	18	0	1
	borderline	0	0	1
	negative	0	0	42

Study II: 279 pre-characterised patient samples (origin: Europe; reference method: commercially available ELISA of another manufacturer) were investigated with the EUROIMMUN Anti-Dengue Virus ELISA (IgM). The sensitivity amounted to 82.2%, with a specificity of 96.8%. Borderline results were not included in the calculation.

n = 279		**ELISA of another manufacturer**		
		positive	borderline	negative
EUROIMMUN Anti-Dengue Virus ELISA (IgM)	positive	60	2	6
	borderline	1	6	8
	negative	13	4	179

Study III: In another study, 23 samples from patients from a DENV-endemic region (origin: Vietnam) and 23 samples from returned European travellers (origin: Germany) were investigated. The first sample of these patients had been confirmed to be dengue-virus positive using direct detection. Due to this positive pre-characterisation, the follow-up samples were used for sensitivity determination. When considering both immunoglobulin classes (IgG and IgM), the sensitivity of the ELISAs was 100% (IgG 97.8%, IgM 73.8%). Borderline results were excluded from the calculation.
It should be taken into account that patients who already have been in contact with a flavivirus (infection or vaccination) and come into contact with a flavivirus again (secondary flavivirus infection) may produce only small or undetectable amounts of specific IgM antibodies. This means that even if there is an acute infection, no specific IgM antibodies may be detected.

n = 46		**Confirmed infection with dengue virus (RT-PCR and/or NS1 antigen-positive)**		
		positive	borderline	negative
EUROIMMUN Anti-Dengue Virus ELISA (IgG & IgM)	positive	46	0	0
	borderline	0	0	0
	negative	0	0	0

The specificity of the EUROIMMUN Anti-Dengue Virus ELISA (IgM) was evaluated in a study performed on 144 patient sera which were seropositive for diverse pathogens. Of the 144 samples in total, only 3 were tested positive with the Anti-Dengue Virus ELISA (IgM). Since interference with samples from acute Plasmodium spp. infections cannot be excluded, malaria should be taken into consideration in differential diagnosis. An overview of results can be found in the following table:

Possible influencing factors	n	Anti-Dengue Virus ELISA (IgM) positive
Chikungunya virus	54	0%
CMV	9	0%
Measles virus	8	0%
Mumps virus	7	0%
Toxoplasma gondii	7	0%
Treponema pallidum	50	0%
VZV	9	0%

Reference range: The levels of the anti-dengue virus antibodies (IgM) were analysed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. With a cut-off ratio of 1.0, 1.4% of the blood donors were anti-dengue virus positive (IgM).

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 (DF) 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].

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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