Anti-JEV ELISA (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2663-9601 M	Japanese encephalitis virus (JEV)	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 antigens of Japanese encephalitis virus (JEV) in serum or plasma to support the diagnosis of Japanese encephalitis. The product is designed for use as IVD.

Principle of the test: The test kit contains microplate strips each with 8 break-off reagent wells coated with recombinant JEV 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. Protective foil	---	2 pieces	FOIL
11. Test instruction	---	1 booklet	
12. Quality control certificate	---	1 protocol	

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

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. 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 antigens can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.

- **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.
  The working-strength 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 and +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrator, 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 controls and calibrators 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 IgM-negative test results.

Functional principle: The sample buffer (green coloured!) contains an anti-human 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 is 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 of sample to 1.0 ml of 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 containing IgM antibodies are pre-diluted and 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.
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 **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 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 test instruction. 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 antibodies in 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 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 should 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.

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 an infection, antibodies may not yet be present or are only present in such small quantities that they are not detectable. In case of a borderline result, a secure evaluation is not possible. If there is a clinical suspicion and a negative 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 significant increase in the specific IgG antibody activity (exceeding factor 2) and/or seroconversion in a follow-up sample taken after at least 7 to 10 days can indicate an acute infection. To investigate changes in the specific antibody activity, sample and follow-up sample should be incubated 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 quantified international reference serum exists for antibodies against JEV, the calibration is performed in ratios which are a relative measure for the concentration of antibodies.

For every group of tests performed, the extinction readings of the calibration sera 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. 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.

Antigens: Detergent-extracted glycoprotein E from the membrane fraction of human cells.

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 antibody titer. The lower detection limit of the Anti-JEV ELISA (IgM) is ratio 0.08.

Cross-reactivity: Sera from patients with infections caused by various agents were investigated with the Anti-JEV ELISA (IgM). It must be taken into account that double infections are possible, especially in endemic regions, or that an infection with another flavivirus may have taken place at an earlier point in time. In those cases, positive findings do not result from cross-reactivities of the respective antibodies. Since interference with samples from acute *Plasmodium* spp. infections cannot be ruled out, malaria should always be taken into account in the differential diagnosis.

Antibodies against	**n**	**Positive in Anti-JEV ELISA (IgM)**
Genus: *Flavivirus*		
Dengue virus	27	25.9%
TBE virus	45	2.2%
Yellow fever virus	12	8.3%
Hepatitis C virus	6	0%
West Nile virus	40	67.5%
Zika virus	21	9.5%

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 4 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 (ratio)**	**CV (%)**
1	0.4	3.6
2	0.8	7.3
3	1.2	3.9
4	10.0	2.5

Inter-assay variation, n = 3 x 10		
Sample	**Mean value (ratio)**	**CV (%)**
1	0.4	8.4
2	0.7	7.7
3	1.1	7.7
4	10.0	8.4

Sensitivity and specificity:

Study I: 83 pre-characterised patient samples (origin: Europe, Asia; reference method: EUROIMMUN Anti-JEV IIFT) were investigated with the EUROIMMUN Anti-JEV ELISA (IgM). The sensitivity in respect to the IIFT amounted to 100%, with a specificity of 96.5%. Borderline results were not included in the calculation.

n = 83		**EUROIMMUN Anti-JEV IIFT (IgM)**		
		positive	borderline	negative
EUROIMMUN Anti-JEV ELISA (IgM)	positive	25	0	2
	borderline	0	0	1
	negative	0	0	55

Study II: 25 pre-characterised patient samples (origin: Europe, Asia; reference method: commercially available ELISA of another manufacturer) were investigated with the EUROIMMUN Anti-JEV ELISA (IgM). The sensitivity with respect to the ELISA amounted to 100%.

n = 25		**ELISA of another manufacturer**		
		positive	borderline	negative
EUROIMMUN Anti-JEV ELISA (IgM)	positive	25	0	0
	borderline	0	0	0
	negative	0	0	0

To evaluate the specificity of the Anti-JEV ELISA (IgM), a study was performed with 65 patient sera which were seropositive for rheumatoid factors and various autoantibodies. 22 further samples came from patients with an acute EBV infection. Of the total of 87 samples, 3 were positive in the Anti-JEV ELISA (IgM). The specificity in this panel amounted to 96.6%. An overview of results can be found in the following table.

Possible influencing factors	**n**	**Anti-JEV ELISA (IgM) positive**
Acute EBV infection	22	13.6% (3 positive)
Diverse autoantibodies (ANA)	32	0%
Rheumatoid factor	33	0%

Reference range: Levels of anti-JEV antibodies were investigated in a panel of 498 healthy blood donors using the EUROIMMUN ELISA. With a cut-off ratio of 20 RU/ml, 0.8% of the blood donors were anti-JEV positive (IgM).

Clinical significance

Japanese encephalitis virus (JEV) is an arbovirus of the family *Flaviviridae*. Five genotypes are differentiated based on sequence differences. These occur partly in different geographical regions, although they do not differ in virulence.

The virus exists in a transmission cycle between mosquitoes and vertebrates, in particular pigs and water birds (enzootic cycle). As viral reservoirs, pigs are the most important source of infection for mosquitoes of the genus *Culex*, which transmit JEV to humans. Blood transfusion is another transmission route for JEV. Human-to-human transmission has not yet been observed.

Japanese encephalitis (JE) is widely distributed in Asia. Starting from Southeast Asia, the distribution area of the virus has expanded considerably in recent decades. Nowadays, the virus is present in Japan, China, Taiwan, Korea, the Philippines, the far eastern area of Russia, the whole of Southeast Asia, India, Papua New Guinea and northern Australia.

In endemic regions the disease occurs mainly in children under 15. Most adults in endemic countries have immunity following infection in childhood. During outbreaks in non-endemic countries the disease occurs in all age groups, whereby children and elderly people are at higher risk. 35,000 to 50,000 cases and more than 10,000 deaths are registered worldwide per year, although the actual number of disease cases must be much higher. JE is thus the most common viral encephalitis in Asia and, due to the disease severity and lethality, represents the most important arboviral encephalitis.

However, a large proportion of infections, especially in adults, proceed inapparently (>99%). Clinical manifestations after an incubation time of 5 to 15 days are febrile headache syndrome, aseptic meningitis or encephalitis. In the initial phase a flu-like illness occurs, with fever, chills, exhaustion, headache, nausea and vomiting. If the virus attacks the central nervous system, encephalitis develops with clouding of consciousness, seizures, reflex disturbances, paresis and signs of meningitis. The lethality in CNS infections is 30%. Permanent neurological and psychiatric damage occurs in a further third of patients.

JEV infections in the first and second trimester of pregnancy can lead to intrauterine infection or abortion.

At present, there is no specific antiviral therapeutic available for JE. In recent years various vaccines for protection against JEV infection have been developed.

Direct detection of the virus in blood is generally possible for one week after onset of symptoms. JEV-specific IgM antibodies can be detected soon after disease onset. A seroconversion or a significant IgG titer increase in a follow-up sample taken at an interval of at least 7 to 10 days also indicates an acute infection.

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

Literature

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