研究用試薬

Anti-TBE Virus ELISA (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2661-9601 M	TBE virus	IgM	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a semiquantitative in vitro assay for human anti-bodies of the IgM class against TBE virus in serum or plasma for the diagnosis of meningoencephalitis, myelitis and neurological failures.

Application: The determination of anti-TBE virus antibodies of classes IgG and IgM helps to clarify suspected cases of TBE virus infections. Pathogen-specific antibodies can be detected 7 to 10 days after infection or at onset of the second disease stage of TBE. A singular, significant increase of values in the IgG and IgM test, or an at least 4-fold IgG titer increase in two successive samples indicate an acute infection. In cases of involvement of the central nervous system, the investigation of specific, intrathecally produced IgM and IgG antibodies in CSF is indicated. For reliable evaluation of TBE virus-specific antibody determination, the vaccination history of the patient should be taken into account.

Principles of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with TBE virus antigens. In the first reaction step, diluted patient samples are incubated with 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. Test instruction	---	1 booklet	
11. Quality control certificate	---	1 protocol	

LOT	Lot description	CE	Storage temperature
IVD	In vitro diagnostic medical device		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.

- **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 bags).
  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 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, 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 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 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 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. 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 of working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Modus").

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-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 the reaction:
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	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 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 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. 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 value of the control or patient sample over the extinction value of calibrator. Use the following formula to calculate the ratio:

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

EUROIMMUN recommends interpreting results as follows:

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

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For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.

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. Significant titer increases (exceeding factor 2) and/or seroconversion in a follow-up sample taken after 7 to 10 days can indicate an acute infection. To investigate titer changes, 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 international reference serum exists for antibodies against TBE virus, results are provided in the form of ratios which are a relative measure for the concentration of antibodies. The calibration is based on internal reference sera used for the evaluation of the test system.

For every group of tests performed, the extinction values 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 values. 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 reagent wells were coated with inactivated TBE virus antigens of the strain "K23" isolated from TBE virus infected PCEC purified chicken embryo 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 detection limit of the Anti-TBE virus ELISA (IgM) is ratio 0.03.

Cross reactivity: Cross reactivities to other flaviviruses cannot be excluded. They were recognised with anti-West Nile positive and anti-Dengue virus positive samples.

Antibodies against	n	Anti-TBE virus ELISA (IgM)
CMV	9	0%
Measles virus	8	0%
Mumps virus	7	0%
Toxoplasma gondii	7	0%
VZV	9	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 sera. 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		
Serum	**Mean value (Ratio)**	**CV (%)**
1	2.8	3.6
2	2.9	5.5
3	4.0	4.2

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (Ratio)**	**CV (%)**
1	2.7	3.9
2	2.7	5.0
3	3.7	5.7

Specificity and sensitivity: 129 clinically characterised patient samples (Interlaboratory test samples from INSTAND, Labquality, ENIVD) were examined with this EUROIMMUN ELISA. The test showed a specificity and a sensitivity of 100%, respectively.

n = 129		**INSTAND / Labquality / ENIVD**		
		positive	borderline	negative
EUROIMMUN Anti-TBE Virus ELISA (IgM)	positive	25	0	0
	borderline	0	0	0
	negative	0	0	104

Immunisation monitoring: 47 patient samples were investigated in a vaccination study with this EUROIMMUN ELISA. Blood was drawn from all patients before and after vaccination. In 44 patients (93.6%) a seroconversion of class IgG and in 40 patients (85.1%) a seroconversion of class IgM occurred. The total results showed that antibodies against TBE virus could be detected in 46 patients (97.9%) after vaccination.

n = 47	IgG positive	IgM positive	IgG and/or IgM positive
before vaccination (neg. patient)	0	0	0
after vaccination	44	40	46

Reference range: The levels of anti-TBE virus antibodies (IgM) were analysed with this EUROIMMUN ELISA in a panel of 300 healthy blood donors. With a cut-off ratio of 1.0, 0.7% of the blood donors were anti-TBE virus positive (IgM) which reflects the known percentage of infections in adults.

Clinical significance

Tick-borne encephalitis (TBE) is a systemic infection caused by the human pathogen TBE virus. The coated, single-stranded RNA virus belongs to the flavivirus family. Three subtypes of TBE virus are currently known: type 1 (Western subtype), type 2 (Siberian subtype) and type 3 (Far Eastern subtype).

The Western subtype, which is found in Europe, is transmitted via bites from infected ticks. The most important vectors are types of the genus Ixodes, with Ixodes ricinus being the most common type in middle Europe. Further types are Ixodes persulcatus, which is predominantly found in East Europe and Russia and is a vector for TBE type 2 virus, and more rarely also the genera Rhipicephalus, Dermacentor, Haemaphysalis, Amblyomma and from the family of soft ticks the genera Argas and Ornithodorus.

The greatest risk of tick bites occurs in bushy areas, on the edge of woods or in high grass. This is because small mammals and deer, which are the main hosts and primary reservoirs of the blood-sucking pathogen, live in these areas. There are no ticks at heights above 1000 meters. In high-risk areas the percentage of ticks infected with TBE is around one to five percent.

With the tick bite the TBE virus is transferred from the tick's salivary gland into the victim's skin, and from here into nearby lymph nodes. From here it moves into further organs, such as connective tissue, skeletal muscle, myocardium, smooth muscle and also monocytes and phagocytes, where the virus multiplies rapidly (primary viraemia). In a second viraemic phase TBE virus moves into the central nervous system, from where it can enter the brain.

Transmission from virus-infected milk products can occur, although very rarely, via milk from infected goats and sheep, and in exceptional cases from cows. Transmission from person to person does not occur.

In a large proportion of patients no verifiable disease symptoms appear. Around 10% to 30% of infected persons show symptoms two to twenty days after infection. These include flu-like symptoms such as fever, head and joint aches, and gastrointestinal complaints such as nausea, vomiting, stomach ache and diarrhoea. These symptoms all clear up within a few days.

In around 10% of symptomatic patients a second phase with infection of the CNS occurs around one week after defervescence. Symptoms are fever peaks of up to 40°C and signs of brain and meningeal involvement such as headache, vomiting and meningeal irritation. If the meningoencephalitis progresses, it can impair consciousness to the point of coma and paralysis. 44% of phase 2 patients develop meningitis, 42% meningoencephalitis and 14% myelitis/radiculitis. These symptoms can last several months. Subtype 2 of the TBE virus generally causes more severe symptoms with a higher mortality than subtype 1.

The number of TBE infections has risen continually in recent years in all affected countries. The frequency of TBE infections occurring per 100,000 inhabitants is currently 0.27 in Germany, 3.4 in Sweden, 6.0 in Austria and Switzerland, 24.6 in the Czech Republic and Slovakia, and 50 to 100 in parts of Russia and West Siberia,. In Europe the greatest affected counties are the Baltic states Lithuania, Estonia and Latvia, where all three subtypes of TBE are in circulation. Because of the often unspecific flu-like symptoms, there are probably a high number of unidentified infections.

The diagnostic method of choice is the demonstration of TBE-virus-specific IgM and IgG in serum or CSF using ELISA. These antibodies can be detected 7 to 10 days after infection or at the start of the second stage of illness. In cases without clear CNS symptoms (meningitis, encephalomyelitis, myelitis) detection of IgM alone in blood is considered insufficiently specific, and demonstration of a 4-fold titer increase between two serum samples is recommended. Early diagnosis is also possible with the detection of low-avidity specific IgG antibodies. Alternatively, simultaneous detection of TBE-specific IgM and IgG in serum increases the specificity. The detection of specific IgM and IgG antibodies in CSF using ELISA and IIFT indicates CNS involvement. With positive serological CSF results, the CSF also shows pleocytosis and an increase in protein after the second fever peak. ELISA is the method of choice for monitoring the human immune reaction following TBE virus vaccination. TBE vaccination usually leads to a positive antibody titer. Detectable specific IgG antibodies persist with lifelong immunity. In endemic areas of Europe they are detectable in 14% to 42% of the population. Special procedures for direct virus detection are the time-consuming cell culture method, nRT-PCR (nested reverse transcriptase polymerase chain reaction) and Westernblot.

When TBE virus is detected directly or indirectly and as long as an acute infection is indicated, the infection is notifiable in Germany. The public health authority also commissions various evaluations itself.

There is no causal treatment for TBE. Specific antiviral medications do not exist. If the disease has manifested, only symptomatic treatment to reduce individual symptoms is possible. In severe cases intensive medical treatment is necessary.

Prognosis is generally good, especially in children and young adults. The majority of cases of the illness heal without any complications. However, in 10% to 30% of symptomatic cases neurological damage of various degrees remains. This includes paralysis, balance difficulties, epilepsy, hearing difficulties and memory and concentration problems. An infection confers lifelong immunity, including against other subtypes of TBE virus. One to two percent of patients with meningoencephalitis die.

Very important in differential diagnostics are encephalitides with symptoms similar to those of TBE infections caused by viruses (e.g. adenovirus, Coxsackie virus, cytomegaly virus, Dengue virus, echovirus, Herpes simplex virus, HIV, Japanese encephalitis virus, mumps virus, Powassan virus, poliomyelitis virus, varicella zoster virus, West Nile virus) and other pathogens (e.g. haemophilus influenzae, neisseria meningitidis, streptococcus pneumoniae, borrelia burgdorferi, eptospira, toxoplasma gondii, trichinella spiralis). Various tests are available for differential diagnosis, particularly BIOCHIP-Mosaics™ for infectious serology (e.g. CNS PROFILE), indirect immunofluorescence (infectious serology), bacteria, microplate ELISA and EUROLINE-WB.

Active immunisation is the most important preventative measure. It is strongly recommended for all people who live in or visit high-risk areas, including children, adolescents and elderly people, because new vaccines without protein-containing stabiliser allow a risk-free application. Passive immunisation is possible up to 3 days following the tick bite (postexposure immunoprophylaxis). A parallel active immunisation is also recommended prophylactically. General preventative protection measures such as exposure prophylaxis should also not be neglected.

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