Anti-Toxoplasma gondii ELISA (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2410-9601 M	Toxoplasma gondii	IgM	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a semiquantitative in vitro assay for human antibodies of the immunoglobulin class IgM against Toxoplasma gondii in serum or plasma for the diagnosis of toxoplasmosis.

Application: Serological detection methods play an important role in the diagnosis of Toxoplasma gondii infections, as the direct detection of Toxoplasma is seldom successful. Specific antibodies of classes IgG and IgM are detectable approximately eight days after infection. While IgM antibodies usually disappear after some months, IgG antibodies persist lifelong. In most cases, the detection of IgG and IgM antibodies allows detecting a recent infection which might constitute a risk for an existing pregnancy. For confirmation purposes the determination of specific IgA antibodies and the avidity deter-mination of specific IgG are performed. A positive IgG detection before pregnancy proves immunity.

Principle of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with Toxoplasma gondii 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. Test instruction	---	1 booklet	
11. Quality control certificate	---	1 protocol	
LOT Lot description IVD In vitro diagnostic medical device	CE 0197		Storage temperature Unopened usable until

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.

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 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.

- **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.

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 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 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 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 of 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 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-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, 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.

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. 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**

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. 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 of the IgM class against Toxoplasma gondii, results are provided in the form of ratios which are a relative measure for the concentration of antibodies in serum or plasma. The calibration is performed with internal reference sera, which were used for evaluation of the test system.

For every group of tests performed, the extinction values of the calibrator and the 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 control sera 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 antigen used is Toxoplasma gondii organisms purified by density gradient centrifugation with detergent extraction.

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-Toxoplasma gondii ELISA (IgM) is ratio 0.1.

Cross reactivity: The quality of the antigen used ensures high specificity of the ELISA. Sera from patients with fresh infections caused by various agents were investigated with the Anti-Toxoplasma gondii ELISA (IgM).

Antibodies against	n	Anti-Toxoplasma gondii ELISA (IgM) positive
Borrelia	10	0%
CMV	19	5.26%
EBV	30	30%
HSV Pool	5	0%
Measles virus	13	0%
Mumps virus	12	0%
Rubella virus	10	10%
VZV	15	0%

From 186 tested serum samples, in 8 patient samples with an acute EBV infection, in 1 patient sample with an acute CMV infection and in 1 patient sample with an acute rubella infection, also antibodies against Toxoplasma gondii were found. It can be assumed that these reactions were caused by a polyclonal B cell stimulation, which is described in the literature in detail. No cross reactions could be determined.

Interference: No interference was observed with haemolytic and icteric samples for concentrations of up to 10 mg/ml for haemoglobin and 0.4 mg/ml for bilirubin. Lipaemic samples of up to 20 mg/ml for triglyceride had an influence on analysis results.

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	1.2	5.3
2	2.6	8.8
3	3.1	3.3

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (Ratio)**	**CV (%)**
1	1.5	7.5
2	2.4	8.5
3	3.1	5.6

Sensitivity and specificity:
Study I: 234 pre-characterised patient samples (origin: Europe, reference method: commercially available ELISA of another manufacturer) were investigated with the EUROIMMUN Anti-Toxoplasma gondii ELISA (IgM). The sensitivity amounted to 100%, with a specificity of 97.2%. Borderline results were not included in the calculation.

n = 234		**ELISA of another manufacturer**		
		positive	borderline	negative
EUROIMMUN Anti-Toxoplasma gondii ELISA (IgM)	positive	63	8	4
	borderline	3	5	13
	negative	0	1	137

Study II: 92 pre-characterised patient samples (origin: Europe, reference method: commercially available ELISA of another manufacturer – FDA-registered assay) were investigated with the EUROIMMUN Anti-Toxoplasma gondii ELISA (IgM). The sensitivity amounted to 100%, with a specificity of 100%. Borderline results were not included in the calculation.

n = 92		**ELISA of another manufacturer (FDA-registered)**		
		positive	borderline	negative
EUROIMMUN Anti-Toxoplasma gondii ELISA (IgM)	positive	17	1	0
	borderline	1	1	1
	negative	0	1	70

Study III: 162 clinically pre-characterised patient samples (INSTAND, Labquality and RfB) were investigated with the EUROIMMUN Anti-Toxoplasma gondii ELISA (IgM). The sensitivity amounted to 98.2%, with a specificity of 97.9%. Borderline results were not included in the calculation.

n = 162		**INSTAND/Labquality/RfB**		
		positive	borderline	negative
EUROIMMUN Anti-Toxoplasma gondii ELISA (IgM)	positive	54	0	2
	borderline	5	1	3
	negative	1	2	94

The specificity of the Anti-Toxoplasma gondii ELISA (IgM) was evaluated in a study of 72 patient sera which were positive for rheumatoid factors and diverse autoantibodies (ANA). From the tested samples only 2 patients with diverse autoantibodies also exhibited IgM antibodies against Toxoplasma gondii. The results are summarised in the following table.

Possible influencing factors	n	Anti-Toxoplasma gondii ELISA (IgM) positive
Diverse autoantibodies (ANA)	35	5.7%
Rheumatoid factor	37	0%

Reference range: Levels of anti-Toxoplasma gondii antibodies were investigated in a panel of healthy blood donors (n = 500, origin: Germany) using the EUROIMMUN ELISA. With a cut-off ratio of 1.0, 0.8% of the blood donors were anti-Toxoplasma gondii positive (IgM).

Clinical significance

The sporozoon Toxoplasma gondii is the causative agent of the worldwide distributed zoonosis toxoplasmosis. The main host animal is the cat. The parasites live in the intestinal cells of the host and cause oocysts to develop (sexual development cycle). During the asexual cycle, the Toxoplasma parasites develop in the brain, muscles, liver, spleen and in other organs of warm-blooded animals, where they become encapsulated. Humans are generally infected perorally by ingestion of oocysts with viable trophozoites, which are contained in the faeces of infected cats or in meat products (raw flesh) from infected animals. Toxoplasma gondii can also be transmitted diaplacentally when a pregnant woman is first infected. In Germany, the risk of infection is particularly high, since 65 to 75% of women of childbearing age are negative for IgG antibodies against Toxoplasma.

Postnatally acquired toxoplasmosis proceeds inapparently in 90% of cases. Cysts containing trophozoites form in the tissues and can persist for years. Acute or previous infections can therefore only be identified serologically. The symptoms of the manifest disease include fever, lymphadenopathy, encephalitis, chorioretinitis, myositis, myocarditis, pneumonia, hepatosplenomegaly and exanthema, depending on the affected organs. In immunocompromised patients (recipients of transplants, tumour patients, HIV-infected patients), a primary infection with Toxoplasma or the reactivation of toxoplasmosis can lead to a life-threatening illness.

After an intrauterine infection with Toxoplasma gondii in the first trimester, placenta and embryo are severely affected, resulting in rejection of the embryo. An infection in the second or third trimester results in foetal symptoms which vary in intensity depending on the time point of infection, the dose of the infection and the immune status of mother and foetus. Among the most important symptoms are the following: hepatosplenomegaly, pneumonia, myocarditis, purpura, hydrocephalus and intracranial anomalies (in particular intracerebral calcification), chorioretinitis and optic nerve oedema with concurrent distant active lesions. Connatally infected children mostly show severe damage, as they are treated too late.

If the Toxoplasma immune status is not known at the start of a pregnancy, the obstetrician should advise the patient on potential infection sources, risks of a potential Toxoplasma infection for the child, preventative measures and the possibility of serological diagnostics. Infections that can present a prenatal risk to the unborn child are combined under the term of TORCH complex: T = toxoplasmosis, O = “other infectious microorganisms“, R = rubella, C = cytomegalovirus infection, H = Herpes simplex. Within TORCH infections in pregnant women the seroprevalence of IgM antibodies against Toxoplasma gondii varies. It is between 15 to 75%, depending on the country.

PCR from blood samples for the diagnosis of acute infection is often not useful in immunocompetent persons because a negative result does not reliably exclude very recent parasitaemia. Positive PCR results from blood samples in the acute phase of infection are mostly incidental findings. Serology is therefore the method of choice for the diagnosis of acute infection.

The standard methods for the serological detection of Toxoplasma-specific IgG and IgM antibodies are IIFT and ELISA. Due to the fact that the diagnostic sensitivity varies and the specificity of serological IgM analysis is generally lower than that of IgG analysis, IgA antibodies and avidity should be in-vestigated in ambiguous cases. The detection of low-avidity antibodies against Toxoplasma gondii in the serum allows the diagnosis of acute Toxoplasma infection.

Interpretation of results:

IgG	IgM	IgG avidity	Probable result
positive	negative	-	inactive latent infection
positive	positive	high	abating or latent (inactive) infection
positive	positive	low	further serological testing/monitoring required

The use of the Anti-Toxoplasma gondii Screen ELISA as a combined test is recommended for a strategic diagnostic approach in the serological diagnosis of Toxoplasma gondii infections. The sensiti-vity is 100%. Antibodies of classes IgG and IgM (sometimes also IgA) can be investigated with one procedure and no loss in specificity. The Anti-Toxoplasma gondii Screen ELISA is particularly re-commended in countries with a low prevalence, such as Germany, to save costs. In regions with a high prevalence and therefore high IgG detection rates, the monospecific standard tests should be carried out. This approach is also recommended by the Robert Koch Institute in Berlin, Germany.

Furthermore, in addition to serum diagnostics, CSF analysis is very important to detect rare cases of specific antibody synthesis in the central nervous system. The CSF/serum quotient allows differentiation between a blood-derived and a pathological, intrathecal antibody fraction in the CSF, taking into account individual changes in the blood/CSF barrier function. The frequently observed discrepancy between anti-body concentrations in serum and CSF is due to local synthesis of antibodies (IgG) against Toxoplasma gondii in the central nervous system, which can persist for several years.

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