Avidity determination of IgG antibodies against Toxoplasma gondii
Test instruction for the ELISA

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2410-9601-1 G	Toxoplasma gondii	IgG	Ag-coated microplate wells	96 x 01 (96)

Background

The differentiation between fresh and long-standing infections is one of the greatest challenges in serology. Until now this was based mainly on determination of specific antibodies of the immunoglobulin class IgM, which generally only appear initially. However, the detection of these antibodies is often unreliable and problematic due to interfering factors such as persistence of the IgM response, too weak or delayed IgM production, and unspecific IgM production through polyclonal B-cell stimulation.

In recent years additional determination of the antibody avidity has become an established method for identification of primary infections. The immune system reacts to an infection by first forming low-avidity antibodies. With continued disease duration, IgG that are more precisely adapted to the antigens are produced – the avidity increases. If high-avidity IgG are detectable in the serum, it can be assumed that the infection is at a late stage.

Contents of the test system: EI 2410-9601-1 G:

Component	Colour	Format	Symbol
1. Test kit Anti-Toxoplasma gondii ELISA (IgG) (IgG, order number EI 2410-9601 G)	---		
2. Positive control HA High-avidity anti-Toxoplasma gondii (IgG, human), ready for use	red	1 x 1.3 ml	POS CONTROL HA
3. Positive control LA Low-avidity anti-Toxoplasma gondii (IgG, human), ready for use	blue	1 x 1.3 ml	POS CONTROL LA
4. Urea solution for Anti-Toxoplasma gondii ELISA, ready for use	yellow	1 x 12 ml	UREA
5. Phosphate buffer ready for use	light blue	1 x 12 ml	PBS BUFFER
6. Test instruction	---	1 booklet	

LOT Lot description — CE 0197 — Storage temperature

IVD In vitro diagnostics — 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.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents should be disposed of according to official regulations.

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.

- **Controls:** Ready for use. The reagents must be mixed thoroughly before use.

- **Urea solution:** Ready for use. The urea solution included in this test kit is only for the use in avidity determination of antibodies against Toxoplasma gondii.

- **Phosphate buffer:** Ready for use.

Warning: The 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

Sample material: 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.

Sample dilution: Patient samples are diluted **1:101** in sample buffer. For example: dilute 10 µl serum to 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

Caution: If the IgG determination for the 1:101 dilution without urea treatment yields an extinction value >1.200 O.D. and there is a resulting borderline or relatively high avidity index, the measurement is to be repeated with a higher sample dilution (e.g. 1:1601 or 1:3201). For the calculation of the relative avidity index, only such dilutions may be used whose extinctions without urea treatment are ≤1.200 O.D. If low avidity of IgG antibodies is already found at extinction values of >1.200 O.D., no further testing is necessary.

NOTE: The controls are prediluted and ready for use, do not dilute them.

Incubation

Sample incubation: (1st step)
Transfer 100 µl of the controls or diluted patient samples as a double value into the individual microplate wells of two different microtiter strips 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 **1 time** using 300 µl of working strength wash buffer.
Automatic: Wash the reagent wells **1 time** 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.
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.

Urea incubation: (2nd step)
Pipette 200 µl of urea solution into each of the microplate wells of the first microtiter strip and 200 µl of phosphate buffer into each of the microplate wells of the second microtiter strip.
Incubate for **10 minutes** at room temperature (+18°C to 25°C).

Washing:
Empty the wells. Wash as described above, but wash **3 times** using working strength wash buffer for each wash.

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 reaction times) can lead to false high extinction values.

Conjugate incubation: (3rd step)
Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgG) 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, but wash **3 times** using working strength wash buffer for each wash.

Substrate incubation: (4th 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.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	**pos HA**	**pos HA**	**P 7**	**P 7**	**P 15**	**P 15**						
B	**pos LA**	**pos LA**	**P 8**	**P 8**	**P 16**	**P 16**						
C	**P 1**	**P 1**	**P 9**	**P 9**	**P 17**	**P 17**						
D	**P 2**	**P 2**	**P 10**	**P 10**	**P 18**	**P 18**						
E	**P 3**	**P 3**	**P 11**	**P 11**								
F	**P 4**	**P 4**	**P 12**	**P 12**								
G	**P 5**	**P 5**	**P 13**	**P 13**								
H	**P 6**	**P 6**	**P 14**	**P 14**								

The above pipetting protocol is an example of the avidity determination of IgG antibodies in 18 patient samples (P 1 to P 18).

Controls (pos HA and pos LA) as well as the patient samples have been incubated in duplicate in one well each of two different microtiter strips. The reagent wells of the microtiter strips 1, 3, 5 etc. are treated with urea solution after the incubation with patients samples, the reagent wells of the microtiter strips 2, 4, 6 etc. are treated with phosphate buffer.
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 minimizes reagent wastage.
Both positive controls with high-avidity and low-avidity antibodies serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

The presence of low avidity antibodies in a patient's serum has been proved if the ELISA extinction value is considerably reduced by urea treatment. For an objective interpretation the relative avidity index (RAI) is calculated and expressed in percent using the extinction values with and without urea treatment.

$$\frac{\textbf{Extinction of the sample with urea treatment} \times 100}{\textbf{Extinction of the sample without urea treatment}} = \textbf{relative avidity index (RAI) in \%}$$

The upper limit of the range of low-avidity antibodies (**cut-off value**) recommended by EUROIMMUN is 40% RAI. Values below the indicated cut-off are to be considered as an indication of low-avidity antibodies, values between 40% and 60% RAI as equivocal, values above 60% as an indication of high-avidity antibodies. If a result is classified as equivocal, it is recommended to collect a second sample not less than 7 days later and to test it together with the first sample.

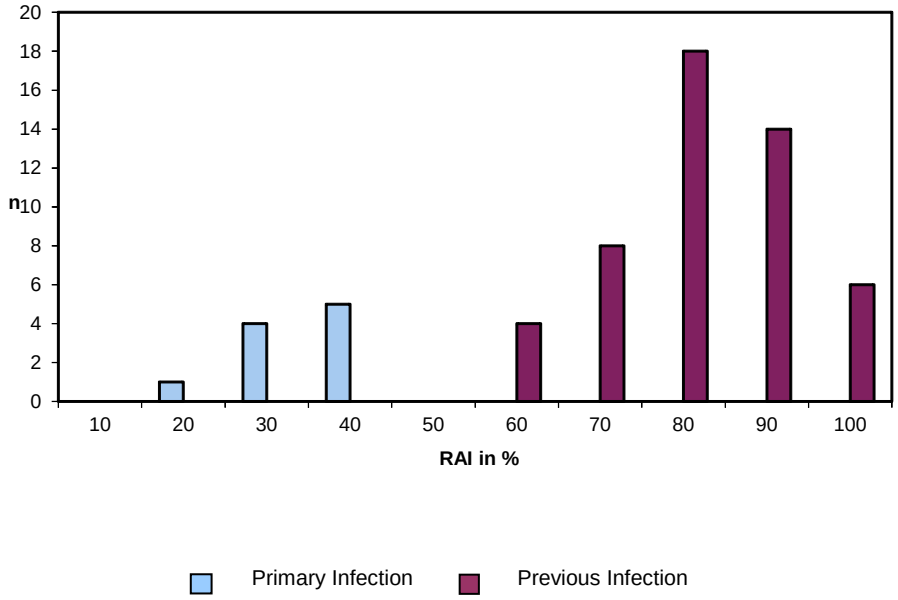
RAI <40%:	Indication of low-avidity antibodies
RAI 40% - 60%:	Equivocal range
RAI >60%:	Indication of high-avidity antibodies

Reliable results in the measurement of IgG antibody avidity can only be yielded if the patient sample contains a diagnostically significant concentration of specific antibodies. Generally, the determination of the relative avidity index is not helpful in samples which have an O.D. of <0.140 after incubation without urea treatment.

For diagnosis, the clinical symptoms of the patient should always be taken into account alongside the serological results.

Attention:
In some patients with an acute Toxoplasma gondii infection, very high titers of IgG antibodies can be found. Even though the specific IgG antibody population is in different maturation stages, both high-avidity and low-avidity, the vacant antigen epitopes are predominantly occupied by high-avidity antibodies in high titer samples. The determination of the avidity of the whole specific IgG antibody population can lead to false high RAI values in results.
False high RAI values were found in some cases of acute infections when the extinction value of the IgG measurement without urea treatment was >1.200 O.D.
It is recommended for samples with extinction values of >1.200 O.D. to repeat the avidity determination with a higher sample dilution (e. g. 1:1.601 or 1:3201). If low avidity of IgG antibodies is already found at extinction values of >1.200, no further testing is necessary.

In a study performed at EUROIMMUN 10 sera of patients with a recent infection showed a relative avidity index below 40% (12% to 37%, mean 28.2%). In each of the 50 serum samples of patients with previous infection the RAI was significantly higher (55% to 96%; mean 76.4%).



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, hepato-splenomegaly 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 micro-organisms”, 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 immuno-competent 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 investigated 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 sensitivity 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 recommended 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 antibody 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.

Literature references

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