Anti-Tetanus Toxoid ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2060-9601 G	Tetanus toxoid	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a quantitative in vitro assay for human antibodies of the immunoglobulin class IgG against tetanus toxoid in serum or plasma for the clarification of an unclear immune status.

Application: The Anti-Tetanus Toxoid ELISA (IgG) is based on inactivated tetanus toxin and is designed for the quantitative determination of human IgG antibodies against tetanus toxoid in serum or plasma. This test is suited for both investigation the immune status and for vaccination control.

Principles of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with tetanus toxoid. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG antibodies (also IgA and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (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 1 5 IU/ml (IgG, human), ready for use	Red coloured in decreasing intensity.	1 x 2.0 ml	CAL 1
3. Calibrator 2 2 IU/ml (IgG, human), ready for use		1 x 2.0 ml	CAL 2
4. Calibrator 3 1 IU/ml (IgG, human), ready for use		1 x 2.0 ml	CAL 3
5. Calibrator 4 0.1 IU/ml (IgG, human), ready for use		1 x 2.0 ml	CAL 4
6. Calibrator 5 0.01 IU/ml (IgG, human), ready for use		1 x 2.0 ml	CAL 5
7. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
8. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
9. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
10. Sample buffer ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
11. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
12. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
13. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
14. Protective foil	---	2 pieces	FOIL
15. Test instruction	---	1 booklet	
16. Quality control certificate	---	1 protocol	

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

- **Calibrators 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.

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.

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

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

Incubation

(Partly) manual test performance

Sample incubation: (1st step)
Transfer 100 µl of the calibrators, 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 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 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.

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

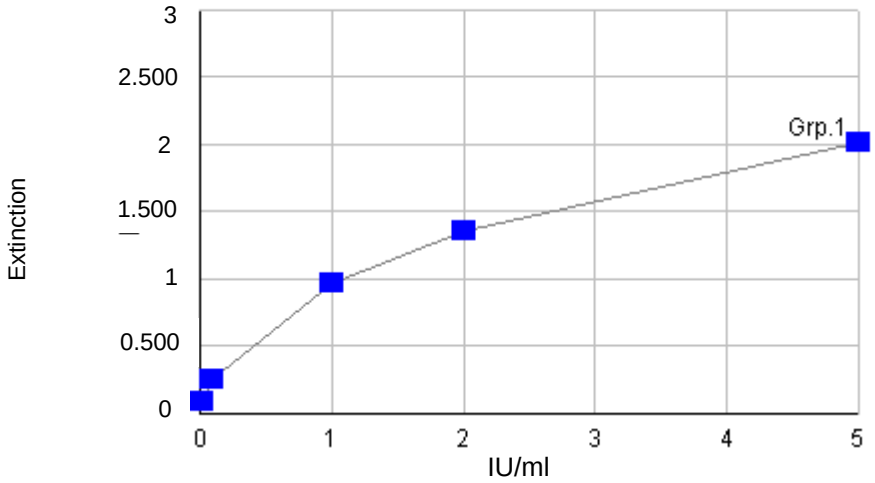
	1	2	3	4	5	6	7	8	9	10	11	12
A	**C 1**	**P 2**	**P 10**	**P 18**								
B	**C 2**	**P 3**	**P 11**	**P 19**								
C	**C 3**	**P 4**	**P 12**	**P 20**								
D	**C 4**	**P 5**	**P 13**	**P 21**								
E	**C 5**	**P 6**	**P 14**	**P 22**								
F	**pos.**	**P 7**	**P 15**	**P 23**								
G	**neg.**	**P 8**	**P 16**	**P 24**								
H	**P 1**	**P 9**	**P 17**									

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

The calibrators (C 1 to C 5), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.
The wells can be broken off individually from the strips. Therefore, the number of tests performed can be matched to the number of samples, minimising 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 standard curve from which the concentration of antibodies in the serum samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 5 calibration sera against the corresponding units (linear/linear). Use “point-to-point” plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.



If the extinction for a patient sample lies above the value of calibrator 1 (5 IU/ml), the result should be reported as “>5 IU/ml”. It is recommended that the sample be retested in a new test run at a dilution of e.g. 1:400. The result in IU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4.

Interpretation of test results

Based on the literature references [4,8,9,13] the following evaluation of the test results is recommended:

<0.1 IU/ml	Insufficient immunity, booster vaccination recommended
0.1-0.5 IU/ml	Immunity given, booster vaccination will provide long-term immunity
>0.5-1.1 IU/ml	Sufficient immunity, booster vaccination in 2 to 5 years
>1.1-5.0 IU/ml	Sufficient immunity, booster vaccination in 5 to 10 years
>5.0 IU/ml	Sufficient immunity, booster vaccination in approx. 10 years

For the interpretation of antibody titers, country-specific recommendations should be considered.

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.

Alongside the serological finding, the vaccination history of the patient must always be taken into account for diagnosis.

Test characteristics

Calibration: The Anti-Tetanus Toxoid ELISA (IgG) was calibrated using the first international WHO standard preparation TE-3 (1st International Standard for Tetanus Immunoglobulin, Human NIBSC Code TE-3).

For every group of tests performed, the extinction values of the calibrators and the international units 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 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 calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The microtiter wells were coated with inactivated Tetanus toxoid antigen.

Linearity: The linearity of the Anti-Tetanus Toxoid ELISA (IgG) was determined by assaying at least 4 serial dilutions of different patient samples. The Anti-Tetanus Toxoid ELISA (IgG) is linear at least in the tested concentration range (0.01 IU/ml to 4.2 IU/ml).

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-Tetanus Toxoid ELISA (IgG) is 0.001 IU/ml.

Cross reactivity: The quality of the antigen used ensures a high specificity of the ELISA. Sera from patients with infections caused by various agents were investigated with the Anti-Tetanus Toxoid ELISA (IgG).

Antibodies against	n	Anti-Tetanus Toxoid ELISA (IgG) positive
Adenovirus	12	0%
Chlamydia pneumoniae	12	0%
CMV	8	0%
EBV-CA	12	0%
Helicobacter pylori	9	0%
HSV-1	12	0%
Influenza A	12	0%
Influenza B	12	0%
Measles virus	12	0%
Mumps virus	12	0%
Mycoplasma pneumoniae	12	0%
Parainfluenza Pool	12	0%
Rubella	12	0%
RSV	12	0%
Toxoplasma gondii	11	0%
VZV	12	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 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 (IU/ml)**	**CV (%)**
1	0.6	2.7
2	0.9	2.2
3	2.0	1.1

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (IU/ml)**	**CV (%)**
1	0.6	6.0
2	1.0	9.7
3	2.0	3.6

Sensitivity and specificity:
Study I: 108 pre-characterised patient samples (origin: Europe; reference method: commercially available ELISA of another manufacturer) were investigated with the EUROIMMUN Anti-Tetanus Toxoid ELISA (IgG). The sensitivity amounted to 98%, with a specificity of 100%.

n = 108		ELISA of another manufacturer	
		positive	negative
EUROIMMUN Anti-Tetanus Toxoid ELISA (IgG)	positive	97	0
	negative	2	9

Study II: 54 clinically pre-characterised patient samples (INSTAND) were investigated with the EUROIMMUN Anti-Tetanus Toxoid ELISA (IgG). The sensitivity amounted to 90%, with a specificity of 100% (cut-off 0.1 IU/ml).

n = 54		INSTAND	
		positive	negative
EUROIMMUN Anti-Tetanus Toxoid ELISA (IgG)	positive	45	0
	negative	5	4

Clinical evaluation:		**n = 54**	**INSTAND**	**EUROIMMUN Anti-Tetanus Toxoid ELISA (IgG)**
< 0.1 IU/ml	Immunity is insufficient, booster is recommended		4	9
>= 0.1-0.5 IU/ml	Immune protection present, booster vaccination gives long-term vaccination protection		10	7
> 0.5-1.1 IU/ml	Immune protection sufficient, booster vaccination in 2 to 5 years		9	8
> 1.1-5.0 IU/ml	Immune protection sufficient, booster vaccination in 5 to 10 years		29	26
> 5.0 IU/ml	Immune protection sufficient, booster vaccination in about 10 years		2	4

Reference range: The levels of anti-tetanus toxoid antibodies (IgG) were analysed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. With a cut-off of 0.1 IU/ml, 97% of the blood donors showed an immunisation protection. 3% of the blood donors required a basic immunisation or a booster.

Clinical significance

The Anti-Tetanus Toxoid ELISA is designed for the detection and quantitative determination of human IgG antibodies against tetanus toxoid in serum or plasma. The pathogenic agent of tetanus, Clostridium tetani (C. tetani), is an obligate anaerobic, motile, gram-positive, spore-forming, rod bacterium of the genus Clostridium, which belongs to the family of Bacillaceae. It is found in soil, in animal corpses, in intestinal content and in faeces of horses, more rarely of cows and other animals. C. tetani spores, which are present ubiquitously in the soil, are resistant to heat and disinfection agents. If they are not exposed to sunlight they can survive for years in the soil. Direct transmission from person to person does not occur.

The precondition for an infection is a wound. The C. tetani spores are carried under the skin via impurities, often together with foreign bodies such as wood splinters, nails or thorns. The wounds do not have to be open.

There is a risk even with tiny wounds that are hardly visible. Conversion of the spores to the vegetative form of C. tetani takes place in the wound. Optimal growth conditions are around 37°C and an anaerobic atmosphere.

C. tetani can produce two exotoxins, tetanolysine and tetanospasmine. The latter, a 150 kDa protein, is a neurotoxin which causes the typical clinical symptoms (mild to severe muscle spasms; generalised, neonatal or local form of tetanus) and in its pathogenic effect is exceeded only by the botulinus toxin.

Tetanus is distributed worldwide with large regional variations. In the industrialised countries of Europe and North America the tetanus incidence is low due to broad vaccination and improved living conditions. In Germany the number of disease cases has decreased from well over 100 (before 1970) to only a few today, although only fatal cases are recorded via death statistics and even this is not done everywhere. In Asia and Africa the incidence is 10 to 50 cases per 100,000 inhabitants. According to estimates by the WHO over a million people die annually from tetanus, in particular infants from tetanus neonatorum with entry via the umbilical cord.

The incubation time is generally 4 to 14 days, occasionally 3 days to 3 weeks. A short incubation (higher amounts of toxin) has an unfavourable prognosis.

The highly potent tetanospasmin, which triggers tonic cramps, and tetanolysin, which has a haemolytical and possibly also a cardiotoxic effect, bind to the receptor gangliosides of the neurons and migrate approximately 5 mm per hour along the peripheral nerves to the CNS. Like strychnine, the exotoxins inhibit the activity of the anterior horn cells of the medulla spinalis and abolish reciprocal innervation, so that outgoing impulses cause an exaggerated reaction.

The generalised form of tetanus is the most common. It begins mostly afebrile or subfebrile with tonic spasms in the skeletal muscle of the face (risus sardonicus with lockjaw, trismus), pharynx (dysphagia), larynx and torso (opisthotonus body posture). Concurrent spasms in flexor and extensor muscles can cause fractures in the area of the spine. Patients remain conscious. Respiratory complications such as obstruction of the airways, congestion of secretions, pneumonia and atelectasis lead to respiratory insufficiency. Involvement of the sympathetic nervous system shows as fluctuations in blood pressure, peripheral circulatory disorders and sweating. With modern intensive therapy the lethality is 10% to 20%, otherwise it is much higher. Causes of death are predominantly respiratory insufficiency and cardiovascular complications.

The neonatal form of tetanus develops in infants who were delivered by mothers with insufficient immunity, and whose umbilicus care was not sufficiently hygienic. The disease generally appears in the first two weeks of life as a generalised form with rigidity, weakness in drinking and cramps.

The local form of tetanus is a rare form which manifests in the muscles surrounding the place of entry. This form mostly occurs in persons with part immunity and has a good prognosis.

Diagnosis of tetanus is made on the basis of typical clinical findings and laboratory diagnostics. Illness is unlikely when the patient has undergone full basic immunisation and timely boosters. A diagnosis can be secured by determining the toxin by means of the neutralisation test in animals (mouse test) using wound material or serum from the patient. Pathogen detection by culture is normally not successful. Various methods are available to determine the tetanus antitoxin titer, such as RIA, FIA, ELISA or microneutralisation test.

The method of choice, which is characterised by high sensitivity, simple performance and the possibility to automate, is ELISA. The Anti-Tetanus Toxoid ELISA (IgG) is available for diagnosing infections and for checking that there are sufficient quantities of protecting antibodies (following immunisation). Since reactions such as polyneuritides and tonic muscle cramps can occur after booster immunisations, it is important to determine the anti-tetanus toxoid titer before the vaccination, in order to see if it is really necessary. Independently of this the WHO recommends that pregnant women be vaccinated against tetanus (including diphtheria and whooping cough) between the 27th and 36th week of pregnancy. The transfer of antibodies to the foetus protects the newborn from C. tetanus infection up until the first tetanus vaccination at the age of 2 months.

Immediate therapeutic measures for patients with a risky wound and no or insufficient immunity (last vaccination more than 10 years ago) are thorough surgical wound care (excision), administration of tetanus immunoglobulin to neutralise unbound toxin and at the same time implementation of tetanus immunoprophylaxis. Missing immunisations should be carried out according to basic vaccination recommendations. Further, the implementation of comprehensive intensive therapy to maintain vital functions and relax the muscles is essential. Keeping the airways free (if necessary tracheotomy and artificial breathing) is often life-saving.

Active immunisation is the method of choice for prophylaxis of tetanus. The vaccine is a tetanus toxoid adsorbate, which is well tolerated. According to recommendations of the Standing Commission for Vaccination at the Robert Koch Institute in Berlin, Germany, active immunisation (in combination with other vaccines) of all infants should be started from the age of 2 months, and then completed according to the vaccination schedule. Furthermore, vaccination is indicated in all persons without or with incomplete basic immunisation, or when the last shot of the basic vaccination or the last booster was more than 10 years ago.

Immune protection is particularly important for elderly people, especially those with circulatory disorders, diabetes mellitus and diseases of the skin surface, e.g. ulcus cruris or open eczema.

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