Anti-Diphtheria Toxoid ELISA (IgG)
Test instruction

ORDER NO.	Determination	IG CLASS	SUBSTRATE	FORMAT
EI 2040-9601 G	Diphtheria 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 diphtheria toxoid in serum or plasma for the clarification of an unknown immune status.

Application: The Anti-Diphtheria Toxoid ELISA (IgG) is based on inactivated diphtheria toxin and is designed for the quantitative determination of human IgG antibodies against diphtheria toxoid in serum or plasma. This test is suited for both investigation of 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 Diphtheria 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 2 IU/ml (human IgG), ready for use	Red coloured in decreasing intensity.	1 x 2.0 ml	CAL 1
3. Calibrator 2 1 IU/ml (human IgG), ready for use		1 x 2.0 ml	CAL 2
4. Calibrator 3 0.1 IU/ml (human IgG), ready for use		1 x 2.0 ml	CAL 3
5. Calibrator 4 0.01 IU/ml (human IgG)		1 x 2.0 ml	CAL 4
6. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
7. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
8. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
9. Sample buffer ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
10. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
11. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
12. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
13. Protective foil	---	2 pieces	FOIL
14. Test instruction	---	1 booklet	
15. Quality control certificate	---	1 protocol	

LOT Lot description
IVD In vitro diagnostic medical device
CE
Storage temperature
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 are to be disposed of according to official 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, opensystem analysis devices is possible. However, the combination should be validated by the user.

Pipetting protocol

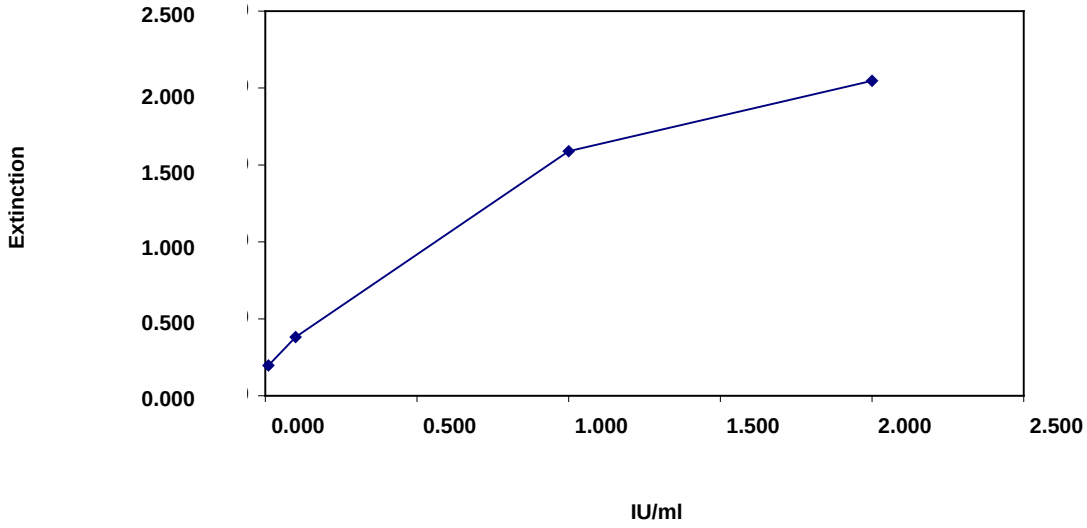
	1	2	3	4	5	6	7	8	9	10	11	12
A	**C 1**	**P 3**	**P 11**	**P 19**								
B	**C 2**	**P 4**	**P 12**	**P 20**								
C	**C 3**	**P 5**	**P 13**	**P 21**								
D	**C 4**	**P 6**	**P 14**	**P 22**								
E	**pos.**	**P 7**	**P 15**	**P 23**								
F	**neg.**	**P 8**	**P 16**	**P 24**								
G	**P 1**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

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 4), 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

Quantitative: 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 4 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 (2 IU/ml), the result should be reported as ">2 IU/ml". It is recommended that the sample be remeasured 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

Recommendations taken from literature for the evaluation of test results (3, 5, 16) and subsequent procedures (13) are summarised below:

<0.01 IU/ml	No protection (individuals without immunisation should receive two vaccinations with a time interval of 4 to 8 weeks and a third vaccination 6 to 12 months after the second one)
0.01-0.099 IU/ml	Uncertain protection
≥0.1 IU/ml	Immunisation protection present
>1.0 IU/ml	Long-term immunisation protection

According to the Standing Committee on Vaccination (STIKO) at the Robert Koch Institute recommendations (13), the determination of the anti-diphtheria toxoid titer is recommended in order to ascertain whether there is sufficient evidence of immunity or whether basic vaccination or a booster shot is required.

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

Test characteristics

Calibration: The controls of the Anti-Diphtheria Toxoid ELISA were calibrated using the international reference preparation NIBSC 10/262 (1st International Standard for Diphtheria Antitoxin Human, National Institute for Biological Standards and Control, Hertfordshire, England).

For every group of tests performed, the extinction values of the calibration sera 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 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 microplate wells were coated with inactivated Diphtheria toxin.

Linearity: The linearity of the Anti-Diphtheria Toxoid ELISA (IgG) was determined by assaying at least 4 serial dilutions of different patient samples. The Anti-Diphtheria Toxoid ELISA (IgG) is linear at least in the tested concentration range (0.06 IU/ml to 1.17 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-Diphtheria 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-Diphtheria Toxoid ELISA (IgG).

Antibodies against	n	Anti-Diphtheria Toxoid ELISA (IgG) positive
Adenovirus	12	0%
Chlamydia pneumoniae	12	0%
CMV	12	0%
EBV-CA	12	0%
Helicobacter pylori	12	0%
HSV-1	12	0%
Influenza A virus	12	0%
Influenza B virus	12	0%
Measles virus	12	0%
Mumps virus	12	0%
Mycoplasma pneumoniae	12	0%
Parainfluenza virus Pool	12	0%
Rubella virus	12	0%
RSV	12	0%
Toxoplasma gondii	12	0%
VZV	12	0%
Yersinia enterocolitica	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	1.3	2.9
2	1.1	2.9
3	1.3	4.2

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (IU/ml)**	**CV (%)**
1	1.5	4.6
2	1.4	5.6
3	1.2	9.2

Sensitivity and specificity:
Study I: 38 pre-characterised patient samples (origin: Germany; reference method: commercially available ELISA of another manufacturer) were investigated with the EUROIMMUN Anti-Diphtheria Toxoid ELISA (IgG). The sensitivity amounted 93.9%, with a specificity of 100%. Borderline results were not included in the calculation.

n = 38		ELISA of another manufacturer	
		positive	negative
EUROIMMUN Anti-Diphtheria Toxoid ELISA (IgG)	positive	31	0
	negative	2	5

Study II: 40 clinically pre-characterised patient samples (INSTAND) were investigated with the EUROIMMUN Anti-Diphtheria Toxoid ELISA (IgG).

n = 40		**INSTAND**	**EUROIMMUN Anti-Diphtheria Toxoid ELISA (IgG)**
EUROIMMUN Anti-Diphtheria Toxoid ELISA (IgG)	Long-term immunisation protection present	5	1
	Immunisation protection present	6	8
	Uncertain protection, re-vaccination required	16	18
	No protection, basic vaccination required	13	13

Reference range: Levels of anti-diphtheria toxoid antibodies were determined in a panel of healthy blood donors (n = 500, origin: Germany) using the EUROIMMUN ELISA. At a cut-off of 0.1 IU/ml, 66.8% of blood donors were anti-diphtheria toxoid positive (IgG).

Clinical significance

The diphtheria toxoid is produced by the pathogenic agent of diphtheria, corynebacterium diphteriae, which is an aerobic, immobile, gram-positive, non-sporogenic bacterium of the Actinomycetales family The cause of diphtheria, an infectious disease which is often accompanied by life-threatening complications and late effects, is not the corynebacterium diphtheria itself but the diphtheria toxoid synthesised by it. The toxoid interrupts protein biosynthesis by inhibiting the elongation factor, thus affecting the stability of the cell membrane and damaging or even destroying the infected cells.

Diphtheria can be found worldwide, more frequently in temperate climatic zones in autumn and winter. Humans are the only reservoir for the pathogen. The disease has been successfully controlled by active immunisation (toxoid vaccine) but not eradicated completely. During the fifties, as many as 4300 people still died of diphtheria in Germany. At present, 60% of Germans between the ages of 30 and 40 have insufficient protection against the disease. Due to these vaccination gaps, diphtheria continues to occur in limited areas. The success of vaccination in western industrial countries is clearly shown by figures: in 1920, the U.S. recorded around 150,000 cases of diphtheria per year, 15,000 of which were fatal. Between 1980 and 1986, only 24 cases, two of them fatal, occurred. In the territory of the former Soviet Union a strong increase in diphtheria cases was found during the first half of the nineties, with for example 50,000 infections and around 2,000 fatalities in each of the years 1994 and 1995.

Since diphtheria still occurs in many developing and some east European countries, the danger of introducing the virus is omnipresent, especially in the U.S., France and Germany.

The disease is transmitted from person to person by aerosols and smear infection, skin diphtheria also by direct contact, but rarely via contaminated objects. People who already suffer from diphtheria carry a higher risk of transmitting the pathogen than individuals who are infected but not yet ill. Contagiousness exists as long as the pathogen is present in secretions and wounds. When treated, diphtheria is only contagious for two to four days. In untreated cases the period of contagiosity is two to four weeks.

The incubation period is on average two to five days, in rare cases eight days. The disease mainly starts with a general feeling of illness, fever (up to 39°C), a sore throat, stomachache and joint pains. Symptoms of pharyngeal and laryngeal diphtheria include pharyngitis and tonsillitis with a grey-white coating (pseudomembrane), which often extends from the tonsils to the palate and the uvula, in rare cases even to the larynx, trachea and bronchi, leading to local tissue necrosis. A sweetish breath, which can be perceived from some distance, is a particular characteristic of the disease. Nasal diphtheria, which mainly occurs in infants and small children, manifests as nasal discharge. Skin diphtheria is predominantly a tropical disease. In western countries, it mainly affects risk groups, such as homeless persons, or alcohol or drug addicts. The symptoms are similar to those of other bacterial skin infections. The pathogen enters the body via skin wounds. Conjunctival diphtheria is characterised by bloody watery secretion and membrane formation at the conjunctiva. Often the cornea is also affected.

During the course of the disease, barking cough with stridor, hoarseness, paralysis of the soft palate, vomiting and lymph node swelling may develop. The toxic effect of the exotoxin secreted by the pathogen (diphtheria toxoid) causes the dreaded life-threatening toxic courses with myocarditis, paralysis of the motoric cerebral nerves and liver and kidney disorders.

An initial diagnosis of diphtheria can often be made from clinical symptoms, enabling treatment to be started immediately. For confirmation of acute diphtheria, direct detection with cultivation of the bacteria is possible, but takes several days. Microscopic diagnosis is insufficient. The identification of corynebacteria types expressing the diphtheria toxoid via detection of the toxoid-encoding gene and the 16S rRNA sequence using PCR is restricted to specialised laboratories.

The determination of human antibodies against diphtheria toxoid is relevant in evaluating an unclear immune status, serologically clarifying the anti-diphtheria toxoid immunity status and monitoring the immune response in certain basic diseases (e.g. malignoma, AIDS, haematological diseases) or therapeutic regimes (e.g. immunosuppression, cytostatic drugs, radiation treatment). Detection of the pathogen, suspected cases of diphtheria, diphtheria infections and fatal cases have been notifiable in Germany since 1st January 2001.

The most important therapeutic measure is the administration of diphtheria antitoxin, a horse immune serum. The antitoxin only neutralises the freely circulating toxin in the blood. The second measure is an antibiotic therapy to prevent the bacteria from spreading. Depending on the complications, intensive medical treatment may be necessary, e.g. timely intubation or operative removal of the pseudomembranes blocking the respiratory tract.

Although the frequency of the disease has been significantly reduced by passive immunisation using serum introduced by the physician and Nobel prize winner Emil von Behring and by active immunisation using diphtheria toxoid introduced by Gaston Ramon, diphtheria has not been eliminated, unlike e.g. smallpox. Therefore, active immunisation remains the main prophylaxis with respect to diphtheria. Only vaccination provides sufficient protection from the disease. Country-specific recommendations for vaccination should be consulted for advice about the recommended time frame and coverage of basic immunisation, booster shots and catch-up vaccinations (for example, in Germany: Standing Vaccination Committee (STIKO) at the Robert-Koch Institute). Almost 90% of children in Germany are protected from diphtheria by vaccination, whereas only a quarter of adults have protection. This also applies for the US. Adults should therefore consider a booster shot when their last vaccination against diphtheria dates back more than ten years. It must be taken into account that even immunity after recovery from the disease does not persist for life.

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