Anti-Bordetella pertussis toxin ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2050-9601 G	Bordetella pertussis toxin	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides quantitative in vitro determination of human antibodies of the immunoglobulin class IgG against Bordetella pertussis toxin in serum or plasma to support the diagnosis of Bordetella pertussis infections and whooping cough.

Application: The Anti-Bordetella pertussis toxin ELISA (IgG) is based on species-specific pertussis toxin (PT). The test is calibrated using the international WHO standard serum and is in accordance with the latest guidelines for the serological diagnosis of B. pertussis infections. Increased anti-PT IgG titers (≥100 IU/ml) are considered as proof of an acute B. pertussis infection. Titers below 40 IU/ml should be further investigated.
Recent vaccinations (<1 year) should be taken into consideration, since these can also cause high anti-PT IgG titers.

Principles of the test: The test kit contains microplate strips each with 8 break-off reagent wells coated with Bordetella pertussis toxin. 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 1 200 IU/ml (human IgG), ready for use	Red coloured in decreasing intensity.	1 x 2.0 ml	CAL 1
3. Calibrator 2 100 IU/ml (human IgG), ready for use		1 x 2.0 ml	CAL 2
4. Calibrator 3 25 IU/ml (human IgG), ready for use		1 x 2.0 ml	CAL 3
5. Calibrator 4 5 IU/ml (human IgG), ready for use		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

Updates with respect to the previous version are marked in grey.

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 and +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 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

For **quantitative analysis** incubate **calibrators 1 to 4** along with the positive and negative controls and patient samples.

(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 for **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 readings.
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 readings.
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, EUROIMMUN 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

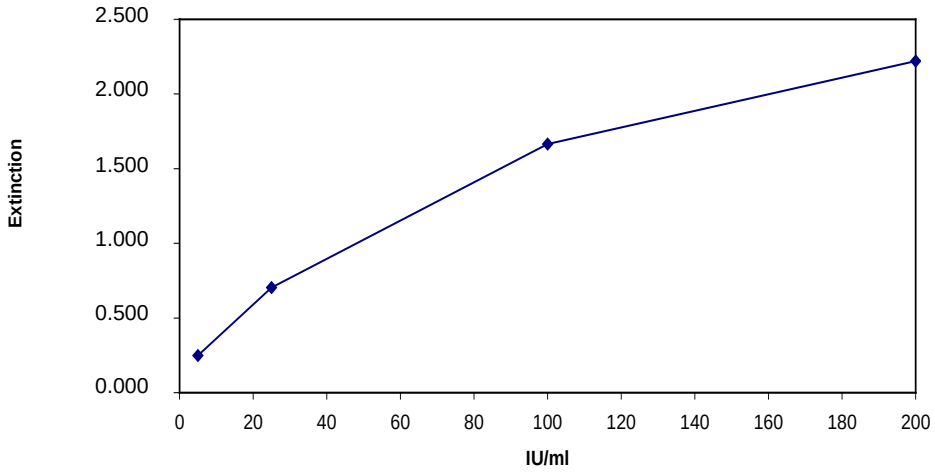
	1	2	3	4	5	6	7	8	9	10	11	12
A	**C 1**	**P 3**	**P 11**	**P 19**								
B	**C 2**	**P 4**	**P 12**	**P 20**								
C	**C 3**	**P 5**	**P 13**	**P 21**								
D	**C 4**	**P 6**	**P 14**	**P 22**								
E	**pos.**	**P 7**	**P 15**	**P 23**								
F	**neg.**	**P 8**	**P 16**	**P 24**								
G	**P 1**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

The pipetting protocol for microplate strips 1 to 4 is an example for the **quantitative analysis** of 24 patient sample (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 readings 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 extinction of calibrator 1 (corresponding to 200 IU/ml = Cal. 1), the result should be reported as ">200 IU/ml". It is recommended that the sample be retested in a new test run at a dilution of e.g. 1:404. The result in IU/ml read from the calibration curve for this sample must then be multiplied by factor 4.

Current publications and guidelines recommend the following interpretation for the evaluation of antibodies against Bordetella pertussis toxin of class IgG:

Anti-PT IgG ≥ 100 IU/ml:	Indication for an acute Bordetella pertussis infection or recent vaccination.
Anti-PT IgG < 40 IU/ml:	No indication for an acute Bordetella pertussis infection.
Anti-PT IgG ≥ 40 - <100 IU/ml:	The results should be verified by further examination or analysis of a second blood sample taken after 7 to 10 days.

For the evaluation of antibodies against Bordetella pertussis toxin of class IgA, no standardised reference values are described. In literature references, the lower detection limit of 12 IU/ml is recommended for the interpretation of anti-PT-IgA antibodies (Riffelmann et al., J. Clin. Microbiol., Vol.48, 2010):

Anti-PT IgA ≥ 12 IU/ml: Indication for an acute Bordetella pertussis infection or recent vaccination.
Anti-PT IgG < 12 IU/ml: No indication for an acute Bordetella pertussis infection.

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.

For diagnosis the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Calibration: The controls of the Anti-Bordetella pertussis toxin ELISA were calibrated using the first International WHO Standard (WHO International Standard Pertussis Antiserum, human, 1st IS NIBSC Code 06/140). Quantification was performed in IU/ml. Additionally, the international reference preparation of the Food and Drug Administration, Bethesda, USA, FDA was used. The results can be given in IU/ml or in FDA-U/ml because the values are identical.

For every group of tests performed, the extinction readings 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 the incubation steps, the greater will be the extinction. 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 Bordetella pertussis toxin antigen.

Antigen	Significance and characteristics
Pertussis toxin (PT) (EI 2050-9601 A or G)	• Anti-PT antibodies are produced specifically after an infection with Bordetella pertussis • Anti-PT antibodies are detectable both after infection and vaccination (PT is contained in many acellular vaccines)
Filamentous haemagglutinin (FHA) (EI 2050-9601-3 A or G)	• Anti-FHA antibodies can be produced after infections with B. pertussis and with B. parapertussis • Anti-FHA antibodies are detectable both after infection and vaccination (FHA is contained in many acellular vaccines)

Linearity: The linearity of the Anti-Bordetella pertussis toxin ELISA (IgG) was determined by assaying at least 4 serial dilutions of different patient samples. The Anti-Bordetella pertussis toxin ELISA (IgG) is linear at least in the tested concentration range (5 IU/ml to 174 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-Bordetella pertussis toxin ELISA (IgG) is 0.2 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-Bordetella pertussis toxin ELISA (IgG).

Antibodies against	n	Anti-Bordetella pertussis toxin 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 virus A	12	0%
Influenza virus B	12	0%
Measles	12	0%
Mumps	12	0%
Mycoplasma pneumoniae	12	0%
Parainfluenza virus Pool	12	0%
Parvovirus B19	12	0%
RSV	12	0%
Rubella virus	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	33	3.3
2	77	1.8
3	115	2,6

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (IU/ml)**	**CV (%)**
1	29	5.5
2	75	5.4
3	113	6.9

Sensitivity and specificity: 92 pre-characterised patient samples (origin: Europe; reference method: commercially available ELISA of another manufacturer) were investigated with the EUROIMMUN Anti-Bordetella pertussis toxin ELISA (IgG). The sensitivity amounted to 95.5%, with a specifity of 100%. Borderline results were not included in the calculation.

n = 92		**ELISA of another manufacturer**		
		positive	borderline	negative
EUROIMMUN Anti-Bordetella pertussis toxin ELISA (IgG)	positive	16	6	3
	borderline	0	0	0
	negative	0	3	64

73 clinically pre-characterised patient samples (INSTAND, Labquality) were investigated with the EUROIMMUN Anti-Bordetella pertussis toxin ELISA (IgG). The sensitivity amounted to 97.8%, with a specificity of 100%. Borderline results were not included in the calculation.

n = 73		**INSTAND/LABQUALITY**		
		positive	borderline	negative
EUROIMMUN Anti-Bordetella pertussis toxin ELISA (IgG)	positive	45	0	0
	borderline	0	0	0
	negative	1	3	24

Reference range: The levels of the anti-Bordetella pertussis toxin antibodies (IgG) were analysed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. 14.8% of the blood donors showed values about 38 IU/ml, wich reflects the known percentage of infections in adults.

Clinical significance

The genus Bordetella (B.) encompasses four known species: B. pertussis, which causes whooping cough, B. parapertussis, which causes a mild whooping cough syndrome, B. bronchiseptica and B. avium. They are distributed worldwide, are highly contagious and are transmitted from person to person by droplet infection.

Pathogenesis: The bacteria bind via various adhesins to the cells of the ciliated epithelium without reaching the epithelium or bloodstream. Besides the capsule, which protects the pathogen from inactivation by complement, there are functionally two groups of virulence factors: Adhesins and toxins:
The two most important adherence factors are filament haemagglutinin (FHA) and pertussis toxin (PT), which can function as an exotoxin or as an adhesin. In contrast to B. pertussis, B. parapertussis does not produce pertussis toxin. B. pertussis and B. parapertussis also possess on their outer membranes the membrane toxins pertactin and BrkA (Bordetella resistance to killing), which play a role in attachment to the host cell.

Further virulence factors are adenylate cyclase toxin, which affects the immune response of the host, and tracheal cytotoxin (TCT) formed from the cell wall peptidoglycan, which causes stasis of cilia movement. The clinical progression of whooping cough depends mainly on the formation of various virulence factors by the pathogen.

After an incubation time of around 7 to 14 days, **Bordetella pertussis infections** begin with an uncharacteristic catarrhal stage, which lasts for about 1 to 2 weeks. Then the convulsive stage develops, lasting for 2 to 3 weeks with typical paroxysmal, staccato coughing attacks, frequently followed by stridor with possible vomiting. Nocturnal attacks are frequent. During both of these stages the pathogen is coughed out. Transmission via contaminated objects cannot be excluded. Following this is the decrimenti stage, which lasts for several weeks, with continual diminishment of coughing attacks.

Mainly in the case of children under the age of 2 years, complications such as secondary pneumonia or otitis media are frequent. There is no difference in morbidity between boys and girls. Season and climate have no influence on the frequency of the disease. An infection confers specific immunity, which reduces after decades. Infections in adulthood are known, but are seldom diagnosed. Reinfections in persons older than 60 years are life-threatening.

Prevention: In Germany, the Standing Commission for Vaccination (STIKO) recommends vaccination at the ages of 2, 3 and 4 months, further vaccination at the age of 11 to 14 months, and booster vaccinations in pre-school and adolescent ages. Furthermore, vaccination of adults, particularly the elderly, with acellular pertussis antigens is indicated (in combination with diphtheria, polio and tetanus vaccines). This “booster” not only protects the individual, but also protects unvaccinated children from potentially becoming infected.

The influence of pertussis vaccination on infections with B. parapertussis is not yet fully understood. Neither whole cell lysates of B. pertussis nor mixtures of different antigens in acellular vaccines appear to protect against infection with B. parapertussis.

Detection of Bordetella pertussis: The identification of cultured bacteria can be achieved microscopically by direct immunofluorescence or by nucleic acid amplification (e.g. PCR) within the first 2 to 3 weeks of illness. Specific antibodies can be detected in serum by IFT, blot methods or ELISA.

ELISA based on pertussis toxin (PT) are recommended for the specific detection of antibodies against Bordetella pertussis, since they allow exclusion of a parapertussis infection and also quantification of the antibody titer. When FHA is used as the antigen, both B. pertussis and B parapertussis infections can be sensitively detected. FHA occurs in all Bordetella species, but also in other bacteria such as Mycoplasma.

The use of an antigen mix (FHA and PT) in ELISA is not recommended. The concentration of Bordetella antibodies should be given in international units (IU/ml), since a WHO reference preparation is available.

The significance of IgA and IgM antibodies is not definitively clarified, since, for example, IgA responses are also induced by vaccination. IgM responses are directed mainly against LPS and fimbriae, which causes problems due to the lack of specificity.

An immune response following vaccination cannot be distinguished from one following infection. The interpretation of results is therefore unreliable for around one year after immunisation with acellular vaccines. The relevance of antibodies against pertactin, which is used in acellular vaccines together with PT and FHA, is being widely discussed in literature. So far, there is no confirmed serological marker for determination of the immunity status. However, various studies showed that antibodies against pertactin have a protective effect against infections with B. pertussis.

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