Anti-Helicobacter pylori ELISA (IgA)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EI 2080-9601 A	Helicobacter pylori	IgA	Ag-coated microplate wells	96 x 01 (96)

Indication: Infection with Helicobacter pylori, chronic active gastritis (antral gastritis), peptic ulcer, duodenal ulcer.

Principles of the test: The ELISA test kit provides a semiquantitative in vitro assay for human antibodies of the IgA class against Helicobacter pylori in serum or plasma. The test kit contains micro-titer strips each with 8 break-off reagent wells coated with Helicobacter pylori antigens. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgA antibodies (also IgG and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgA (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 (IgA, human), ready for use	dark red	1 x 2.0 ml	CAL
3. Positive control (IgA, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
4. Negative control (IgA, human), ready for use	colourless	1 x 2.0 ml	NEG CONTROL
5. Enzyme conjugate peroxidase-labelled anti-human IgA (rabbit), ready for use	orange	1 x 12 ml	CONJUGATE
6. Sample buffer ready for use	light blue	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 — CE — 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 be 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.

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 crystallization 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 deionized 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.

Warning: Calibrators and controls used have been tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays and indirect immunofluorescence methods. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the toxic agent sodium azide. 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 in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

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

Incubation

(Partly) manual test performance

Sample incubation: (1st step)
Transfer 100 µl 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 reagent wells 3 times with 450 µl working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Modus").

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) remaining 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.
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 IgA) 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 the reaction:
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 the 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 inquiry.
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 minimizes 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. Values above the indicated cut-off are to be considered as positive, those below as negative.

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 calibrator. Use the following formula to calculate the ratio:

$$\frac{\textbf{Extinction of the control or patient sample}}{\textbf{Extinction of calibrator}} = \textbf{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <0.8:	**negative**
Ratio ≥0.8 to <1.1:	**borderline**
Ratio ≥1.1:	**positive**

In cases of borderline test results, an additional patient sample should be taken 7 days later and re-tested in parallel with the first patient sample. The results of both samples allow proper evaluation of titer changes.

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.

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

Test characteristics

Calibration: As no international reference serum exists for antibodies of against Helicobacter pylori, results are provided in the form of ratios which are a relative measure for the concentration of antibodies.

For every group of tests performed, the extinction values of the calibrator and the ratios of 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 activity of the enzyme used is temperature-dependent and the extinction values may vary if a thermostat is not used. The higher the room temperature during substrate incubation, 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 antigen source is a bacterial lysate from the Helicobacter pylori strain "ATCC43504".

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 detection limit of the Anti-Helicobacter pylori ELISA (IgA) is ratio 0.1.

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-Helicobacter pylori ELISA (IgA).

Antibodies against	n	Anti-Helicobacter pylori ELISA (IgA)
Adenovirus	10	0%
Bordetella FHA	10	0%
Bordetella pertussis	10	0%
Brucella abortus	5	0%
Chlamydia pneumoniae	10	0%
Chlamydia trachomatis	10	0%
EBV-CA	10	0%
HSV Pool	10	0%
Influenza virus A	10	0%
Influenza virus B	10	0%
Legionellen pneumoniae	10	0%
Mycoplasma pneumoniae	10	0%
Parainfluenza virus Pool	10	0%
RSV	10	0%
Toxoplasma gondii	10	0%
VZV	10	0%
Yersinia enterocolitica	10	0%

Interference: Haemolytic, lipaemic and icteric samples showed no influence at the result up to a concentration of 10 mg/ml for hemoglobin, 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 using 3 sera. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed on 6 different days.

Intra-assay variation, n = 20		
Serum	**Mean value (Ratio)**	**CV (%)**
1	1.2	9.0
2	1.7	3.9
3	1.9	7.0

Inte-assay variation, n = 4 x 6		
Serum	**Mean value (Ratio)**	**CV (%)**
1	1.2	6.8
2	1.5	5.8
3	1.9	6.7

Specificity and sensitivity: 59 pre-characterized patient sera (INSTAND, quality assessment, Germany) were examined with the EUROIMMUN Anti-Helicobacter pylori ELISA (IgA). The specificity was 97.0% and the sensitivity 92.9%.

n = 59		**INSTAND**		
		positive	borderline	negative
EUROIMMUN Anti-Helicobacter-pylori-ELISA (IgA)	positive	13	1	1
	borderline	3	0	2
	negative	1	6	32

Correlation of the ELISA with an Helicobacter Urease Test: Sera from 26 patients whose bioptical material was positive in an Helicobacter Urease Test were investigated with the EUROIMMUN Anti-Helicobacter pylori ELISA (IgA and IgG).

n = 26	**ELISA positive**		
	IgA	IgG	IgA or IgG
Helicobacter Urease Test positive	65%	100%	100%

Reference range: The levels of the anti-Helicobacter pylori antibodies (IgA) were analyzed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. With a cut-off ratio of 1.0, 19.0% of the blood donors were anti-Helicobacter pylori (IgA) positive.

Clinical significance

Helicobacter pylori (synonyms: Campylobacter pylori and C. pyloridis) is the causative agent of the second most common bacterial infectious disease in humans. It takes a mostly chronic course, with a world-wide annual death rate of 700,000 cases through stomach cancer, and affects men three times more frequently than women. Helicobacter pylori was discovered in 1983 by B. Marshall and J. Robin Warren of Perth, Western Australia. They also succeeded in cultivating Helicobacter pylori from the stomach mucous membranes of patients with chronic gastritis.

Helicobacter pylori, the only globally occurring human-pathogenic species of the genus Helicobacter, is a gram-negative bacterium of spiral form with an extremely high urease production and lives intra-cellularly on the luminal side of the epithelial cells of the stomach mucous membrane. Two morpho-logical forms of the bacterium exist; a spiral form proven to be infectious, and a long-lived, coccoid form.

Humans and animals shed the bacteria in their faeces. H. pylori has the ability to survive in water. With an average world-wide prevalence of 50%, the infection rate in developing countries is considerably higher (up to 70%) than in industrial countries. In Germany, a total of about 33 million persons are infected with H. pylori, of whom about 10 - 20% develop a peptic ulcer. Antibodies to H. pylori occur in about 70% of patients with chronic active gastritis, and in 60 to 90% of cases are associated with ulcerous conditions.

Biopsies mainly show a colonization in the mucous membrane of the antrum. In rare cases, H. pylori can be found in the corpus mucous membranes or, in cases of gastric metaplasia, in the duodenum. H. pylori infections do not heal spontaneously. The agent can persist life-long. H. pylori are considered to be the causative agent of type B chronic gastritis. The clinical manifestation is dyspepsia with corresponding pain in the upper abdomen. Histologically, a superficial gastritis with atrophy of the mucous membranes might be visible. However, the large majority of infections take a clinically asympto-matic course.

With 1.65 million base pairs, the relatively small genome of H. pylori codes for 1,500 proteins. Some of these are special virulence factors and adhesion proteins which are characteristic for the individual H. pylori strains. Isolates of Helicobacter pylori can be broadly divided into two types, the spiral strain of type I, which can express the specific antigen protein p95 (VacA vacuolating cytotoxin) and its associated protein p120 (CagA – cytotoxin associated antigen), as well as H. pylori strains of type II, which do not synthesise these proteins. Other, less toxic H. pylori proteins are, for example, protein p33, protein p30 (OMP, outer membrane protein) and protein p19 (OMP). As the CagA protein itself can be involved in the genesis of the tumour, it is also referred to as a bacterial oncoprotein. Alongside gastritis and the formation of ulcers, the clinical symptoms include possible late consequences, such as MALT lymphomas and adenocarcinomas. Infections with the type I agent seem to be associated with a higher pathogenicity.

The direct detection of H. pylori involves taking samples from the lower third of the stomach; detection is then possible in the subsequent investigation by microscopy. The sample can also be tested for the presence of urease, and therefore indirectly for the presence of H. pylori. In the case of a recent infection, it is also possible to determine H. pylori in the faeces. H. pylori can be detected indirectly with a high degree of probability using a breath test, but this requires special laboratory equipment.

After contact with Helicobacter pylori, IgA, IgG and IgM antibodies to Helicobacter pylori can appear in the serum:

IgA antibody titres are mostly found after a few weeks and can still be detected over a considerable period. A positive IgA result correlates well with the activity of the gastritis. IgA antibodies are formed locally, but are not detected in the serum in every case.
IgG antibody titres frequently cannot be found until after the IgM titre has fallen. An increase in the serum IgG titre is an indication of an ongoing infection. IgG antibody titres can persist over many years. Elevated IgG antibody titres are considered to be a marker for a chronic infection.
IgM antibodies are found within a few days after contact with Helicobacter pylori. Specific IgM antibodies are no longer found after just a few weeks.

The persistence of H. pylori promotes relapses through recolonization by residual agents from the crypts of the mucous membrane. A complete and permanent eradication of the bacteria in a diagnosed H. pylori infection in children, adolescents and adults leads to a reduction in the recurrence rate of 80% in the case of peptic ulcers and 20% in duodenal ulcers. The test for specific IgG antibodies against Helicobacter pylori is a suitable indicator for the complete eradication of the agent as a part of therapy monitoring. A significant fall in the IgG antibody titre after about six weeks of treatment is a sign of success.

Table: **Test characteristics**
of the recommended methods for the diagnosis of an H. pylori infection

Invasive methods (requiring gastroscopy)	**Sensitivity (%)**	**Specificity (%)**
Culture	70-90	100
Histology	80-98	90-98
Urease rapid test	90-95	90-95
PCR	90-95	90-95
Non-invasive methods (not requiring gastroscopy)		
Urease Breath Test (UBT)	85-95	85-95
Stool antigen test (using only monoclonal antibodies)	85-95	85-95
IgG antibody detection in serum	70-90	70-90

In comparison with other test methods, serological tests are less stressful for the patient. It must also be considered when performing and interpreting the various test methods that a low colonization density (even if only transient) can lead to false-negative results in the UBT, in the stool antigen test and in the culture or histology, with the exception of serology. Low colonization densities mainly occur under treatment with protein pump inhibitors (PPI), antibiotics, after a partial gastric resection, in cases of mucous membrane atrophy as well as in stomach cancers and MALT. The guidelines therefore recommend that for reliable H. pylori diagnostics, the following minimum times should be observed without H. pylori-suppressive treatment: two weeks after termination of PPI treatment and four weeks after a preceding eradication or other antibiotic treatment.

Hence, the serological investigation of antibodies to Helicobacter pylori using the Anti-Helicobacter pylori ELISA (IgA, IgG, IgM), the Anti-Helicobacter pylori IIFT (IgA, IgG or IgM) or the Anti-Helicobacter pylori EUROLINE-WB (IgA, IgG), by means of which cytotoxins can be clearly differentiated from other disease-relevant proteins, represents the diagnostically most reliable and inexpensive method for the diagnosis of Helicobacter infections and for confirming the success of treatment after eradication, and is much less stressful for the patient, particularly for children.

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