Anti-Helicobacter pylori CagA ELISA (IgA)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EI 2081-9601 A	Helicobacter pylori CagA antigen	IgA	Ag-coated microplate wells	96 x 01 (96)

Principles of the test: The ELISA test kit provides a semiquantitative in vitro assay for human antibodies of the IgA class against Helicobacter pylori CagA antigen in serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with recombinant Helicobacter pylori CagA antigen. 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), which is capable of promoting 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	green	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. Protocol with target values	---	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 the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips shoud 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) around 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 a minimum of 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 microplatestrip: 5 ml concentrate plus 45 ml water.
  The ready-to-use diluted wash buffer is stable for 1 month 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: The controls and calibrators used have 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 contact with the skin.

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** 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).

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

Incubation

Sample incubation: (1. 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 400 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 falsely 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 falsely 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: (2. 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: (3. 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 of 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	**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 results, an additional patient sample should be taken 7 days later and retested in parallel with the first patient sample. The result 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 alongside the serological results.

Test characteristics

Calibration: As no international reference serum exists for antibodies against Helicobacter pylori CagA antigen, results are evaluated in the form of ratios which are a relative measurement of the antibody concentration in serum or plasma.

For every group of tests performed, the extinction values of the calibrator and ratios of the positive and negative controls must lie within the limits stated for the relevant test kit lot. A protocol containing these target 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 microplate wells were coated with recombinant Helicobacter pylori CagA antigen. The corresponding cDNA was expressed in E. coli as a full length protein.

Detection limit: The detection limit is defined as a value of three times the standard deviation of an analyte-free sample and is the smallest detectable antibody titer. The detection limit of the Anti- Helicobacter pylori CagA ELISA (IgA) is approximately ratio 0.03.

Cross reactivity: This ELISA showed no cross reactivity.

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 (CV) 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	2.0	8.3
2	2.8	9.9
3	3.7	4.6

Inter-Assay Variation, n = 4 x 6		
Serum	**Mean value (Ratio)**	**CV (%)**
1	1.8	7.4
2	2.4	7.3
3	3.1	8.4

Specificity and sensitvity: 15 uncharacterized sera were investigated with the EUROIMMUN Anti-Helicobacter pylori CagA ELISA (IgA) and the EUROIMMUN Anti-Helicobacter pylori Westernblot (IgA) was used as a reference. The test showed a specificity and a sensitivity of 100% each with regard to the Westernblot.

n = 15		**Westernblot: anti-CagA**		
		positive	borderline	negative
ELISA	positive	10	0	0
	negative	0	0	5

Reference range: The levels of anti-Helicobacter pylori CagA antibodies (IgA) were analyzed with this EUROIMMUN ELISA in a panel of 300 healthy blood donors. With a cut-off ratio of 1.0, 19.5% of the blood donors were anti-Helicobacter pylori CagA positive (IgA).

Clinical significance

Helicobacter pylori (synonyms Campylobacter pyloris or C. pyloridis) was first cultured from the stomach mucosa of patients with chronic gastritis in 1982. The presence of bacteria in the stomach mucosa has been known for about 100 years and a connection with gastritis and stomach ulcers has been suspected since the forties.

Today, Helicobacter pylori is considered the etiological agent of chronic gastritis type B. Biopsies show mainly colonization of the antrum mucosa, while the bacterium is rarely detected in the corpus mucosa or the duodenum. Diseases associated with a H. pylori infection include, in addition to gastritis, stomach and duodenum ulcers and MALT lymphoma of the stomach. Infections are also associated with an increased risk of stomach adenocarcinoma. The majority of infections proceed clinically inapparently. H. pylori infections do not heal spontaneously and the pathogen can persist lifelong. Recurrent infections result from recolonization by pathogens persisting in the mucous membrane cryptae. Full and lasting eradication of the bacteria in diagnosed H. pylori infections reduces recurrence by 80% for peptic ulcers and 20% for duodenal ulcers. 50% of people worldwide are infected with H. pylori, and epidemiologically, an increase in infections with age has been determined.

Helicobacter pylori is a gram-negative, spiral-shaped bacterium with unusually strong urease production. It colonizes the epithelial cells on the luminal side of the stomach mucosa intercellularly. The bacterium appears in two forms: a bent spiral form which is proven to be infectious and a long-lived coccoid form. H. pylori is the only human pathogen of the genus Helicobacter and it is found worldwide. Clinical isolates of H. pylori can be divided into two types: H. pylori strains of type I can express a specific vacuolating cytotoxin (VacA) and an associated protein (CagA). Strains which are not capable of synthesising these proteins are classified as type II.

Infections with type I pathogens appear to be associated with higher pathogenicity: 60% of patients with gastritis have type I infections, while in patients with duodenal ulcers this can be up to 90%. In this respect, an infection with CagA-positive H. pylori strains can increase the risk of developing carcinoma three to six fold. Therefore, infections with type 1 pathogens (CagA positive) and type II pathogens (CagA negative) need to be differentiated. Since the CagA protein is highly immunogenic, non-invasive serological detection of antibodies against CagA is highly suitable for differentiating between the two subtypes.

Following contact with H. pylori, antibodies of classes IgA, IgG and IgM against the pathogen appear in the serum. The specific IgM disappear after a week, while antibodies of class IgA remain detectable for a longer time period. Increased IgG titers are often found only after the IgM titer has decreased, and they can persist for years. Antibodies of class IgA are formed locally and are not always detectable in patient serum. Positive IgA results correlate well with gastritis activity, while increased IgG antibody titers are considered a marker for chronic infection.

Antibodies against H. pylori occur in 70% of patients with chronic active gastritis, and in 60-90% of cases they are associated with ulcerous complaints. 80% of stomach and intestinal ulcers heal after consequent therapy with suitable antibiotics. The determination of specific IgG antibodies against H. pylori is suitable for demonstrating complete eradication of the pathogen. A significant redution in the IgG titer around 6 weeks after treatment shows that the therapy has been successful.

Reference list

1. Barthel JS, Everett ED. **Diagnosis of Campylobacter pylori Infections: The "Gold Standard" and the Alternatives.** Rev. Infect. Dis. 12 (Suppl. 2): 107-114 (1990).
2. Kuipers EJ, Schenk BE, Meuwissen SGM. **Helicobacter pylori: who is positive and who not?** Europ. J. Gastroent. Hepatol. 7: 533-536 (1995).
3. Zöller L, Sobe D, Faulde M. **Nachweis von Helicobacter pylori-Infektion: Rolle der Immundiagnostik.** Klin. Lab. 39: 45-54 (1993).
4. Mobley HLT, Hu LT, Foxall PA. **Helicobacter pylori Urease: Properties and Role in Pathogenesis.** Scand. J. Gastroent. 26 (Suppl. 187): 39-46 (1991).
5. Bölin I, Lönroth H, Svennerholm A-M. **Identification of Helicobacter pylori by Immunological Dot Blot Method Based on Reaction of a Species-Specific Monoclonal Antibody with a Surface-Exposed Protein.** J. Clin. Microbiol. 33: 381-384. (1995).
6. Faulde M, Cremer J, Zöller l. **Humoral immune response against Helicobacter pylori as determined by immunoblot.** Electrophoresis 14: 945-951 (1993).
7. Dunn BE. **Proteins, antigens, and typing methods for Helicobacter pylori.** In Helicobacter pylori: Biology and Clinical Practice. 191-207. Edited by C.S. Goodwin and B.W. Worsley. CRC Press (1993).
8. Blaser MJ, Perez-Perez GI, Kleanthous H et al. **Infection with *Helicobacter pylori* strains possessing CagA is associated with an increased risk of developing adenocarcinoma of the stomach.** Cancer Res. 55: 2111-2115 (1995)
9. Parsonnet J, Friedmann GD, Orentreich N, Vogelman H. **Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection.** Gut 40: 297-301 (1997)
10. Demirturk L, Ozel AM, Yazgan Y, Solmazgul E, Yildirim S, Gultepe M, Gutbuz AK. **CagA status in dyspeptic patients with and without peptic ulcer disease in Turkey: association with histopathologic findings.** Helicobacter 6: 163-168 (2001)