Anti-CCP ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	Ig CLASS	SUBSTRATE	FORMAT
EA 1505-9601 G	cyclic citrullinated peptides (CCP)	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: Rheumatoid arthritis

Principle of the test: The ELISA test kit provides a semi-quantitative or quantitative in vitro assay for the determination of human autoantibodies of the IgG class against cyclic citrullinated peptides (CCP). The test kit contains microtiter strips each with 8 individual break-off reagent wells coated with synthetic cyclic citrullinated peptides. In the first reaction step, diluted patient samples (serum or EDTA, heparin or citrate plasma) are incubated in the wells. In the case of positive samples, the specific IgG antibodies (also IgA and IgM) will bind to the corresponding antigenic site. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate), which is capable of promoting a colour reaction.

Contents of the test kit:

Description	Colour	Format	Symbol
1. Microplate wells coated with antigens coated with antigens: 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 x 8	STRIPS
2. Calibrators 1 to 5 1, 5, 20, 100, 200 RU/ml (IgG, human), ready for use	red	5 x 2.0 ml	CAL 1-5
3. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
4. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
5. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
6. Sample buffer ready for use	violet	1 x 100 ml	SAMPLEBUFFER
7. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASHBUFFER 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 reference values	---	1 protocol	
LOT Lot description		CE	⚡ Storage temperature
IVD In vitro determination			⌛ 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 should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Preparation and stability of reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. Unless stated otherwise, the reagents after initial opening are stable until the expiry date when stored between +2°C and 8°C and protected from contamination.

- **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.
  Note: The sample buffer provided in this test kit must only be used together with the Anti-CCP ELISA.

- **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 amount 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.
  Example: For 1 microplate strip use 5 ml concentrate plus 45 ml water.
  The ready-to-use diluted 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 bottle immediately after use, as the contents are sensitive to light. The 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 are contain the toxic agent sodium azide. Avoid contact with the skin.

Preparation and stability of the serum or plasma samples

Samples: Human serum or EDTA, heparin or citrate plasma. Do not use heat-inactivated samples, as they can lead to false positive results.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples must be incubated within one working day.

Sample dilution: Patient samples are diluted to be investigated are diluted **1:101** with sample buffer.
Example: Add 10 µl of serum to 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

Note: Calibrators and controls are prediluted and ready for use, do not dilute them.

Incubation

For **qualitative/semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1 to 5** along with the positive and negative controls and patient samples.

(Partly) manual test performance

Sample incubation: (1. 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. The pipetting should not take longer than 15 minutes. Incubate for **60 minutes** at room temperature (+18°C to 25°C).
Wash:	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 of working strength wash buffer (programme 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 <u>and</u> 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. Attention: 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 (2. 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).
Wash:	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 **30 minutes** at room temperature (+18°C to 25°C), protect from direct sunlight.
Stop:	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, carefully 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 and the Analyzer I-2P 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 2	P 6	P 14	P 22			C 1	P 2	P 10	P 18		
B	pos.	P 7	P 15	P 23			C 2	P 3	P 11	P 19		
C	neg.	P 8	P 16	P 24			C 3	P 4	P 12	P 20		
D	P 1	P 9	P 17	P 25			C 4	P 5	P 13	P 21		
E	P 2	P 10	P 18				C 5	P 6	P 14	P 22		
F	P 3	P 11	P 19				pos.	P 7	P 15	P 23		
G	P 4	P 12	P 20				neg.	P 8	P 16	P 24		
H	P 5	P 13	P 21				P 1	P 9	P 17	P 25		

The pipetting protocol for microtiter strips 1-4 is an example for the **qualitative/semiquantitative analysis** of 25 patient sera (P 1 to P 25).
The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 25 patient sera (P 1 to P 25).

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 further improved by duplicate determinations for each sample. 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

Qualitative/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 2. Calculate the ratio according to the following formula:

$$\frac{\textbf{Extinction of the control or patient sample}}{\textbf{Extinction of calibrator 2}} = \textbf{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio ≤1.0: negative
Ratio >1.0: positive

Quantitative: The standard curve from which the concentration of anti-CCP antibodies in the serum samples can be taken is obtained by plotting the extinction values measured for the 5 calibration sera (linear, y-axis) against the corresponding concentrations (logarithmic, x-axis). The standard curve can be calculated by computer using one of the following curve-fitting techniques: 4-parameter logistic, 5-parameter logistic, spline fits, log/logit curve and lim/limit curve. 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.
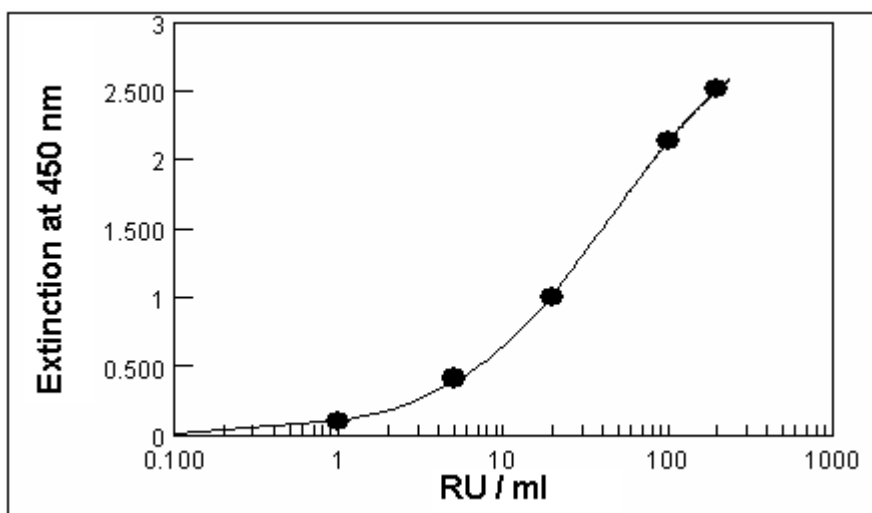
If the extinction for a patient sample lies above that of calibrator 5 (200 RU/ml), the result should be reported as ">200 RU/ml". It is recommended that the sample be remeasured in a new test run at a dilution of e.g. 1:400. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4. The upper limit of the normal range (cut-off) recommended by EUROIMMUN is 5 relative units (RU) /ml. EUROIMMUN recommends interpreting results as follows:

≤5 RU/ml: **negative**
>5 RU/ml: **positive**

The recommendation is based on data yielded in a ROC analysis using the results of 419 samples of patients with rheumatoid arthritis and 1144 control samples. According to the analysis, the specificity was 98% at a cut-off of 4.2 RU/ml. The 99th percentile based on 400 healthy blood donors was also 4.2 RU/ml (q.v. respective paragraphs under "Test characteristics").

Please note that 11 of 21 (52%) positive results from the control panel samples (n = 1144) but only 16 of 329 (5%) positive results in patients with rheumatoid arthritis ranged between 5 RU/ml and 10 RU/ml. Results from a weak positive range of 5 RU/ml to 10 RU/ml should be interpreted with prudence and possibly verified with a new patient sample taken several weeks later.

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 CCP, the calibration is performed in relative units (RU/ml).

For every group of tests performed, the relative units or ratio values determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A protocol 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 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 reagent wells are coated with synthetic cyclic citrullinated peptides (CCP) which contains modified arginine residues.

Linearity: The linearity of the ELISA was determined by assaying serial dilutions of 6 serum samples. The average concordance of dilution-factor-corrected results for the serum samples amounted to 103% (86% – 125%). The Anti-CCP ELISA is linear in the tested concentration range (3 RU/ml to 196 RU/ml).

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 lower detection limit of the Anti-CCP ELISA is 0.3 RU/ml.

Cross reactivity: The ELISA presented here specifically detects IgG class antibodies directed against CCP. There were no cross reactions with other autoantibodies in samples of patients with the following diseases: SLE (n = 6), Scleroderma (n = 5), Sjögren's syndrome (n = 5) and IgM rheumatoid factor-positive rheumatoid arthritis (n = 10).

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 using 4 sera. 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		
Serum	Mean value (RU/ml)	CV (%)
1	18	5.9
2	20	4.0
3	26	3.6
4	52	3.4

Inter-assay variation, n = 4 x 6		
Serum	Mean value (RU/ml)	CV (%)
1	19	6.3
2	23	6.5
3	35	7.2
4	63	6.8

Clinical sensitivity and specificity: Sera from 419 patients with rheumatoid arthritis, a control panel of 744 patients with other diseases and 400 healthy blood donors were analysed using the EUROIMMUN Anti-CCP ELISA. The sensitivity of the ELISA for rheumatoid arthritis was 78.5% with a specificity of 98.2%.

Panel	Anti-CCP ELISA	
	n	**positive**
Sensitivity for rheumatoid arthritis	**419**	**329 (78.5%)**
Asymptomatic blood donors	400	2 (0.5%)
Psoriatic arthritis	28	0
Other arthritides	35	3 (8.6%)
SLE	108	3 (2.8%)
Sjögren's syndrome	106	2 (1.9%)
Scleroderma	98	3 (3.1%)
Autoimmune thyroiditis	159	4 (2.5%)
Wegener's granulomatosis	25	1 (4.0%)
Anti-parvovirus B19-positive	126	3 (2.4%)
Viral hepatitides	54	0
Anti-HIV-positive	5	0
Specificity for rheumatoid arthritis	**1144**	**21 (98.2%)**

In the case of anti-CCP positive results in the control panel it can neither be excluded that the patients have rheumatoid arthritis in addition to the underlying illness nor that they are at an early, symptom-free stage of RA. Various studies have shown that a majority of anti-CCP positive test persons without characteristic RA symptoms developed RA within a few years after the serological analysis.

In a ROC analysis of the results of 419 RA patients samples and 1144 control samples listed in the above table the following characteristics were determined:

cut-off	specificity	sensitivity
2.6 RU/ml	95.0%	81.4%
4.2 RU/ml	98.0%	79.0%
8.0 RU/ml	99.0%	75.4%

Reference range: Levels of anti-CCP antibodies were analysed in 400 sera from healthy blood donors of between 18 and 68 years of age (149 women, 251 men) using the EUROIMMUN ELISA. No differences with respect to age or gender were observed. The mean concentration of antibodies against CCP was 1.2 RU/ml (± 0.8 RU/ml of standard deviation) and the values ranged from 0.2 to 8.0 RU/ml. With a cut-off of 5 RU/ml, 0.5% of the blood donors were anti-CCP positive.

cut-off	percentile
2.6 RU/ml	95.0%
3.3 RU/ml	98.0%
4.2 RU/ml	99.0%

Clinical significance

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases and also the most frequent chronic inflammatory arthropathy. The disease affects around 1% of the world population, 75% of which are female. It is characterised by inflammation of the synovial membrane, which spreads symmetrically from the small to large joints leading to the destruction of the joints in the late phase accompanied by a systemic involvement of the soft tissue. Initial symptoms include painful swelling of the metacarpophalangeal joints with morning stiffness in the joints [1]. Reliable and earliest possible diagnosis is indispensable to keep the disease under control with suitable therapy and to avoid irreversible joint damage [2, 3, 4, 5].

The most commonly performed serological test in suspected RA cases was until now the determination of rheumatoid factors (RF) in addition to general inflammatory parameters. RF are antibodies (predominantly of class IgM) which react with gamma globulins and occur in 60-80% of RA patients. RF are a sensitive, but not very specific marker for RA since they also occur in healthy individuals, e.g. in various infections or other autoimmune diseases such as systemic lupus erythematosus, Sjögren's syndrome and scleroderma [6, 7, 8, 9].

40-60% of RA patients also exhibit autoantibodies against epidermal filaggrin (RA keratin, anti-perinuclear factor) in their serum. Filaggrin is a protein of the epidermis, which links keratin filaments to one another. Autoantibodies against filaggrin are detected by indirect immunofluorescence: the antigen substrate rat oesophagus shows staining of the stratum corneum (RA keratin) on the luminal side; anti-perinuclear factors (APF) are apparent in the cytoplasmic inclusion bodies of human epithelial cells of the oral mucosa [2, 10, 11, 12].

In recent years it has been shown that the rare amino acid citrulline, which is present in filaggrin, is a substantial component of the antigenic epitope. A direct comparison study demonstrated that the sensitivity can be increased from 49% to 68% by using cyclic citrullinated peptides instead of linear citrullinated peptides as an ELISA substrate [13]. Autoantibodies against cyclic citrullinated peptides (CCP) are therefore a new, highly specific marker for rheumatoid arthritis [2, 13, 14, 15, 16].

Antibodies against CCP occur independently of rheumatoid factors. The term "seronegative RA" for RF-negative cases is outdated and should not be used anymore. It could be demonstrated in many studies that 20% to 57% of all RF-negative RA patients have antibodies against CCP. Thus, the parallel determination of both antibodies increases the serological hit rate in RA patients [8, 13, 17, 18, 19, 20]. The CCP titer generally correlates with the activity of the disease [2, 14, 15, 16, 21, 22].

Antibodies against CCP are predominantly of class IgG and have a specificity of 95% for RA [9, 13]. They are a predictive marker since they can be found in the serum and the synovial liquid of 70%-80% of patients very early during the development of the disease, often even many years before the onset of the first symptoms [8, 18, 22, 23, 24, 25, 26, 27, 28]. The earlier the diagnosis is established, the earlier a suitable therapy can be started. With respect to disease prognosis, radiological examinations show that severe joint damage is found significantly more often in patients with anti-CCP antibodies than in anti-CCP negative patients [13, 18, 19, 20, 22, 27, 29, 30, 31, 32, 33]. This fact emphasises the importance of CCP antibodies as prognostic marker for the development and progression of the disease.

The great importance of the Anti-CCP antibody determination in the diagnosis of rheumatoid arthritis is limited in suspected cases of juvenile idiopathic arthritis (JIA) and in the monitoring of RA therapy: The prevalence of antibodies against CCP in patients with JIA is as low as 2% to 12%. Therefore, the determination of antibodies against CCP in suspected JIA cases plays only a minor role [34, 35, 36, 37]. Due to partially discrepant research results, the determination of antibodies against CCP can only be of limited use in the monitoring of therapy measures [38, 39, 40, 41].

The importance of antibodies against CCP as a serological marker becomes apparent in comparison with rheumatoid factors (RF) which have a significantly lower specificity (anti-CCP: 96%-100%, RF: 63%) at almost the same sensitivity (anti-CCP: 80%, RF: 79%) [13, 42]. Antibodies against CCP can also be used as a marker in differential diagnostics, e.g. in the differentiation of hepatitis-associated arthropathies from rheumatoid arthritis (e.g. anti-CCP negative and RF positive in HCV infections) [43, 44].

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