Anti-CEP-1 ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EA 151b-9601 G	CEP-1	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human autoantibodies of the IgG class against CEP-1 in serum or plasma for the diagnosis of rheumatoid arthritis.

Application: Supplementary serological parameter for the diagnosis of rheumatoid arthritis and to investigate the pathogenesis and possible subtyping of the disease.

Principle of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with CEP-1. 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 RU/ml (IgG, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 20 RU/ml (IgG, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 RU/ml (IgG, human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
8. Sample buffer ready for use	violet	1 x 100 ml	SAMPLE BUFFER
9. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
10. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
11. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
12. Test instruction	---	1 booklet	
13. Quality control certificate	---	1 protocol	

LOT Lot description
IVD In vitro diagnostic medical device
CE
Storage temperature
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 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.

- **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.

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

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

Incubation

For **semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1, 2 and 3** 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 or negative control or diluted patient samples into the individual microplate wells according to the pipetting protocol. Incubate for **60 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 of 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 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 **30 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 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 and the Analyzer I-2P 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 2**	**P 6**	**P 14**	**P 22**			**C 1**	**P 4**	**P 12**	**P 20**		
B	**pos.**	**P 7**	**P 15**	**P 23**			**C 2**	**P 5**	**P 13**	**P 21**		
C	**neg.**	**P 8**	**P 16**	**P 24**			**C 3**	**P 6**	**P 14**	**P 22**		
D	**P 1**	**P 9**	**P 17**				**pos.**	**P 7**	**P 15**	**P 23**		
E	**P 2**	**P 10**	**P 18**				**neg.**	**P 8**	**P 16**	**P 24**		
F	**P 3**	**P 11**	**P 19**				**P 1**	**P 9**	**P 17**			
G	**P 4**	**P 12**	**P 20**				**P 2**	**P 10**	**P 18**			
H	**P 5**	**P 13**	**P 21**				**P 3**	**P 11**	**P 19**			

The pipetting protocol for microtiter strips 1-4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 3), 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

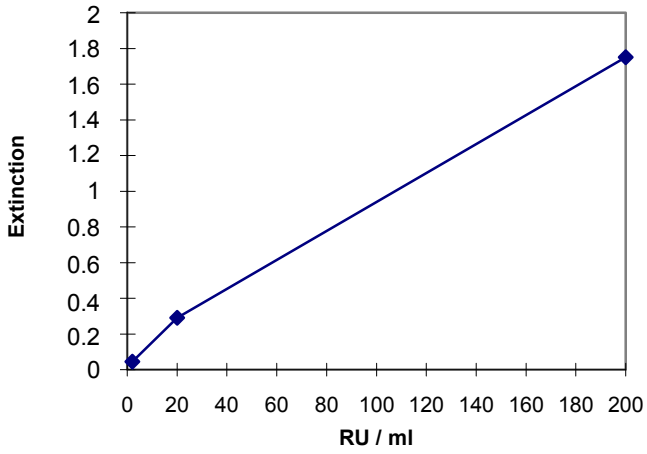
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator 2. Calculate the ratio according to the following formula:

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

EUROIMMUN recommends interpreting results as follows:

Ratio <1.0: **negative**
Ratio ≥1.0: **positive**

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 3 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 (200 RU/ml), the result should be reported as “>200 RU/ml“. It is recommended that the sample be retested 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 20 relative units (RU) /ml. EUROIMMUN recommends interpreting results as follows:

<20 RU/ml:	**negative**
≥20 RU/ml:	**positive**

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 against CEP-1, 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 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 during the 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 antigen used is citrullinated α-enolase peptide 1 (CEP-1).

Linearity: The linearity of the Anti-CEP-1 ELISA (IgG) was determined by assaying 4 serial dilutions of different patient samples. The coefficient of determination R^2 for all sera was > 0.95. The Anti-CEP-1 ELISA (IgG) is linear at least in the tested concentration range (3 RU/ml to 200 RU/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-CPE-1 ELISA is 0.4 RU/ml.

Cross reactivity: This ELISA specifically detects autoantibodies of class IgG against CEP-1. Cross reactions with other autoantibodies were not found in samples from patients with the following diseases: spondylarthritis (SpA) (n = 10) and fibromyalgia (FM) (n = 10).

Interference: Haemolytic and icteric samples showed no influence on the result up to a concentration of 10 mg/ml for haemoglobin and 0.4 mg/ml for bilirubin in this ELISA. The presence of triglyceride in the sample may influence the test. We therefore recommend to refrain from using lipaemic samples.

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 3 determinations performed in 10 different test runs.

Intra-assay variation, n = 20		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	16.3	3.6
2	67.3	2.4
3	170.3	1.5

Inter-assay variation, n = 3 x 10		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	16.5	7.6
2	67.5	5.7
3	170.9	2.5

Specificity and sensitivity: Samples from 110 patients with rheumatoid arthritis, a control panel of 238 patients with other diseases and 500 healthy blood donors were analysed using the EUROIMMUN Anti-CEP-1 ELISA. The sensitivity of the ELISA for rheumatoid arthritis was 43.6% with a specificity of 97.6%.

Panel	**Anti-CEP-1 ELISA**	
	n	**positive**
Sensitivity for rheumatoid arthritis	**110**	**48 (43.6%)**
SLE	120	5
SS	30	1
SCL	30	2
FM	20	0
SpA	20	1
PsA	2	0
Infectious diseases	16	1
Healthy blood donors	500	8
Specificity for rheumatoid arthritis	**738**	**18 (97.6%)**

Reference range: Levels of anti-CEP-1 antibodies were analysed in 300 samples from healthy blood donors using the EUROIMMUN ELISA. The mean concentration of antibodies against CEP-1 was 4 RU/ml and the values ranged from 0.9 to 53.6 RU/ml. With a cut-off of 20 RU/ml, 1.7% of the blood donors were anti-CEP-1 positive.

cut-off	percentile
16.1 RU/ml	97.0%
19.5 RU/ml	98.0%
23.8 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 the large joints. This leads to the destruction of the joints in the late phase accompanied by a systemic involvement of soft tissue.

In suspected cases of RA, mostly rheumatoid factors (RF) and autoantibodies against cyclic citrullinated peptide (CCP) are determined serologically alongside general inflammation parameters. RF are found in 70 to 90% of sera from RA patients, however, they also occur in patients with other autoimmune and infectious diseases. The determination of autoantibodies against CCP which was optimised for high specificity (97%) and sensitivity (72%) is a standard test. It employs a mixture of synthetic peptides which do not present homologies with synovial proteins, but were chosen due to their reactivity with sera from RA patients. In addition to the Anti-CCP ELISA, autoantibodies against citrullinated protein/peptide antigens (ACPA), can be detected by means of further artificial antigens, e.g. mutated citrullinated vimentin (MCV).

Citrullinated proteins are produced physiologically with the help of the enzyme peptidylarginindesiminase (PAD) by desimination of the amino acid arginin to citrullin. Whilst citrullinated proteins can also occur in inflammation and some other processes which are not caused by the disease (e.g. in keratinisation of the skin), autoantibodies against citrullinated proteins are specific for RA.

The tests described above do allow the detection of ACPA, but do not give insight into the nature of the autoantibodies. Therefore, the reactivity to citrullinated proteins which actually occur in RA patients needs to be investigated. In this context, the citrullinated antigens fibrinogen/fibrin, vimentin, collagen type II, fibronectin and α-enolase were described over the last years.

Citrullinated α-enolase was identified as an autoantigen in RA in 2005. The epitope within the α-enolase responsible for the autoimmune reaction, was identified by means of a peptide screening and given the name "citrullinated α-enolase peptide 1" (CEP-1). This peptide reacts with 37 to 62% of sera from RA patients, but only with 2 to 3% of sera from healthy blood donors or patients from a control panel. Approx. 60% of the anti-CCP-positive patients also show autoantibodies against CEP-1.

Alongside the serological confirmation of an RA diagnosis, autoantibodies against CEP-1 are suited for a subclassification of RA: In anti-CCP-positive RA patients, the risk factors for RA (smoking, the HLA-DRB1 "shared epitope“ alleles and the PTNP22 polymorphism) occur mostly in combination with autoantibodies against CEP-1. Moreover, autoantibodies against CEP-1 could also be detected in cases of *Porphyromonas gingivalis* infections which constitutes the main cause of parodontitis. An association between RA and parodontitis was already decribed. Alongside an increased occurrence of RA cases in patients with parodontitis and vice versa, both diseases present a similar pathophysiology and similar risk factors. It could also be shown that *P. gingivalis* expresses an own PAD enzyme which enables it to citrullinate endogen and human proteins, and that antibodies against CEP-1, which are isolated from the serum of RA patients, cross-react with the citrullinated enolase of *P. gingivalis*. For this reason, it is assumed that an infection with *P. gingivalis* is responsible for triggering of autoimmune processes in RA though citrullination of human and/or bacterial proteins and the related generation of autoantigens. Current studies are investigating whether the determination of autoantibodies against CEP-1 might also be suitable for predictions for the treatment response and the disease course and therapy monitoring.

Literature references

1. Fisher BA, Plant D, Brode M, van Vollenhoven RF, Mathsson L, Symmons D, Lundberg K, Rönnelid J, Venables PJ. **Antibodies to citrullinated α-enolase peptide 1 and clinical and radiological outcomes in rheumatoid arthritis.** Ann Rheum Dis 70 (2011) 1095-1098.
2. EUROIMMUN AG. Stöcker W, Schlumberger W, Krüger C. Alle Beiträge zum Thema Autoimmundiagnostik und Labordiagnostik der Infektionskrankheiten. In: Gressner A, Arndt T (Hrsg.) **Lexikon der Medizinischen Laboratoriumsdiagnostik.** 2. Auflage. Springer Medizin Verlag, Heidelberg (2012).
3. Kinloch A, Tatzer V, Wait R, Peston D, Lundberg K, Donatien P, Moyes D, Taylor PC, Venables PJ. **Identification of citrullinated alpha-enolase as a candidate autoantigen in rheumatoid arthritis.** Arthritis Res Ther 7, 6 (2005) R1421-9.
4. Kinloch A, Lundberg K, Wait R, Wegner N, Lim NH, Zendman AJ, Saxne T, Malmström V, Venables PJ. **Synovial fluid is a site of citrullination of autoantigens in inflammatory arthritis.** Arthritis Rheum 58 (2008) 2287-2295.
5. Lundberg K, Kinloch A, Fisher BA, Wegner N, Wait R, Charles P, Mikuls TR, Venables PJ. **Antibodies to citrullinated alpha-enolase peptide 1 are specific for rheumatoid arthritis and cross-react with bacterial enolase.** Arthritis Rheum 58 (2008) 3009-3019.
6. Lundberg K, Wegner N, Yucel-Lindberg T, Venables PJ. **Periodontitis in RA-the citrullinated enolase connection.** Nat Rev Rheumatol 6, 12 (2010) 727-30.
7. Mahdi H, Fisher BA, Källberg H, Plant D, Malmström V, Rönnelid J, Charles P, Ding B, Alfredsson L, Padyukov L, Symmons DPM, Venables PJ, Klareskog L, Lundberg K. **Specific interaction between genotype, smoking and autoimmunity to citrullinated α-enolase in the etiology of rheumatoid arthritis.** Nature Genetics 41 (2009) 1319-1324.
8. Montes A, Perez-Pampin E, Calaza M, Gomez-Reino JJ, Gonzalez A. **Association of anti-citrullinated vimentin and anti-citrullinated α-enolase antibodies with subsets of rheumatoid arthritis.** Arthritis Rheum 64 (2012) 3102-3110.
9. Snir O, Widhe M, Hermansson M, von Spee C, Lindberg J, Hensen S, Lundberg K, Engström A, Venables PJ, Toes RE, Holmdahl R, Klareskog L, Malmström V. **Antibodies to several citrullinated antigens are enriched in the joints of rheumatoid arthritis patients.** Arthritis Rheum 62 (2010) 44-52.
10. Wegner N, Lundberg K, Kinloch A, Fisher B, Malmström V, Feldmann M, Venables PJ. **Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis.** Immunol Rev233 (2010) 34-54.