IgA Rheumatoid Factor ELISA
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EA 1814-9601 A	Human IgG	IgA	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides semiquantitative or quantitative in vitro determination of human IgA rheumatoid factors (autoantibodies of the IgA class against human IgG) in serum or plasma to support the diagnosis of autoimmune diseases, especially cases of suspected rheumatoid arthritis and further rheumatic diseases.

Application: Enzyme immunoassays enable differential determination of rheumatoid factors of isotypes IgA, IgG and IgM. A positive result is considered a classification criterion by the American College of Rheumatology (ACR) and the European League against Rheumatism (EULAR) for the diagnosis of rheumatoid arthritis. In suspected cases of rheumatoid arthritis, the determination of rheumatoid factors is often performed in parallel to the detection of antibodies against citrullinated peptides (anti-CCP antibodies).

Principles of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with IgG. 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 1 200 RU/ml (IgA, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 20 RU/ml (IgA, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 RU/ml (IgA, human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (IgA, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control (IgA, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgA (rabbit), ready for use	orange	1 x 12 ml	CONJUGATE
8. Sample buffer ready for use.	light blue	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	CE	Storage temperature
IVD	In vitro diagnostic medical device		Unopened usable until

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.

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 **1:201** in sample buffer. For example: dilute 5 µl sample to 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 **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 **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 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) remaining 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 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:
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, 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 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 to 4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7 to 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

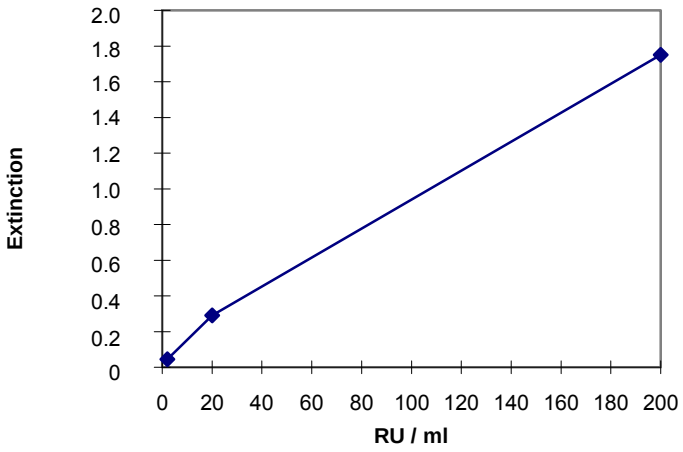
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction of the control or patient sample over the extinction of calibrator 2. Calculate the ratio according to the following formula:

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

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 readings measured for the 3 calibrators 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 RU/ml), the result should be reported as “>200 RU/ml”. It is recommended that the sample be re-tested at a dilution of e.g. 1:800. The result in RU/ml read from the calibration curve for this sample must then be multiplied by factor 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, EUROIMMUN recommends retesting the samples.

For the medical diagnosis, the clinical symptoms of the patient and, if available, further findings should always be taken into account alongside the serological result. A negative serological result does not exclude the presence of a disease.

Test characteristics

Calibration: As no international reference serum exists for rheumatoid factors, the calibration is performed in relative units (RU).

For every group of tests performed, the extinction readings of the calibrators and the relative units and/or ratios 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 activitiy 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 Fc fragments of human IgG isolated from human serum by delipidisation, ion exchange chromatography, papain degradation and molecular sieve chromatography.

Linearity: The linearity of the IgA Rheumatoid Factor ELISA was determined by assaying at least 4 serial dilutions of different patient samples. The IgA Rheumatoid Factor ELISA is linear at least in the tested concentration range (9 RU/ml to 184 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 IgA Rheumatoid Factor ELISA is 1 RU/ml.

Cross reactivity: This ELISA showed no cross reactivity.

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 (RU/ml)**	**CV (%)**
1	54	4.3
2	76	4.0
3	121	3.7

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (RU/ml)**	**CV (%)**
1	53	8.2
2	80	5.7
3	123	4.7

Prevalence and specificity: The prevalence of IgA rheumatoid factors in a panel of 105 clinically characterised patients with rheumatoid arthritis was 70.5%. The specificity in a control panel of healthy blood donors (n = 200) was 97.5%.

Reference range: The levels of IgA rheumatoid factors were analysed with this EUROIMMUN ELISA in a panel of 200 healthy blood donors. With a cut-off of 20 RU/ml, 2.5% of the blood donors were IgA rheumatoid factor positive.

Clinical significance

Rheumatoid factor (RF), sometimes also used in plural (rheumatoid factors), is a group of autoantibodies, predominantly of the immunoglobulin classes IgA, IgG and IgM. Their autoimmunological activity is directed against the Fc fragment of the human IgG molecule.

Along with the investigation of inflammation parameters, the most commonly performed test in suspected rheumatoid arthritis (RA) is the detection of RF, which can be found in the serum of 60% to 80% of RA patients. RF is mainly produced in plasma cells of the synovial membrane. It can therefore be found earlier and in higher concentration in synovial liquid than in serum.

RA is both one of the most common autoimmune disorders and also the most common chronic inflammatory joint disease. Around 1% of the world population is affected, around 75% of which are women. Its peak incidence is at around 50 years of age. The initial symptoms of this systematic disease include painful swelling of the finger basal joints with morning ankylosis. Characteristic is an infection of the synovial membrane, which symmetrically spreads from the smaller to the larger joints and destroys the joints in the late stage. Depending on the degree of severity, extraarticular manifestations may occur, which afflict the skin, the vessels and the internal organs. If insufficiently treated, RA will, over the long term, lead to a significant decline in the quality of a person’s life. Both morbidity and mortality will increase.

To delay — by means of adequate therapy — the disease from progressing to the point where irreversible damage to the joints occurs, it is imperative to secure a diagnosis as early as possible. In addition to case history, clinical findings and imaging procedures, serological laboratory testing is another, often decisive pillar when diagnosing rheumatic diseases.

The detection of RF is not very specific for RA since RF also occurs in non-RA patients, e.g. in other autoimmune diseases, tumours and various infectious diseases as follows:

1. Frequency of RF in rheumatic diseases:

Rheumatic disease	**Frequency of RF**
Rheumatoid arthritis (RA)	60%-80% (50%-90%)
Systemic lupus erythematosus (SLE)	15%-35%
Sjögren's syndrome	75%-95%
Systemic sclerosis (Ssc)	20%-30%
Polymyositis / dermatomyositis	5%-10%
Cryoglobulinaemia	40%-100%
Mixed connective tissue disease	50%-60%

2. Occurrence of RF in other diseases such as
- primary biliary cholangitis (PBC)
- autoimmune hepatitis (AIH)
- lymphoma
- sarcoidosis
- interstitial lung diseases
- subacute/acute bacterial endocarditis
- tuberculosis
- lues/syphilis
- malaria
- mononucleosis (EBV infection)
- hepatitis (HBV/HCV infection)

3. Detection of RF in around 10% of healthy blood donors, for example following vaccination or blood transfusion.
Beside semiquantitative agglutination methods (Waaler-Rose and latex tests), which only allow detection of RF IgM, rheumatoid factor IgA, IgG, IgM ELISA is the most frequently performed serological assay in RA. A high titer of RF is associated with a severe and progressing course.
The investigation of autoantibodies against cyclic citrullinated peptide (CCP), a marker of RA, provides highly specific and highly sensitive serodiagnostic results. CCP antibodies, which belong primarily to the IgG class and possess a specificity of more than 95% for RA, are also of significance for differential diagnosis; for instance, for distinguishing patients with reactive arthritis (e.g. hepatitis-associated arthropathies) from patients with RA (e.g. anti-CCP negative and RF positive with HCV infection).

Another significant autoantibody with a specificity of nearly 100% for patients with RA in Europe, Asia and America is the anti-Sa antibody. Citrullinated vimentin expressed in synovial membrane is the target antigen of this autoantibody. Although anti-Sa antibodies exhibit a lower sensitivity (anti-Sa ELISA 40% to 50%), their prognostic value for a severe form of RA progression is unsurpassed. Their high predictive value of as much as 99% for RA is closely associated with severe joint involvement as well as extraarticular manifestations. The detection of anti-Sa among healthy individuals indicates a risk for development of RA. It is quite possible for 10 to 15 years to elapse before those persons become ill with RA; the higher the anti-Sa titer, the shorter the interval will be.

Literature references

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