Anti-Sa ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EA 151a-4802 G	Sa	IgG	Ag-coated microplate wells	48 x 02 (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 Sa. The test kit contains microtiter strips each with 8 individual break-off reagent wells coated with Sa. In the first reaction step, diluted patient samples (serum) 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 6 microplate strips Sa and 6 microplate strips Blank each containing 8 individual break-off wells in a frame, ready for use	---	6 x 8 each	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	WASHBUFFER 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. 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: Undiluted patient samples and incubated microplate strips should be handled as infectious waste. Other reagents do not need to be collected separately, unless stated otherwise in official 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. 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 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.
  For example: 1 microplate strip use 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 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 contain the toxic agent sodium azide. Avoid skin contact.

Preparation and stability of the serum or plasma samples

Samples: Human serum

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: The **patient samples** 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 3** along with the positive and negative controls and patient samples.

Sample incubation: (1. step)
Transfer 100 µl of the calibrators, positive and negative controls or diluted patient samples into the individual microplate wells (Sa and Blank parallel) 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).

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 (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 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.

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).

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 **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.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	**C 2**	**C 2**	**P 6**	**P 6**			**C 1**	**C 1**	**P 4**	**P 4**		
B	**pos.**	**pos.**	**P 7**	**P 7**			**C 2**	**C 2**	**P 5**	**P 5**		
C	**neg.**	**neg.**	**P 8**	**P 8**			**C 3**	**C 3**	**P 6**	**P 6**		
D	**P 1**	**P 1**	**P 9**	**P 9**			**pos.**	**pos.**	**P 7**	**P 7**		
E	**P 2**	**P 2**	**P 10**	**P 10**			**neg.**	**neg.**	**P 8**	**P 8**		
F	**P 3**	**P 3**	**P 11**	**P 11**			**P 1**	**P 1**	**P 9**	**P 9**		
G	**P 4**	**P 4**					**P 2**	**P 2**	**P 10**	**P 10**		
H	**P 5**	**P 5**					**P 3**	**P 3**	**P 11**	**P 11**		
	Sa 1	**Blank 1**	**Sa 2**	**Blank 2**	**Sa 3**	**Blank 3**	**Sa 4**	**Blank 4**	**Sa 5**	**Blank 5**	**Sa 6**	**Blank 6**

The pipetting protocol for microtiter strips 1-4 is an example for the **qualitative/semiquantitative analysis** of 11 patient sera (P 1 to P 11).
The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 11 patient sera (P 1 to P 11).

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 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 O.D. value of the control or patient sample over the extinction value of calibrator 2. The extinction values of the Blank wells have to be subtracted from the extinction values of the corresponding Sa well. Calculate the ratio according to the following formula:

$$\frac{\text{O.D. Sa - O.D. Blank of the control or patient sample}}{\text{O.D. Sa - O.D. Blank of calibrator 2}} = \text{Ratio}$$

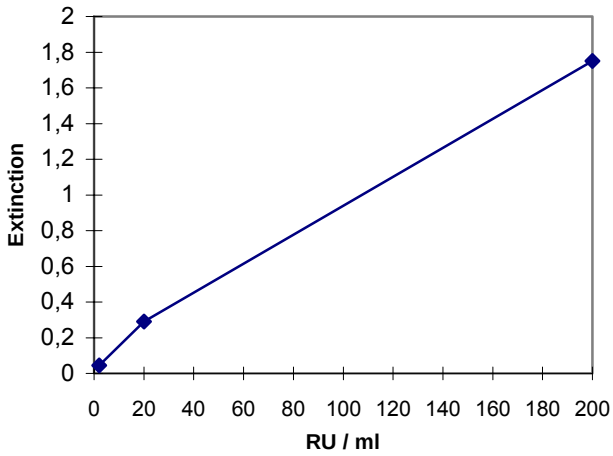
EUROIMMUN recommends interpreting results as follows:

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

Quantitative: The extinction value for each calibrator, control and patient sample has to be calculated according to the following formula:

O.D. Sa - O.D. Blank = calculated O.D.

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 calculated O.D. 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 of a serum sample is above the value of calibrator 1 (200 RU/ml), the result should be given as ">200 RU/ml". It is recommended that the sample be re-measured 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.
Note: Calculated extinctions and antibody concentrations (RU/ml) may be below 0. In this case, use 0 RU/ml for evaluation.

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**

The recommendation is based on data yielded in a ROC analysis using the results of 237 samples of patients with rheumatoid arthritis and 589 control samples. According to the analysis, the specificity was 99% at a cut-off of 16.7 RU/ml. The 99th percentile based on 408 healthy blood donors was also 16.3 RU/ml (q.v. respective paragraphs under "Test characteristics").

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 Sa, the calibration is performed in relative units (RU).

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 ELISA was developed in cooperation with Prof. Menard (McGill University, Montreal, Canada). The reagent wells are coated with citrullinated and non- citrullinated Sa antigen (Blank).

Linearity: The linearity of the ELISA was determined by assaying 4 serial dilutions of 6 serum samples. The calculated linear regression R^2 was > 0.95 for all samples. The Anti-Sa ELISA (IgG) is linear at least in the tested concentration range (2 RU/ml to 179 RU/ml) linear.

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-Sa ELISA is 0 RU/ml.

Cross reactivity: The ELISA specifically detects IgG class antibodies directed against Sa. There were no cross reactions with other autoantibodies in samples of patients with SLE (n = 15), scleroderma (n = 5) and Sjögren's syndrome (n = 5).

Interference: Haemolytic, lipaemic and icteric samples showed no influence at 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 3 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	31	8.9
2	119	3.5
3	160	2.4

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	28	9.1
2	112	8.0
3	165	4.3

Clinical sensitivity and specificity: Sera from 237 patients with rheumatoid arthritis, 55 patients with juvenile idiopathic arthritis, 181 patients with psoriasis arthritis and 408 healthy blood donors were analysed using the EUROIMMUN Anti-Sa ELISA. The sensitivity of the ELISA for rheumatoid arthritis was 48.1 %, at a specificity of 99.5 %.In the juvenile idiopathic arthritis panel 1.8 % of patients were tested positive.

Panel	**Anti-Sa ELISA**	
	n	**positive**
Rheumatoid arthritis	**237**	**114 (48.1 %)**
Juvenile idiopathic arthritis	55	1 (1.8 %)
Psoriasis arthritis	181	0 (0,0 %)
Asymptomatic blood donors	408	3 (0.7 %)
Sensitivity for rheumatoid arthritis	**237**	**114 (48.1 %)**
Specificity for rheumatoid arthritis	**589**	**3 (99.5 %)**

A ROC analysis (AUC value: 0.977) based on the results of 237 samples from patients with rheumatoid arthritis and 589 control samples shown in the table yielded the following data:

Cut-off	Specificity	Sensitivity
5.9 RE/ml	98 %	57 %
16.7 RE/ml	99 %	53 %

Reference Range: Levels of anti-Sa antibodies were determined in 408 sera from healthy blood donors of between 17 and 67 years of age (148 women, 260 men) using the EUROIMMUN ELISA. With a cut-off value of 20 RU/ml, 0.7 % of blood donors were anti-Sa positive.

Cut-off	Percentile
3.7 RE/ml	98.
16.3 RE/ml	99.

Clinical significance

Serological antibody diagnosis is critically important for the early diagnostic detection and confirmation as well as assessment of progression, therapy and prognosis of rheumatoid arthritis (RA). In addition to the traditional identification of rheumatoid factors (RF) as a possible indication of RA, autoantibodies against citrullinated antigens (CCP, cyclic citrullinated peptides) contribute substantially to the diagnosis of RA. Both the qualitative and quantitative detection of anti-Sa (Sa = Mrs Savoie, a RA patient in whom the antibodies against citrullinated vimentin were first discovered) can also objectify the activity (progression, therapeutic success) of RA.

RA is both one of the most common autoimmune disorders and the most common chronic inflammatory joint disease. It affects approximately 1% of the world population— approximately 75% of whom are female. Its peak incidence is around 50 years of age. The initial symptoms of this systemic disease include painful swelling of the finger joints with morning stiffness [1]. Characteristic is an inflammation of the synovial membrane, which symmetrically spreads from the smaller to the larger joints and leads to the destruction of the joints in the late stage. Depending on the degree of severity, extraarticular manifestations may occur, which affect the skin, the vessels and the internal organs. Therefore, if insufficiently treated, RA will generally – over the long term – lead to a significant decline in the quality of a person's life. Both morbidity and mortality will increase. To retard – 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 [2, 3, 4, 5] and identify early those patients with the worse prognosis. In addition to case histories and clinical findings, as well as imaging procedures, the serological lab test is the third and, indeed, most decisive pillar when diagnosing that rheumatic disease.

Apart from examining general inflammation parameters, the determination of rheumatoid factors (RF) and of antibodies against CCP are the most common serological tests carried out when RA is suspected. RF are antibodies (predominantly of class IgM) that react with IgG and are detectable in the serum of 60-80% of RA patients. RF are indeed sensitive yet they are not very specific markers for RA, given the fact that they also occur in non-RA patients, e.g. patients with various infections or other autoimmune diseases, such as systemic Lupus erythematosus, Sjögren's syndrome, scleroderma, and in up to 30% of healthy individuals [6, 7, 8, 9, 10, 11].

Considerably better results are provided by the detection of autoantibodies against cyclic citrullinated peptides (CCP) as a highly specific marker for RA, with a comparable sensitivity to RF [2, 12, 13, 14, 15, 16]. CCP antibodies belonging primarily to the IgG class and possessing a specificity of more than 95% for RA are, among others, also of significance in terms of a differentiated diagnosis; for instance, when it comes to distinguishing patients with reactive arthritis (e.g. hepatitis-associated arthropathies) from patients with RA (e.g. anti-CCP negative and RF positive with HCV infections) [11, 17, 18].

The detection of anti-CCP among healthy individuals means that there is a risk of them having RA [8]. It is quite possible for 10-15 years to elapse before those persons become ill with RA; and, indeed, the higher the anti-CCP titre, the shorter the interval will be [19, 20, 21].

An autoantibody with a high specificity of nearly 100% for patients with RA in Europe, Asia and America is the anti-Sa antibody [7, 22, 23, 24]. Citrullinated vimentin expressed in synovial membrane is a target antigen of this autoantibody [24]. If anti-Sa antibodies also exhibit a lower sensitivity (anti-Sa WB 40%, anti-Sa ELISA 55-60%), its prognostic value for a severe form of RA progression is unsurpassed [23, 25, 26, 27]. Its high predictive value of as much as 99% for RA is closely associated with severe joint involvement as well as extraarticular manifestations [7, 24, 25, 28, 29]. Autoantibodies against CCP and against Sa can be detected among approximately 75%, or roughly 60% of RA patients, respectively, very early in the progression of the disease— often even many years before the first symptoms arise for the former, and, indeed, both in the serum as well as in the synovial fluid [7, 10, 25, 26, 27, 28]. As a result, the diagnosis can be made earlier and an adequate therapy can ensue more quickly [30]. With regard to prognosis, radiological examinations indicate that among patients with anti-CCP antibodies, and more so with anti-Sa antibodies, severe joint damage occurs significantly more frequently than in anti-CCP negative or anti-Sa negative patients [7, 25, 29, 31]. This increases the significance of detecting both anti-CCP and anti-Sa as a prognostic marker for the development and progression of RA.

The generally high significance of anti-CCP antibody detection for the diagnosis of RA is limited when it comes to monitoring therapy in adult RA and checking the suspicion of juvenile idiopathic arthritis (JIA). Antibodies against CCP only occur with a prevalence of 2-12% in patients with JIA, which means that identification of autoantibodies against CCP plays a minor role in cases of JIA [32, 33, 34]. Thus far, the detection of anti-CCP – as a means of documenting the efficacy of rheumatic therapies – has only been employed with reservations due to the partially contradictory study results [35, 36, 37, 38].

A significant correlation has been demonstrated between the level of the anti-Sa antibody titre and the degree of severity of RA, as well as its disease activity (disease activity score: DAS28 = internationally accepted composite score for the evaluation of RA disease activity). Consequently, the anti-Sa ELISA is quite suitable for the stratification of RA [39].

Patients with active RA exhibit significantly higher anti-Sa antibody titres compared to patients with mild RA. As with anti-CCP antibodies, anti-Sa antibodies are well suited for the early diagnosis of RA, albeit with approximately 5% lower sensitivity [25]. Both assays (Anti-CCP ELISA and Anti-Sa ELISA) are in equal measure predictive of the radiological outcome, i.e. equally associated with the radiological progression [25, 28, 29, 40].

Because Anti-CCP ELISA and Anti-Sa ELISA overlap and confirm one another in part in terms of their validity, and in particular complement one another (with regard to early detection, confirmation, progression, therapeutic success and prognosis of RA), the parallel implementation of these two serological tests currently offers the greatest possible diagnostic security [7, 25, 26, 41].

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