Anti-SS-A ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EA 1595-9601 G	SS-A	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The enzyme immunoassay (ELISA) provides semiquantitative or quantitative in vitro determination of human antibodies of the immunoglobulin class IgG against SS-A in serum or plasma to support the diagnosis of Sjögren's syndrome and systemic lupus erythematosus. The product is designed for use as IVD.

Application: The Anti-SS-A ELISA is designed for the detection of anti-SSA antibodies which mainly occur in the diseases Sjögren's syndrome and systemic lupus erythematosus (SLE).

Principle of the test: The test kit contains microplate strips each with 8 break-off reagent wells coated with SS-A. 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	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

IVD In vitro diagnostic medical device

CE

Storage temperature

Unopened usable until

Updates with respect to the previous version are marked in grey.

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 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 of 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 **semiquantitative 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 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 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 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 **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, EUROIMMUN Analyzer I-2P or the DSX from Dynex 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 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 microplate strips 1 to 4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microplate 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

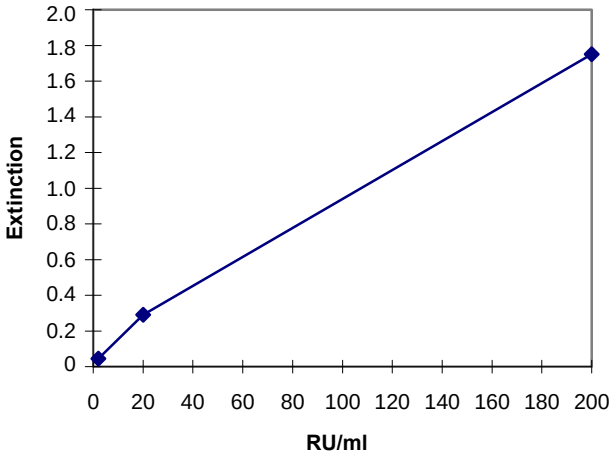
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction of the control or patient sample over the extinction of calibrator 2. Calculate the ratio according to the following formula:

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

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 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 extinction of calibrator 1 (corresponding to 200 RU/ml), the result shold be reported as ">200 RU/ml". It is recommended that the sample be re-tested at a dilution of 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 the quantitative detection of antibodies against SS-A, the calibration is performed in relative units (RU)/ml. The reactivity of the Anti-SS-A ELISA was verified using the human reference serum CDC-ANA #7 of the "Centers for Disease Control" (Atlanta, USA).

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 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 (+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 SS-A (60 kDa) purified by affinity chromatography from calf thymus.

The SS-A antigen is localized in the cell nucleus and is involved in the processing of mRNA to translationally active molecules. It is a small ribonucleoprotein consisting of an RNA molecule (Y1, Y2, Y3, Y4 or Y5 RNA: 80-112 bases in length) and two different proteins, which are the targets for antibodies against SS-A. Initially, in 1984, a protein of 60 kDa was described as a component of ribonucleoproteins. In 1988, antibodies against a further protein with a molecular weight of 52 kDa (Ro-52) were detected in anti-SS-A-positive sera by means of Westernblot. However, Ro-52 does not appear to be a stable component of the native ribonucleoprotein particle.
Only test systems which use native SS-A 60 kDa as the antigen should be used for the detection of autoantibodies against SS-A in SLE or Sjögren's syndrome (SS). Test systems which include Ro-52 in the antigen substrate are not recommended, since antibodies against Ro-52 are frequently also found in myositis patients. Thus, the specificity of these test systems for SLE and SS is reduced. All anti-SS-A positive sera from SLE or SS patients can be identified using native SS-A 60 kDa as the antigen.

Linearity: The linearity of the Anti-SS-A ELISA (IgG) was determined by assaying 4 serial dilutions of different patient samples. The Anti-SS-A ELISA (IgG) is linear at least in the tested concentration range (13 RU/ml to 185 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-SS-A ELISA (IgG) 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	92	3.0
2	113	1.9
3	114	4.2

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (RU/ml)**	**CV (%)**
1	92	3.1
2	116	4.1
3	121	3.0

Reference range: The levels of the anti-SS-A antibodies (IgG) were analysed with this EUROIMMUN ELISA in a panel of 206 healthy blood donors. With a cut-off of 20 RU/ml, all blood donors were anti-SS-A negative.

Clinical significance

Antibodies (AAb) against nuclear antigens (ANA) are directed against various cell nuclear components. Among the most important nuclear antigens, including cytoplasmic antigens, are nRNP/Sm, Sm, SS-A (Ro), SS-B (La), Scl-70, PM-Scl, Jo-1, centromeres, PCNA, dsDNA, nucleosomes, histones and ribosomal P-proteins. They are mainly components of functional nuclear particles, are bound to nucleic acids or fulfil functions in the cell cycle, e.g. in transcription or translation.

The investigation of ANA and subsequent differentiation within the ANA (or ENA) spectrum contributes greatly to establishing a diagnosis, particularly in the following rheumatic diseases:
- systemic lupus erythematosus (SLE),
- Sharp syndrome (mixed connective tissue disease = MCTD),
- Sjögren's syndrome (SS),
- systemic sclerosis (SSc), and
- poly-/dermatomyositis (PM/DM).

Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disease of the exocrine glands which can be found in one to four million people in the US alone. Nine out of ten patients are women. The main clinical feature of primary SS is ocular and oral dryness as a result of the destruction of lachrymal and salivary glands by lymphocytic infiltration. The pancreatic glands, the mucous secreting glands of the intestine, bronchia or vagina and the sudoriferous glands may also be affected. Around 5% of SS patients develop malignant lymphoma. In secondary SS, primary SS symptoms accompany rheumatoid arthritis (RA), SSc, SLE, PM/DM, primary biliary cholangitis or autoimmune hepatitis.

Anti-SS-A are detected in 40% to 95% of SS cases. They mostly occur in parallel with autoantibodies against SS-B (anti-La). Autoantibodies against SS-A are also found in 20% to 60% of SLE patients and in neonatal lupus erythematosus (neonatal LE syndrome) with a prevalence of 100%. The antibodies are transmitted diaplacentally to the foetus and often cause congenital AV block in addition to inflammatory reactions when the mother is anti-SS-A or anti-SS-B positive (level I-III).

Note: Differentiation of anti-SS-A antibodies from those against the so-called Ro52 antigen (52 kDa protein, RING dependent E3 ligase) is of decisive diagnostic importance, since antibodies against Ro52 are not disease-specific, but are also detected in myositis, systemic sclerosis, neonatal lupus erythematosus and other collagenoses, primary biliary cholangitis, autoimmune hepatitis and viral hepatitis.

Antibodies against	Disease	Prevalence
SS-A (Ro)	Sjögren's syndrome (SS) Systemic lupus erythematosus (SLE) Neonatal lupus erythematosus	40% - 95% 20% - 60% 95% - 100%

Literature references

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