研究用試薬

Anti-ENA Pool ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EA 1590-9601-9 G	nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, ribosomal P proteins	IgG	Ag-coated microplate wells	96 x 01 (96)

Indications: Sharp syndrome (MCTD), Lupus erythematosus disseminatus, Sjögren's syndrome, Progressive systemic sclerosis.

Principles of the test: The ELISA test kit provides a semiquantitative in vitro assay for human autoantibodies of the IgG class in serum or plasma against seven different antigens**, nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70 and ribosomal P proteins**. The test kit contains microtiter strips each with 8 reagent wells coated with a pool of these antigens. In the first reaction step, diluted patient samples are incubated with 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	Symbole
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 (IgG, human), ready for use	dark red	1 x 2.0 ml	CAL 1
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	light blue	1 x 100 ml	SAMPLE BUFFER
7. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 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 target values	---	1 protocol	

LOT	Lot description		Storage temperature
IVD	In vitro diagnostics	CE	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 should be disposed of according to official 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 a minimum of 4 months.

- **Calibrator 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 crystallization 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 deionized 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: Calibrator 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 azide. Avoid skin contact.

Preparation and stability of the patient samples

Sample material: 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 10 µl serum in 2.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: Calibrator and controls are prediluted and ready for use, do not dilute them.

Incubation

(Partly) manual test performance

Sample incubation: (1. step)
Transfer 100 µl of the calibrator, 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 reagent wells 3 times with 450 µl 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.

Attention: Residual liquid (> 10 µl) 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) intoeach 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 **15 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 intro- duced.

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 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**	**P 6**	**P 14**	**P 22**								
B	**pos.**	**P 7**	**P 15**	**P 23**								
C	**neg.**	**P 8**	**P 16**	**P 24**								
D	**P 1**	**P 9**	**P 17**									
E	**P 2**	**P 10**	**P 18**									
F	**P 3**	**P 11**	**P 19**									
G	**P 4**	**P 12**	**P 20**									
H	**P 5**	**P 13**	**P 21**									

The above pipetting protocol is an example of the semiquantitative determination of antibodies in 24 patient sera (P 1 to P 24).

Calibrator (C), positive (pos.) and negative (neg.) controls and the serum samples have been incubated in one well each. The reliability of the ELISA test can be improved by duplicate determinations of each sample. The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and minimizes reagent wastage.
Both positive and negative control serum serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

Semiquantitative: The extinction value of the calibrator defines the upper limit of the reference range of healthy subjects (**cut-off**) recommended by EUROIMMUN. Values above the indicated cut-off are to be considered as positive, those below as negative. Besides this qualitative interpretation a semiquantitative evaluation of the result is possible by calculating a ratio according to the following formula:

$$\frac{\textbf{Extinction of control or patient samples}}{\textbf{Extinction of the calibrator (cut - off)}} = \textbf{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

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

For duplicate determination 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.

In the case of positive results in the **EUROIMMUN Anti-ENA Pool ELISA** we recommend differentiation of the antibodies with the respective **monospecific EUROIMMUN test systems**.

Test characteristics

Calibration: For every group of tests performed, the extinction values of the calibrator and the ratios of the positive and negative controls must lie within the limits stated for the relevant test kit lot. A protocol containing these target 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 activity of the enzyme used is temperature-dependent and the extinction values may vary if a thermostat is not used. The higher the room temperature (+18°C to +25°C) during substrate incubation, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrator is subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigens: The microplate wells were coated with an antigen pool consisting of the following antigens:

nRNP/Sm: Native U1-nRNP purified by affinity chromatography from calf and rabbit thymus. U1-nRNP contains the RNP specific proteins 70K, A and C as well as the Sm specific proteins B, B‘, D, E, F, G.

Sm: Native Sm antigen purified by affinity chromatography from bovine spleen and thymus.

The antigens nRNP and Sm belong to a group of small ribonucleoproteins (snRNP, small nuclear ribonucleoproteins) which consist of low molecular weight RNA with a high uridine content (U-RNA) complexed with various proteins (molecular weights 9 - 70 kDa). The RNA component is termed U1 to U6, depending on its behaviour in chromatography. Besides the particular RNA, the particles of U-nRNP contain six different core proteins (B, B', D, E, F, G), U1-nRNP additionally contains particle-specific proteins (70K, A, C). Antibodies to U1-nRNP are directed against one or more of the particle-specific proteins 70K, A or C. In contrast, antibodies to Sm can also react with one or more core proteins. The U- nRNP particles are involved in splicing of the pre-mRNA (pre-messenger RNA) - they split off the non- coding mRNA sequences (introns) and insert the coding mRNA sequences (exons) to recreate the messenger RNA.

SS-A/Ro: Native SS-A antigen purified by affinity chromatography from bovine spleen and thymus.
The SS-A/Ro antigen is localized in the cell nucleus and is involved in the processing of mRNA to translationally active molecules. It is a small ribonucleoprotein which consists of one RNA molecule (Y1-, Y2-, Y3-, Y4- or Y5-RNA; 80-112 bases) and a 60 kDa protein. A 52 kDa protein (Ro-52) is also associated with the SS-A/Ro complex, but whether this protein is a component of the SS-A/Ro complex is controversially discussed in the literature. Anti-SS-A positive patient samples contain antibodies against the native SS-A (60 kDa protein) and might additionally react with the Ro-52 protein. Antibodies exclusively against **Ro-52** are not specific for Sjögren’s syndrome or SLE and can be found in a number of different disease conditions.

Ro-52: Recombinant Ro-52 (52 kDa). The relevant human cDNA was expressed with baculovirus vectors in insect cells.

SS-B: Native SS-B antigen purified by affinity chromatography from calf and rabbit thymus.
The SS-B antigen is a phosphoprotein with a molecular weigth of 48 kDa. It functions in the cell nucleus as a helper protein for RNA polymerase III.

Scl-70: Native Scl-70 antigen purified by affinity chromatography from bovine and rabbit thymus.
The Scl-70 antigen has been identified as the enzyme DNA Topoisomerase-I. The molecular weight of the native antigen is 100 kDa. Originally, only a metabolic product of molecular weight 70 kDa was found in the western blot. The DNA Topoisomerase-I is situated in the nucleoplasm and, in a particularly high concentration, in the nucleolus. The enzyme participates in the replication and transcription of the DNA double helix.

Ribosomal P proteins: Ribosomal P proteins purified by affinity chromatography from bovine and rabbit thymus.
The ribosomal P antigen consists of 3 proteins of the 60S ribosomal subunit. These proteins are designed P0 (molecular weight 38 kDa), P1 (19 kDa) and P2 (17 kDa). The major immunoreactive epitope is localized to the carboxy terminus of all 3 protein and consists of an identical sequence of 17 amino acids.

Detection limit: 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-ENA Pool-ELISA is a ratio value of 0.1.

Cross reactivity: No cross reactions were found in antibodies against M2, CCP and dsDNA in patient sera when tested.

Interference: Haemolytic, lipaemic and icteric samples showed no influence at the result up to a concentration of 10 mg/ml for hemoglobin, 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 (ratio)**	**CV (%)**
1	1.3	2.4
2	2.1	2.7
3	10.2	2.4

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (ratio)**	**CV (%)**
1	1.4	8.0
2	2.1	6.4
3	9.8	6.2

Reference range: The level of ANA (IgG) were analyzed with this EUROIMMUN ELISA in a panel of 200 healthy blood donors. With a cut-off of ratio 1.0, 0.5% of the blood donors were ANA (IgG) positive.

Clinical significance

High titres of **antibodies against U1-nRNP** are characteristic of mixed connective tissue disease (MCTD, **Sharp syndrome**). The prevalence is 95% - 100%. The antibody titre correlates with the clinical activity of the disease. Antibodies against U1-nRNP are also found in 15% to 40% of patients with **systemic lupus erythematosus**.

Antibodies against Sm have a high specificity for **systemic lupus erythematosus**. Together with antibodies to dsDNA, they can be considered pathognomonic for this condition, but occur in only 5% to 30% of patients.

Antibodies against SS-A and **Ro-52** are associated with various autoimmune diseases. The most common occurence is in patients with **Sjögren's syndrome** (40% to 80% of cases), but also in systemic lupus erythematosus (30% to 40%), and primary biliary cirrhosis (20%). Apart from this, antibodies to SS-A can be found in practically 100% of cases of **neonatal lupus erythematosus**. They are transmitted diaplacentally to the foetus and cause an inflammatory reaction. They can also cause a **congenital heart block** in newborn babies.
It has been shown in various studies that anti-SS-A positive sera always contain antibodies against native SS-A (60 kDa protein) and may additionally exhibit antibodies against Ro-52. For example, in a Japanese study (EUROIMMUN) sera from 103 patients with SLE and Sjögren's syndrome (SLE n=26, Sjögren's syndrome n=77), which were characterized as anti-SS-A positive by double immunodiffusion, were investigated. 102 sera reacted with native SS-A, and 90 sera reacted additionally with the Ro-52 protein. But no serum showed only a reaction with Ro-52. This study demonstrates that antibodies against SS-A can be reliably detected using the native SS-A. In rare cases and in suspected cases of neonatal lupus syndrome, the Ro-52 band may provide important supplementary information.

Antibodies against SS-B are found almost exclusively in women (29:1) in cases of **Sjögren's syndrome** (40% to 80% of cases) and systemic lupus erythematodes (10% to 20%). In Sjögren's syndrome, combined SS-A and SS-B antibodies mainly occur.

Antibodies against Scl-70 are found in 25% to 75% of patients with **progressive systemic sclerosis** (diffuse form), depending on the test methods used and the degree of activity of the disease - (Scl = scleroderma). They do not occur in circumscriptive scleroderma.

Antibodies against ribosomal P proteins are specific for **systemic lupus erythematosus**. The prevalence is 10%.

Literature references

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