Anti-ENA PoolPlus ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EA 1590-9601-7 G	nRNP/Sm, Sm, SS-A, SS-B, Scl-70, Jo-1	IgG	Ag-coated microplate wells	96 x 01 (96)

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

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 six different antigens: nRNP/Sm, Sm, SS-A, SS-B, Scl-70 and Jo-1. The test kit contains microtiter strips each with 8 break-off 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), which is capable of promoting 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 (IgG, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Positive control serum (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
4. Negative control serum (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	SAMPLEBUFFER
7. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASHBUFFER 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		storage temperature
IVD	In vitro determination	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 shoud 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) around 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 ready-to-use diluted wash buffer is stable for 1 month 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 tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays or 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 contact with the skin.

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: The serum or plasma samples are diluted 1:201 sample buffer. For example: dilute 5 µl 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

Sample incubation: (1. step)
Transfer 100 µl sample buffer (blank), 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 400 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.

Note: Residual liquid (> 10 µl) remaining in the reagent wells after washing can interfere with the substrate and lead to falsely 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 falsely 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 bis 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 bis 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 of 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.

Pipetting protocol

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

The above pipetting protocol is an example of the quantitative analysis of antibodies in 24 patient samples (P 1 to P 24).

Sample buffer (Bl), calibrator (C), positive (pos.) and negative (neg.) control as well as the patient 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

Quantitative: If the photometer has no automatic blank adjustment, then the blank value must first be subtracted from all other measured values. The extinction values for the positive and the negative controls and for the patient samples can be converted to relative units (RU) using the values for the calibrator as follows:

$$\frac{\textbf{Extinction of the sample x 100}}{\textbf{Extinction of the calibrator}} = \textbf{Value in RU/ml}$$

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 alongside the serological results.

In the case of positive results in the Anti-ENA PoolPlus ELISA we recommend a differentiation of the antibodies with the EUROIMMUN Anti-ENA Profile ELISA (assay for antibodies against nRNP/Sm, Sm, SS-A, SS-B, Scl-70 and Jo-1; the test kit contains microplate strips with wells individually coated with these six antigens) or with the monospecific EUROIMMUN ELISA.

An indirect immunofluorescence test should always be performed in parallel with the determination of cell nucleus antibodies by ELISA. On the one hand, this provides a check on plausibility as a safeguard against false-positive ELISA results, on the other hand, by using EUROIMMUN Hep-2 cells, and in particular in combination with frozen sections of primate liver, immunofluorescence permits the detection

of a wider range of cell nucleus antibodies, as not all cell nucleus antigens are presently available in the ELISA substrate.

Test characteristics

Calibration: The Anti-ENA PoolPlus ELISA is calibrated with a mixed serum. As no international reference serum exists for antibodies against ENA, the calibration is performed in relative units (RU/ml).

For every group of tests performed, the extinction values of the blank and the calibrator and the relative units determined for 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 calibration and 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 calibration sera are 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: 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.

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.

Jo-1: Native Jo-1 antigen (histidyl-tRNA synthetase) purified by affinity chromatography from calf and rabbit thymus.

The Jo-1 antigen is identical to histidyl-tRNA synthetase, a cytoplasmic phosphoprotein with a molecular weight of 50 kDa. It joins the amino acid histidine in the cytoplasm to its corresponding tRNA.

Detection limit: The detection limit is defined as a value of three times the standard deviation of an analyte-free sample and is the smallest detectable antibody titer. The detection limit of the Anti-ENA PoolPlus ELISA (IgG) is 1 RU/ml.

Cross reactivity: This ELISA showed no cross reactivity.

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 (CV) using 3 sera with values at different points on the calibration curve. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed on 6 different days.

Intra-Assay-Variation, n = 20		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	33	3.2
2	77	4.7
3	107	3.8

Inter-Assay-Variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	36	8.1
2	89	6.9
3	111	4.9

Reference range: The levels of anti-ENA antibodies (IgG) were analyzed 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-ENA negative.

Clinical significance

High titres of **antibodies to 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 to U1-nRNP are also found in 30% - 40% of patients with **disseminated lupus erythematosus**.

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

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

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

Antibodies to Jo-1 are found in polymyositis with a prevalence of 25% - 35%. They are often associated with a concurrent interstitial fibrosis of the lung.

Literature references

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