Anti-SLA/LP ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EA 1302-9601 G	SLA/LP (soluble liver antigen/ liver-pancreas antigen)	IgG	Ag-coated microplate wells	96 x 01 (96)

Indications: Increase in transaminases for unclear reasons, suspected autoimmune hepatitis.

Principles of the test: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human autoantibodies of the IgG class against SLA/LP (soluble liver antigen/liver-pancreas antigen) in serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with SLA/LP. 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) 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 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	SAMPLEBUFFER
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 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.

- **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 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: 5 ml concentrate plus 45 ml water.
  The ready-to-use diluted 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: Calibrators and controls used have 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 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: Patient samples are diluted **1:101** sample buffer. For example: dilute 10 µl serum in 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 **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.

Sample incubation: (1. step)
Transfer 100 µl 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 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 micro-plate 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**	**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 1-4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7-10 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. 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 controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

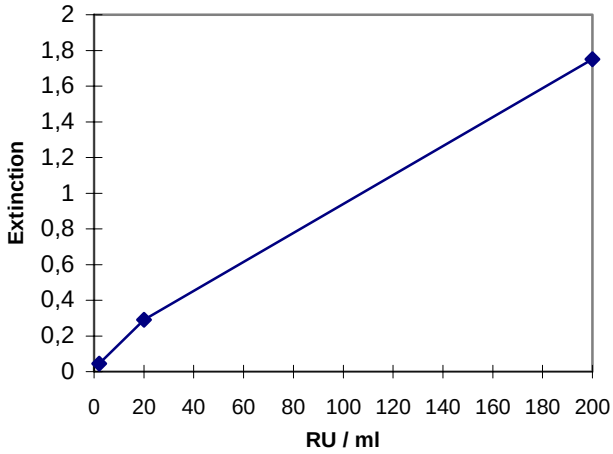
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator 2. Calculate the ratio according the following formula:

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

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 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 lies above the value of calibrator 1 (200 RU/ml). The result shold be given as ">200 RU/ml". It is recommended that the sample be re-tested at a dilution of 1:400. The result in IU/ml read from the calibration curve for this sample must then be multiplied by a factor of 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 the sample should be retested.

For diagnosis, the clinical symptoms of the patient should always be taken into account alongside the serelogical results.

Test characteristics

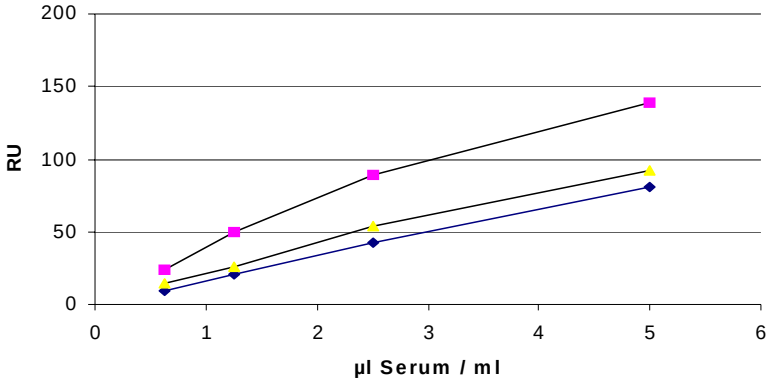
Calibration: As no international reference serum exists for antibodies against SLA/LP, the calibration is performed in relative units (RU).

For every group of tests performed, the extinction values 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 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 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 recombinant SLA/LP. The corresponding human cDNA was expressed in E. coli. Identification of SLA/LP at the DNA level succeeded in 1998 by cloning the target antigen (A. W. Lohse, University Hospital of Mainz). SLA/LP is probably a cytoplasmic molecule with a molecular weight of 50 kDa which is involved in the regulation of the protein biosynthesis (an UGA-suppressor-tRNA associated protein). Previous descriptions of SLA as being the liver cytokeratins 8 and 18 or glutathione-S-transferase were apparently wrong.

Linearity: The linearity of the test was investigated by assaying serial dilutions of patient samples with high antibody concentrations. The chart below shows the typical linearity of samples on the basis of 3 patient sera. The Anti-SLA/LP ELISA (IgG) is linear in the measurement range 2 - 200 RU/ml.



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-SLA/LP ELISA (IgG) is approximately 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	41	2.6
2	110	2.3
3	154	3.7

Inter-Assay Variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	44	4.4
2	118	3.6
3	169	3.8

Prevalence and specificity: Sera from 454 patients with autoimmune hepatitis, 165 patients with other liver diseases and 200 healthy blood donors were examined with the Anti-SLA/LP ELISA. The prevalence anti-SLA/LP antibodies in non-Japanese AIH patients was in the range between 15% and 19%. The test showed a specificity of 100%.

Patient group (n=819)	Origin of sera	n	Anti-SLA/LP-positive
Autoimmune hepatitis	Prof. Lohse, Univ. Mainz	108	21 (19%)
	Brasilian panel	154	25 (16%)
	Japanese panel	43	2 (5%)
	American panel	149	23 (15%)
AIH (anti-LKM-1 positive)	Dr. Gruber, Univ. München	18	0
Primary biliary cirrhosis	Dr. Gruber, Univ. München PD Dr. Wick, Klinikum Großhadern	30	0
Hepatitis-B infection	Labor Dr. Stöcker, Lübeck	40	0
Hepatitis-C infection	Prof. Lohse, Univ. Mainz Labor Dr. Stöcker, Lübeck	39	0
Steatohepatitis	Prof. Lohse, Univ. Mainz	24	0
Toxic liver damage	Prof. Lohse, Univ. Mainz	14	0
Blood donors	Med. Univ. Lübeck	200	0

Reference range: The levels of the anti-SLA/LP antibodies (IgG) were analyzed with this EUROIMMUN ELISA in a panel of 200 healthy blood donors. With a cut-off of 20 RU/ml, all blood donors were anti-SLA/LP negative.

Clinical significance

The determination of autoantibodies against soluble liver antigen/liver-pancreas antigen (SLA/LP) is a new and important component in the diagnostics of autoimmune diseases of the liver.

Autoimmune diseases of the liver include
- autoimmune hepatitis (AIH),
- primary biliary liver cirrhosis (PBC) and
- primary sclerosing cholangiitis (PSC).

Mainly women are affected by **autoimmune hepatitis** (**AIH**, earlier designations: lupoid hepatitis, chronically active hepatitis). The disease manifests itself through increased levels of bilirubin, liver enzymes and immunoglobulins, through typical histological changes (liver biopsies show necrosis of the parenchymal cells with lymphocyte and plasma cell infiltrates) and through the occurrence of various autoantibodies. The disease can occur from infancy until the old age, however, it affects most frequently young adults.
The incidence of AIH in western Europe is 1.9 cases per 100,000 inhabitants per year. Untreated, autoimmune hepatitis soon develops into liver cirrhosis. However, if immunosuppressive low-dosed therapy is commenced in good time and continued consistently throughout the patient's life, the patient has a normal life expectancy. For differential diagnosis, a current infection with hepatitis viruses must be ruled out by investigation of the appropriate serological parameters.

Circulating autoantibodies have gained great significance for the diagnosis of AIH. They occur in the majority of patients, but their role in the pathogenesis is questionable. Also, there is no clear correlation between the activity or the prognosis of the disease and the antibody titre. Besides antibodies against SLA/LP the following autoantibodies are associated with AIH: antibodies against cell nuclei (**ANA**), **nDNA**, smooth muscle (**SMA**, with the most important target antigen being **F-actin**), liver-kidney microsomes (**LKM-1**, target antigen: cytochrome P450 IID6) and granulocytes (**pANCA**). The **autoantibodies against SLA/LP** that can today be measured by EUROIMMUN enzyme immunoassays probably have the highest diagnostic accuracy of all antibodies involved in autoimmune hepatitis. Anti-SLA/LP occur in AIH either singly or together with other autoantibodies. Although their prevalence is only between 10% and 30%, the predictive value is almost 100%: Every positive result essentially provides evidence of autoimmune hepatitis (provided that the corresponding clinical symptoms are present).

Investigations of antibodies against SLA/LP have up until now only been possible in a few special laboratories. It was earlier suspected that the SLA antigen is identical with the liver cytokeratins 8 and 18 or with the enzyme glutathione-S-transferase. It has only recently been found that this is apparently not the case. The newly discovered target antigen SLA/LP has presently only been identified reliably at DNA level. It is probably a cytoplasmic molecule which is involved in the regulation of the protein biosynthesis (an UGA-suppressor-tRNA associated protein). The great diagnostic value of autoantibodies against SLA/LP has now been clearly proved.

Autoantibodies against cell nuclei (ANA) and against smooth muscle (SMA) are frequent in AIH, but also occur in 10% to 20% of patients with chronic viral hepatitis and in other diseases. Autoantibodies against LKM-1 can only be demonstrated in about 1% of adult AIH patients, but they are more frequent in children. Antibodies against LKM-1 can also be found 1% to 2% of patients with a positive hepatitis C serology. As opposed to all other autoantibodies, antibodies against SLA/LP are highly specific for AIH and have not been described in viral hepatitis.

Some authors classify autoimmune hepatitis in accordance with the autoantibody status: Subtype I (ANA, SMA), Subtype II (antibodies against LKM-1) and Subtype III (antibodies against SLA/LP). This classification has probably neither a clinical nor a therapeutic and prognostic significance.

The diagnosis of AIH is based on the clinical picture, the biochemical and serological test results and the histological evidence of an inflammation reaction. It requires the exclusion of other causes of chronic hepatitis, such as viruses, alcohol and drugs, and a delimitation to the other autoimmune liver diseases (primary biliary liver cirrhosis and primary sclerosing cholangiitis). The diagnosis AIH is appropriate when 4 of the following 5 main criteria are fulfilled. If all 5 criteria are present, the diagnosis is definite. The diagnosis is confirmed if the patient responds to immunosuppressive therapy.

Main criteria for the diagnosis of autoimmune hepatitis:
1. Histological evidence of hepatitis
2. Detection of autoantibodies (ANA, SMA, LKM, SLA/LP)
3. Hypergammaglobulinaemia
4. Negative HBV and HCV serology
5. Detection of the HLA antigens B8, DR3 or DR4

The serological determination of **autoantibodies against SLA/LP** provides an exact delimitation to viral hepatitis in many patients with AIH. The autoimmune serology of hepatitis has thus been enriched by a further parameter whose significance must be rated more highly than that of antibodies against cell nuclei, DNA, smooth muscle and liver-kidney microsomes (**ANA, nDNA, ASMA, LKM**). Testing for these antibodies has considerable consequences for the hepatology clinic: the consequence of incorrect treatment of AIH with interferon can be just as fatal as the immunosuppressive therapy of the virus infection.
Primary biliary cirrhosis (PBC) is a liver cirrhosis which takes the form of a non-suppurative, destructive inflammation of the biliferous ducts. It occurs in woman ten times more frequently than in men. In the foreground of the clinical picture is cholestasis. In Europe, 13 out of every 100,000 persons develop this disease each year. As a cirrhosis does not occur in every case, and mostly not until the late stage, this disease is better described as **“chronic non-suppurative destructive cholangiitis”**. Pathognomonic is the serological detection of autoantibodies against the mitochondrial fraction M2 (**AMA M2**) and against **Nuclear Dots** (acid protein SP100). Approximately 10% to 20% of PBC patients develop a secondary autoimmune hepatitis (also called **overlap syndrome**). In these cases, as in AIH, autoantibodies can frequently be detected. Antibodies against SLA/LP indicate a secondary AIH (overlap syndrome) which requires an immunosuppressive therapy.

The incidence of **primary sclerosing cholangiitis (PSC)** is quoted as being 4 cases in every 100,000 inhabitants per year. The clinical picture is also characterised by cholestasis. Diagnostic pointers are the relevant laboratory findings, the histology and ERCP (endoscopic retrograde cholangiopancreaticography). Men are mainly affected, and in half of the patients a concomitant Colitis ulcerosa is present (inversely, the prevalence of PSC in cases of Colitis ulcerosa is 4%). Most patients with PSC display autoantibodies against granulocytes (**pANCA**). Occasionally, pANCA can also occur in AIH and PBC, and their value for the differential diagnosis is limited in this respect - however, pANCA are an indication

of an autoimmune disease of the liver and can possibly be used as a means of delimitation to infectious forms of hepatitis. Even though overlaps between PSC and AIH have been reported, antibodies against SLA/LP have not been found in such patients.

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