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Instruction For Use
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ORG 722 Liver-9-Line 2nd Generation

NAME AND INTENDED USE

Liver-9-Line 2nd Generation Immunoblot assay is a membrane based enzyme immunoassay for the semi-quantitative measurement of IgG class autoantibodies to AMA-M2, Sp100, gp210, SLA/LP, LKM-1, LC1, F-Actin, Actinin and Tropomyosin in human serum or plasma. The assay is intended for professional in vitro diagnostic use only.

SYMBOLS USED

IVD	In vitro diagnostic medical device		
	Manufacturer		
REF	Catalogue number	BLOT STRIPS	Blot strips
Σ	Sufficient for	DILUENT	Sample Buffer
LOT	Batch code	CONJUGATE	Enzyme Conjugate
	Use by	WASH	Wash Buffer
2°C 8°C	Temperature limitation	BCIP	BCIP Substrate
	Consult instructions for use	RTU	Ready to use
	Keep away from sunlight		
	Do not reuse		
	Date of manufacture		

SUMMARY AND EXPLANATION OF THE TEST

Detection of autoantibodies plays a pivotal role in the diagnosis of liver disorders, namely autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC) variants in adults and children. Autoimmune hepatitis (AIH) is a chronic liver disease of unclear aetiology. AIH is a very rare disease (estimate: 50 to 200 infected per 1 million). Women are more often affected than men are. Two types of the disease, type I AIH (AIH-1) and type 2 AIH (AIH-2) can be distinguished, whereas AIH-1 affects all age groups, AIH-2 is typically found in children and young adults. AIH-2 represents only a small fraction of all AIH cases, but progression of this disease is very grave.

Elevated transaminase levels, hypergammaglobulinemia, and elevated titres of antinuclear antibodies (ANA) and/or smooth muscle antibodies (SMA) are typical of this disease. Histologically, the disease is characterised by interface hepatitis and plasma cell infiltrates in the portal fields. If untreated, the disease progresses to cirrhosis. At this point, mortality is high. In a substantial fraction of patients, the disease is often diagnosed in the process of investigating elevated liver values in a clinically asymptomatic stage of the disease. Because the latest treatments may significantly improve the prognosis, timely diagnosis of autoimmune hepatitis is of great importance. In addition to AIH, PBC and PSC, there are also overlap syndromes involving these diseases and hepatitis C. Associations with other, extrahepatic autoimmune diseases are also frequently observed.

Liver-9-Line 2nd Generation from ORGENTEC is an immunoblot for the identification of a differentiated serological profile for autoimmune liver diseases. The simultaneous detection of autoantibodies against nine different antigens makes it possible to differentiate AIH subtypes and to rule out other liver diseases.

AMA-M2

Anti-mitochondrial antibodies (AMA) are a heterogeneous group of autoantibodies directed against various proteins of the outer and inner mitochondrial membrane. AMA of the M2 subtype are directed against epitopes of the pyruvate dehydrogenase complex and indicate primary biliary cirrhosis (PBC): They are detected in 90 to 95 % of PBC patients.

SP100 and gp-210

Two special types of antibodies to nuclear components are specific for PBC: anti-Sp100 and anti-gp210. They are mainly reacting with the nuclear body antigen Sp100 and the nuclear pore antigen gp210 respectively. They are especially important in AMA-M2 negative PBC patients. They only rarely occur in association to AIH or PSC. Antibodies to gp210 are associated with extrahepatic manifestations. They correlate with disease severity and may be a marker of poor prognosis.

SLA/LP

Autoantibodies against the soluble liver antigen (SLA) and the liver-pancreas antigen (LP) are highly specific markers for AIH-1. They bind to the UGA tRNA suppressor associated antigenic protein (tRNP(Ser)Sec). They are also called anti-SLA/LP antibodies and have been proposed as specific markers of a third type of severe AIH seronegative for the conventional AIH-1 autoantibodies. Similarities in the clinical profile between patients with AIH-1 (ANA and/or ASMA positive) and AIH patients with anti-SLA alone in addition with an approximately 30 % seropositivity overlap between anti-SLA and ASMA and/or ANA suggest that anti-SLA is rather an additional important marker for the diagnosis of AIH-1, than a marker of a third type of AIH. Anti-SLA antibodies denote patients with a more severe course of AIH.

Cryptogenic autoimmune hepatitis is characterised by a clinical picture that is indistinguishable from autoimmune hepatitis. ANA, ASMA, and anti-LKM-1 are negative at disease onset and may appear late in the disease course. Anti-SLA may be positive in 12-30 % of patients with cryptogenic hepatitis.

LKM-1 und LC1

Antibodies against type 1 liver-kidney mircosomes (LKM-1) and/or against the liver cystolic antigen type 1 (LC1) are serological markers for the diagnosis of AIH-2. AIH-2 patients with anti-LC1 antibodies have histologically more severe disease compared to those without anti-LC1 antibodies. LKM-1-antibodies bind to cytochrome P450 IID6 (CYP2D6); the enzyme formiminotransferase cyclodeaminase (FTCD) is the molecular target of anti-LC1 antibodies. Anti-LC1 occurs in association with anti-LKM-1 in 25-48 % of patients with AIH-2, or in isolation in 22 %.

Autoantibodies against LC1 were initially diagnosed on the basis of their specific fluorescence pattern. They showed an intense homogeneous cytoplasmic fluorescence, similar to LKM-1 autoantibodies, especially in the periportal hepatocytes, sparing the perivenous hepatocytes around the central vein. In contrast to LKM-1 autoantibodies, they do not react with the proximal tubules of the rat kidney. When LKM-1 and LC1 appear simultaneously the characteristic fluorescence pattern of LC1 is masked by LKM-1 fluorescence, and the detection of anti-LC1 via IFT is no longer successful.

Chronic hepatitis C virus (HCV) infection is closely related to autoimmune hepatitis. Anti-LC1 antibodies have been

found sporadically in anti-LKM-1 antibody positive patients suffering from chronic hepatitis C virus infection, mostly in association with anti-LKM-1. A recent study showed that 50 % of patients with anti-LKM-1 and anti-LC1 antibodies were infected with HCV.

F-Actin, actinin and tropomyosin

ASMAs are typical markers for AIH-1. They are a heterogenic population of antibodies and recognise a variety of different antigens of the smooth muscle. Among them are the proteins F-actin, alpha-actinin and tropomyosin, the building blocks of the microfilaments. The differentiation of individual antigens of smooth muscle F-actin, alpha-actinin and tropomyosin allows for a better characterization of these diagnostically relevant biomarkers.

Actin is a globular multi-functional protein that forms a linear polymer called F-actin. Two helical, interlaced polymer chains of F-actin build a microfilament. The F-actin filament also contains a filamentary tropomyosin molecule, which is wrapped around the F-actin helix. Similar to tropomyosin alpha-actinin also belongs to the group of actin-binding proteins. It contributes to the formation of filament bundles and to crosslinking of the filaments.

Anti-F-actin and anti-alpha-actinin antibodies are detected simultaneously in more than 60 % AIH-1 patients. The combination of both antibody specificities reflects high disease activity. F-actin antibodies are positive in patients with active advanced disease. Low titers of antibodies against alpha-actinin at baseline appear to predict treatment response and decrease of anti-alpha-actinin titres may be used for monitoring treatment outcome in AIH-1.

In addition to F-actin and actinin, tropomyosin is one of the most important targets for autoantibodies in autoimmune liver diseases. Approximately one third of patients with AIH also form antibodies against tropomyosin. In a subset of patients with PBC antibodies against tropomyosin have also been detected.

PRINCIPLE OF THE TEST

Highly purified antigens AMA-M2, Sp100, gp210, SLA/LP, LKM-1, LC1, F-Actin, Actinin and Tropomyosin as well as three control antigens for CO Cut-off Control, EC Enzyme Conjugate Control and SC Serum Control are bound to nitrocellulose membrane blot strips.

Autoantibodies present in serum or plasma bind to the immoblizied antigen. Washing of the blot strips removes unbound antibodies and unspecific sample components. Alkaline phosphatase conjugated anti-human IgG detect the bound sample antibodies forming a conjugate/antibody/antigen complex. Washing of the blot strips removes unbound conjugate. The substrate BCIP/NBT is hydrolized by bound enzyme conjugate to form an insoluble blue-violet product. Washing of the blot strips removes unhydrolyzed substrate and stopps the reaction. The amount of color is directly proportional to the concentration of IgG antibodies present in the original sample.

WARNINGS AND PRECAUTIONS

- All reagents of this kit are intended for professional in vitro diagnostic use only.
- Bovine serum albumin (BSA) used in components has been tested for BSE and found negative.
- Avoid contact with the substrate BCIP/NBT.
- Sample buffer and wash buffer contain sodium azid 0.09% as preservative. This concentration is classified non-hazardous.
- Enzyme conjugate contains 0.05% ProClin as preservative. This concentration is classified as non-hazardous.

During handling of all reagents, controls and serum samples observe the existing regulations for laboratory safety regulations and good laboratory practice:

- First aid measures: In case of skin contact, immediately wash thoroughly with water and soap. Remove contaminated clothing and shoes and wash before reuse. After contact with the eyes carefully rinse the opened eye with running water for at least 10 minutes. Get medical attention if necessary.
- Personal precautions, protective equipment and emergency procedures:

Observe laboratory safety regulations. Avoid contact with skin and eyes. Do not swallow. Do not pipette by mouth. Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled. When spilled, absorb with an inert material and put the spilled material in an appropriate waste disposal.

- Exposure controls / personal protection: Wear protective gloves of nitril rubber or natural latex. Wear protective glasses. Used according to intended use no dangerous reactions known.

- Conditions to avoid: Since substrate solution is light-sensitive. Store subtrate solution in the dark.
- For disposal of laboratory waste the national or regional legislation has to be observed.

Observe the guidelines for performing quality control in medical laboratories by assaying control sera.

SPECIMEN COLLECTION, STORAGE AND HANDLING

- Collect whole blood specimens using acceptable medical techniques to avoid hemolysis.
- Allow blood to clot and separate the serum by centrifugation.
- Test serum should be clear and non-hemolysed. Contamination by hemolysis or lipemia is best avoided, but does not interfere with this assay.
- Specimens may be refrigerated at 2-8 °C for up to five days or stored at -20 °C up to six months.
- Avoid repetitive freezing and thawing of serum samples.
- Testing of heat-inactivated sera is not recommended.

CONTENTS OF THE KIT

▽16	ORG 722-16	Sufficient for 16 determinations
▽8	ORG 722-08	Sufficient for 8 determinations
BLOT STRIPS	1x/2x	8 antigen coated nitrocellulose strips. Ready to use. 1 pre-developed calibration strip (coded CAL) for semiquantitative evaluation. Ready to use.
DILUENT	1x 20 ml	Sample Buffer PB, containing PBS, BSA, detergent, preservative sodium azide 0.09%, yellow. Ready to use.
CONJUGATE	1x 20 ml	Enzyme Conjugate containing anti-human IgG antibodies, alkaline phosphatase labelled; PBS, BSA, detergent, preservative ProClin 0.05%, light red. Ready to use.
WASH	1x 20 ml	Wash Buffer WB, containing Tris, detergent, preservative sodium azide 0.09%; 50 x conc.
BCIP	1x/2x 10 ml	BCIP Substrate; containing BCIP/NBT. Ready to use.
		Incubation tray
	1x	Instruction for Use: ELISA Mini-DVD
	1x	Certificate of Analysis

MATERIALS REQUIRED

- Pipettes for 10 µl and 1000 µl
- Distilled or deionised water
- Graduated cylinder for 1000 ml
- Laboratory timing device
- Rocking platform
- Tweezers

STORAGE AND STABILITY

- Store the kit at 2-8 °C.
- Keep nitrocellulose strips carefully sealed in the original plastic tube with desiccants provided.
- Important: The calibration strip is very light-sensitive. Store in the dark!
- Do not expose test reagents to heat, sun or strong light during storage and usage.
- The unopened test kit is stable for 18 months from day of production. See expiry date on outer labels for individual batches.
- Diluted wash buffer is stable for at least 30 days when stored at 2-8°C. We recommend consumption on the same day.

PROCEDURAL NOTES

- Do not use kit components beyond their expiration dates.
- Do not interchange kit components from different lots.
- All materials must be at room temperature (20-28 °C).
- Have all reagents and samples ready before start of the assay. Once started, the test must be performed without interruption to get the most reliable and consistent results.
- Perform the assay steps only in the order indicated.
- Always use fresh sample dilutions

- To avoid carryover contamination, change the tip between samples.
- All incubation steps must be accurately timed.
- Control sera should routinely be assayed as unknowns to check performance of the reagents and the assay.
- Nitrocellulose strips must be handled with gloves or tweezers.
- It is important to make sure, that air-bubbles do not interfere with the strip during incubation. This could cause irregularities in coloration of developing bands and can lead to wrong results.

PREPARATION OF REAGENTS

WASH

Dilute the contents of one vial of the buffered wash solution concentrate (50x) with distilled or deionised water to a final volume of 1000 ml prior to use.

DILUENT

Ready to use.

Preparation of samples
Sample dilution see test procedure. Effective dilution during test is 1:101.

TEST PROCEDURE

Using tweezers insert one nitrocellulose strip into one chamber of the incubation tray:

- Add **1000 µl sample buffer** to the strip in the chamber.
- Allow to equilibrate for 5 minutes with gentle agitation.
- Add **10 µl of patient sample** directly to the chamber.
- Incubate for **60 minutes** at room temperature (20-28 °C) with gentle agitation.
- Remove the diluted sample completely from the chamber.
- Add 2000 µl wash buffer to the chamber, incubate for 5 minutes.
- Remove wash buffer completely. Repeat this procedure twice.

- Add **1000 µl enzyme conjugate** to each strip in the chamber of the incubation tray.
- Incubate for **30 minutes** at room temperature with gentle agitation.
- Remove the conjugate completely from the chamber.
- Add 2000 µl wash buffer to the chamber, incubate for 5 minutes.
- Remove wash buffer completely. Repeat this procedure twice.

- Add **1000 µl substrate** to each strip in the chamber of the incubation tray.
- Incubate for **10 minutes** at room temperature with gentle agitation.
- Remove the substrate completly.
- Add 1000 µl distilled water to the chamber, incubate for 5 minutes.
- Remove water completely. Repeat this procedure twice.

Carefully dry the strips on a tissue paper. Allow strips to air dry before evaluating with the calibration strip.

VALIDATION

The assay is valid if all three control lines (**CO** Cut-off Control, **EC** Enzyme Conjugate Control and **SC** Serum Control) show a turn-over of substrate in terms of blue-violet lines! If this criterion is not met the assay is invalid and should be repeated.

Note: Borderline samples should be repeated or tested using an alternative procedure. Samples from patients diagnosed with autoimmune diseases often show multiple autoantibody specificities. Such samples may show a positive reaction with more than one antigen line.

CALCULATION OF RESULTS

The intensity of a **blue-violet line** at the position of the coated antigen is directly proportional to the concentration of IgG antibodies present in the sample tested.

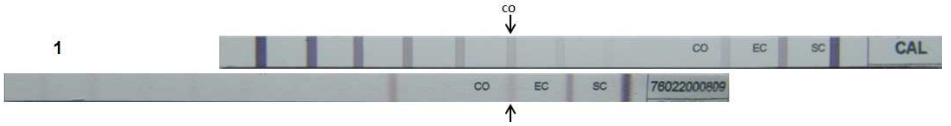
Semi-quantitative evaluation of sample strip:

negativ	intensity of patient sample line weaker than intensity of CO-line
borderline	intensity of patient sample line eqivalent to intensity of CO-line
weak positive	intensity of patient sample line up to 1 level stronger than intensity of CO-line

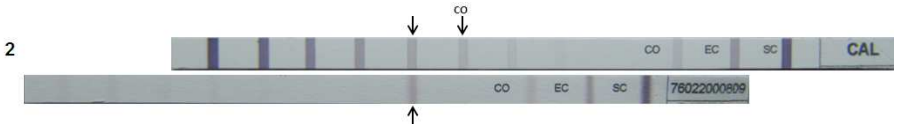
positive	intensity of patient sample line up to 2 levels stronger than intensity of CO-line
strong positive	intensity of patient sample line ≥3 levels stronger than intensity of CO-line

Interpretation of the intensity of blue-violet lines:

(1) Compare intensity of the **CO-line of the sample strip** to the intensity of the lines of the calibration strip.
Example:



(2) Compare the intensity of the **patient sample line** to the intensity of the lines of the calibration strip.
Example: Interpretation of intensity of patient sample line is "weak positive"



PERFORMANCE CHARACTERISTICS

CALIBRATION

The sensitivity, specificity and dose response of the Liver-9-Line 2nd Generation immunoblot was evaluated using clinically defined in house quality control sera containing varying relative amounts of sera with known specificity.

Measuring range

The evaluation of the intensity of the blue lines as described above allows a semi-quantitative determination of IgG class autoantibodies in the sample tested into quantification ranges:
negative, borderline, weak positive, positive, strong positive

Expected values

In a normal range study with samples from healthy blood donors the following ranges have been established with this assay. Cut-off: borderline

Interpretation of results

normal:	negative
elevated:	weak positive, positive, strong positive

Linearity

Patient samples containing high levels of specific antibody were serially diluted in sample buffer. Activity of each dilution step was determined using the calibration strip.

Linearity				
Sample	Dilution	Observed	Expected	O/E
1	1:100	strong positive	strong positive	PASS
.	1:200	positive	positive	PASS
.	1:400	weak positive	weak positive	PASS
.	1:800	borderline	borderline	PASS
.	1:1600	negative	negative	PASS
2	1:100	strong positive	strong positive	PASS
.	1:200	positive	positive	PASS
.	1:400	weak positive	weak positive	PASS
.	1:800	borderline	borderline	PASS
.	1:1600	negative	negative	PASS

Sensitivity

This immunoblot assay is a semi-quantitative assay method. Any reactivity less than borderline is considered

negative and cannot be quantified any further.

Reproducibility

Intra-assay precision: Coefficient of variation (CV) was calculated for each of three samples from the results of 24 determinations in a single run. Results for precision-within-assay are shown in the table below.
Inter-assay precision: Coefficient of variation (CV) was calculated for each of three samples from the results of 6 determinations in 5 different runs. Results for run-to-run precision are shown in the table below.

Intra-Assay		
Sample	Mean	Result
1	negative	PASS
2	weak	PASS
3	positive	PASS

Inter-Assay		
Sample	Mean	Result
1	negative	PASS
2	weak	PASS
3	positive	PASS

Interfering substances

No interference has been observed with haemolytic (up to 1000 mg/dl) or lipemic (up to 3 g/dl triglycerides) sera or plasma, or bilirubin (up to 40 mg/dl) containing sera or plasma. Nor have any interfering effects been observed with the use of anticoagulants (Citrate, EDTA, Heparine). However for practical reasons it is recommended that grossly hemolyzed or lipemic samples should be avoided.

Study results

Study population	n	n pos	%
Autoimmune hepatitis AIH	59	51	86.4
Primary biliary cirrhosis PBC	21	21	100.0
PBC overlap syndrome	9	9	100.0
Rheumatoid Arthritis RA	25	2	8.0
SLE	19	0	0.0
Thrombosis	16	1	6.3
Normal human sera	69	0	0.0

		Clinical Diagnosis		
		Pos	Neg	
ORG 722	Pos	81	3	
Liver-9-Line 2nd Generation	Neg	8	126	
		89	129	218
Sensitivity:	91.0	%		
Specificity:	97.7	%		
Overall agreement:	95.0	%		

LIMITATIONS OF THE PROCEDURE

This assay is a diagnostic aid. A definite clinical diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical and laboratory findings have been evaluated concerning the entire clinical picture of the patient. Also every decision for therapy should be taken individually.

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1. Add **blot strip** into the incubation tray
   → Add **1000 µl** sample buffer per strip into the incubation tray
   → Shake **5 minutes** while incubating

2. Add **10 µl** patient sample and resuspend
   → Shake **60 minutes** while incubating
   → Discard content and wash 3 times for **5 minutes** with **2000 µl** wash buffer, discard wash

3. Add **1000 µl** enzyme conjugate solution per strip
   → Shake **30 minutes** while incubating
   → Discard content and wash 3 times for **5 minutes** with **2000 µl** wash buffer, discard wash

4. Add **1000 µl** substrate per strip
   → Shake **10 minutes** while incubating
   → Discard content and wash 3 times for **5 minutes** with **1000 µl distilled water**, dry blot strips. Read after complete drying, only